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L'effet du chauffage, de la glycation et de mutations sur structure,interactions et sur l'allergénicité de la ß-lactoglobuline bovine

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Abstract

β-Lactoglobulin (β-Lg) is a lipocalin, which is the major whey protein of cow milk and the milk of other mammals. However, it is absent from milk of primates. This globular protein of about 18 kDa is folded forming a β-barrel (or calyx) structure. Each monomer contains two disulphide bonds and one cysteine at position 121 (Cys121). This free thiol plays an important role in the heat-induced aggregation of β-Lg, and, possibly, in the maintenance of its conformational stability. The biological function of β-Lg is not clear, but its potential role in carrying fatty acids through the digestive tract has been suggested. β-Lg is also one of major allergens in milk.

Heating is one of the most common technologic treatments applied during many milk transformations. Despite the frequency of use of heating, the effects of heating β-Lg and its denaturation on its recognition by IgE of patients are not fully described. Binding of IgE from patients with cow milk allergy to native β-Lg and to heated β-Lg in the temperature range 65-95°C was measured by ELISA. Since β-Lg can be subjected to modifications during heating by the existing reducing sugars in the milk through Maillard reaction, binding of IgE of patients to glycated β-Lg was also measured. Loss of tertiary and secondary structures of β-Lg by heating above 75°C resulted in a decrease in its recognition by IgE, whose reactivities differ between the patients. The intensity of the decrease of IgE binding was also observed in case of intensively glycated β-Lg**.**

The expression in the yeast *Pichia pastoris* of a mutant bovine β-Lg, in which Cys121 was changed into Ser (Cys121Ser) was accomplished during this study. The Cys121Ser mutation blocks completely the irreversible aggregation induced by heat treatment. Binding of IgE from patients with cow milk allergy to native β-Lg, wild-type β-Lg and Cys121Ser mutant β-Lg was aslo measured by ELISA.

β-Lg has been found in complexes with lipids such as butyric and oleic acids and has a high affinity for a wide variety of compounds. Serotonin (5-hydroxytryptamine, 5-HT), an important compound found in animals and plants, has various functions, including the regulation of mood, appetite, sleep, muscle contraction, and some cognitive functions such as memory and learning. In the last part of this study, the interaction of serotonin and one of its derivatives, arachidonyl serotonin (AA-5HT), with β-Lg was investigated using circular dichroism (CD) and fluorescence intensity measurements. These two ligands interact with β-Lg forming equimolar complexes. The binding constant for the serotonin/β-Lg interaction is between 10^5 and 10^6 M^{-1}, while for the AA-5HT/β-Lg complex it is between 10^4 and 10^5 M^{-1} as determined by measurements of either protein or ligand fluorescence. The observed binding affinities were higher in hydroethanolic media (25 % EtOH). The interactions between serotonin/β-Lg and AA-5HT/β-Lg may compete with self-association (micellization) of both the ligand and the protein. According to far-and near-UV CD results, these ligands have no apparent influence on β-Lg secondary structure, however they partially destabilize its tertiary structure. Their binding by β-Lg may be one of the peripheral mechanisms of the regulation of the content of serotonin and its derivatives in the bowel of milk-fed animals.

Keywords : beta-lactoglobulin, interaction, allergy, modification, serotonin.

Résumé

La β-lactoglobuline (β-Lg) est une protéine la plus abondante du lactosérum du lait de vache et des autres mammifères. Elle appartient à la famille des lipocalines. Toutefois, elle est absente dans le lait de primates. C'est une protéine globulaire d'environ 18 kDa formant une structure en feuillet β (appelé calice). Chaque monomère contient deux ponts disulfures et une cystéine en position 121 (Cys121). Ce groupement thiol libre joue un rôle important dans l'agrégation de la β-Lg induite par la chaleur et, éventuellement, dans le maintien de sa stabilité de conformation. La fonction biologique de la β-Lg n'est pas claire, mais son rôle potentiel dans le transport des acides gras dans le tube digestif a été suggéré. La β-Lg est connue comme l'un des allergènes majeurs dans le lait.

Parmi les traitements technologiques le chauffage est le plus couramment appliqué au cours des procédés de transformation du lait. Malgré la fréquence d'utilisation du chauffage, les effets des traitements thermiques de la β-Lg sur sa dénaturation et sa reconnaissance par les IgE de patients ne sont pas décrits. La fixation des IgE de patients allergiques au lait de vache à la β-Lg native, et à la β-Lg chauffée dans la gamme de température de 65-95 °C, a été étudiée par ELISA. La β-Lg a été soumise aux modifications au cours du chauffage en présence des sucres réducteurs par la réaction de Maillard. La fixation des IgE de patients à la β-Lg glycosylée a également été mesurée. La perte de structures tertiaires et secondaires de β-Lg par chauffage au dessus de 75 °C a entraîné une diminution de sa reconnaissance par les IgE, dont les réactivités différent entre les patients. L'intensité de la baisse de la liaison IgE a également été observée dans le cas des β-Lg fortement glycosylées.

L'expression au niveau la levure *Pichia pastoris* d'un mutant de l'espèce bovine β-Lg, dans lequel le groupement Cys121 a été changé en Ser (Cys121Ser) a été réalisée au cours de cette étude. La mutation Cys121Ser bloque complètement l'agrégation irréversible induite par un traitement thermique. La fixation des IgE, obtenus de patients allergiques au lait de vache, avec la β-Lg native, β-Lg type sauvage et la β-Lg mutée Cys121Ser a également été étudiée par ELISA.

La β-Lg peut complexer des lipides tels que les acides butyrique et l'acide oléique ainsi que d'autres variété de ligands. La sérotonine (5-hydroxytryptamine, 5-HT) est un composé important qu'on trouve chez les animaux et les plantes. Elle est connue par ses diverses fonctions, y compris la régulation de l'humeur, l'appétit, le sommeil, la contraction musculaire, et certaines fonctions cognitives comme la mémoire et l'apprentissage.

Dans la dernière partie de cette étude, l'interaction de la sérotonine et la sérotonine arachidonyl (AA-5HT) avec β-Lg a été étudiée par dichroïsme circulaire (CD) et par fluorescence. Ces deux ligands interagissent avec β-Lg pour former des complexes équimolaires. La constante d'affinité du complexe sérotonine/β-Lg est compris entre 10^5 et 10^6 M^{-1}, tandis que celle du complexe AA-5HT/β-Lg est comprise entre 10^4 et 10^5 M^{-1}. Les constantes d'affinités observées sont plus élevés en milieu hydro-éthanolique (25 % EtOH). Selon les résultats des UV proche et lointain en CD, ces ligands n'ont aucune influence apparente sur la structure secondaire de la β-Lg, mais ils déstabilisent partiellement la structure tertiaire. La liaison avec la β-Lg pourrait expliquer l'un des mécanismes périphériques de la régulation de la sérotonine et de ses dérivés au niveau de l'intestin des animaux nourris au lait.

Mots-clés : beta-lactoglobulin, interaction, allergie, modification, serotonin.

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Abbreviations

AGE	Advanced Glycation End products
α-La	alpha-Lactalbumin
Ara	Arabinose
AA-5HT	Arachidonyl serotonin
β-Lg	beta-Lactoglobulin
BCA	Bicinchoninic Acid
BSA	Bovine Serum Albumin
CD	Circular Dichroism
C-ELISA	Colorimetric ELISA
cDNA	Complementary DNA
CMA	Cow Milk Allergy
CMP	Cow Milk Proteins
CMC	Critical Micellization Concentration
Cys121Ser	Cysteine 121 Serine
ELISA	Enzyme-Linked Immunosorbent Assay
EAACI	European Academy of Allergy and Clinical Immunology
F-ELISA	Fluorescent ELISA
Gal	galactose
Glu	glucose
HPLC	High Performance Liquid Chromatography
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IUIS	International Union of Immunological Societies
kDa	kilo Dalton
Lac	lactose
LCPL	Left-handed Circularly Polarised Light
LB	Luria-Broth
β-ME	2-mercaptoethanol
4-MUP	4-methylumbelliferyl phosphate
MW	Molecular Weight
DMMAC	N-dimethyl-2-mercaptoethylammonium chloride
NPN	Non-Protein Nitrogen
TCA	trichloroacetic acid
TEMED	N,N,N,′N′-tetramethylethylenediamine
OPD	Ortho-phenylene diamine
OPA	Ortho-phthaldialdehyde
PBS	Phosphate Buffered Saline
PVA/T	Polyvinyl Alcohol/Tween
PPs	Proteose peptones
RP	Reverse Phase
Rib	ribose
RCPL	Right-handed Circularly Polarised Light
RBP	Retinol Binding Protein
Rha	rhamnose

5-HT	serotonin
SEC	Size Exclusion Chromatography
SDS	Sodium Dodecyl Sulfate
Th1	T helper 1 cells
Th2	T helper 2 cells
WPC	Whey Protein Concentrate
WT	Wild Type
WHO	World Health Organization

Publications and Communications

I-Publications

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II-Communications

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Introduction

1. Introduction

The World Health Organization (WHO) considered that food allergies are the sixth problem of human health in the human pathologies categorization. In the recent years, food allergy patients are increased. For example, in USA approximately 4.5 to 8% and in France, about 4% of children less than two years old have symptoms of allergic reactions to food and they are suffering from food allergies.

The third food allergen among the top six food allergies is Cow Milk Allergy (CMA), concerning 9 % of the overall quantity of food allergy patients. Approximately 80 % of CMA patients outgrow their allergy when they are three years old and 20 % of patients have symptoms of allergic reactions to bovine milk at older ages. Milk allergy symptoms differ in all CMA patients and frequently consist of gastrointestinal symptoms, such as asthma, systemic anaphylaxis, rhinitis, dermatologic symptoms, nausea and diarrhea.

The relationships of dairy proteins with bovine milk allergic reactions have been intensively studied. β-lactoglobulin (β-Lg), α-lactalbumin (α-La) and a mixture of caseins dominates in casein fractions (α_{s1}-, α_{s2}-, β-and κ-casein) are the most abundant proteins in bovine milk. β-Lg is the main protein in bovine whey with 162 amino acid residues, which is classified in the lipocalin family (lipocalin proteins have the same secondary and tertiary structures and they can bind a large number of small hydrophobic ligands as their function). β-Lg has been also suggested as one of the most allergen proteins in the mammalian milk.

It is focalized in this research on the major whey protein β-lactoglobulin. Several linear epitopes present in the β-Lg recognized by IgE were identified but nothing is known about exact antigenic determinants of these epitopes neither about their recognition nor fate (after digestion). When one is analyzing entire protein (recognition of an epitope is dependent on secondary and tertiary structure) and the forms of

denatured β-Lg formed during technologic treatments (interactions with IgE, other dairy proteins and small important ligands change). Almost nothing is known about the recognition of epitopes masked or transformed during the technologic treatment or after the interactions with other components of food matrix and with small ligands. The recognition of epitopes will depend on the exposition of different proteic surfaces to the hydrolysis by the major digestive proteases like pepsin, trypsin, chymotrypsin and after on the recognition by the proteases from cellular lysosomes of lymphocytes B and Th.

Knowing that the some of the interactions can be also the causes of the dissimulation of potential sites of alimentary transformations of dairy proteins and β-Lg in particular, modifying their hydrolysis, the study of interactions of dairy globular protein β-Lg in respect with their antigenicity and allergenicity is much needed.

1.1. Milk in general

Milk is secreted by the females of all mammals, primarily to provide the full nutritional requirements of the newborn. The principal nutritional requirements are for energy (provided by lactose, lipids and proteins), essential amino acids and amino groups for the biosynthesis of non-essential amino acids (supplied by proteins), essential fatty acids, vitamins, inorganic elements and water. Because the nutritional requirements of the neonate depend on its maturity at birth, its growth rate and its energy requirements (depending mainly on environmental temperature), large inter-species differences has been found in the gross composition of milk [1, 2]. Milk also plays a number of physiological functions, mainly thanks to dairy proteins and peptides, including immunoglobulins, enzymes, enzyme inhibitors, growth factors, hormones and antibacterial agents.

Of the 4500 species of mammal, the milk of only about 180 species has been analyzed. The data for only about 50 species are considered to be reliable (i.e. a

sufficient number of samples, samples taken properly, representative sampling, and adequate coverage of the lactation period). Milk from the commercially important species such as cow, goat, sheep, buffalo, yak, horse and pig is quite well characterized. Human milk is also well characterized, as is the milk of experimental laboratory animals, especially rats and mice. Reviews on non-bovine milks include: general [3, 4], buffalo [5], goat [6], sheep [7], sheep and goats [8, 9], camel [10, 11], horse [12, 13], human [14-16], and sow [17].

In many parts of the world, the major components of the human food have been composed from milk and dairy products. Recorded milk production today is about 600×10^6 tones per year, about 85 % of which is bovine milk, 11 % is buffalo and about 2 % each is ovine, with small amounts produced from horses, goats, donkeys, camels, yaks and reindeer. Milk and dairy products are consumed throughout the world but are particularly important in Europe, the USA, Canada, Argentina, India, Australia and New Zealand. The contribution of milk and dairy products to dietary intake varies widely in different regions of the world, e.g. the kilocalories per day supplied by milk consumption range from 12 in China to 436 in Ireland. In UK, milk and dairy products supply ≈ 30 % of dietary protein consumed by young children, ≈ 27 % of dietary lipids and ≈ 65 % of calcium [18].

1.1.1. Composition of milk

Milk is extremely complex liquid containing a wide variety of molecular species. Its principal constituents are water, lipids, sugar (lactose) and proteins. In addition, it contains numerous minor constituents, mostly at trace levels, e.g. minerals, vitamins, hormones, enzymes and miscellaneous compounds. The chemistry of these compounds is generally similar across mammal species but in many cases their structure differs in detail, reflecting evolutionary changes. The concentration of the principal

constituents varies widely among animal species: lipids, 2-55 %; proteins, 1-20 %; lactose, 0-10 %, reflecting mainly the energy requirements (lipids and lactose) and growth rate (mainly proteins) of their neonates. The concentrations of the minor constituents also vary widely.

Within any mammal species, the composition of milk varies among individual animals, between breeds, with the stage of lactation, feed and health of the animal and with many other factors. The fat content of bovine milk shows large inter-breed differences and within any breed there is a wide range of fat and protein content for individual animals; similar differences occur in the milk of the sheep, goat and buffalo.

Reflecting mainly the nutritional and physiological requirements of the neonate, the composition of milk, and even the profile of constituents therein, changes markedly during lactation. The changes are most marked during the first few days post-partum, especially in the immunoglobulin fraction of proteins. In marsupials, the milk changes from a high-carbohydrate (mainly oligosaccharides) secretion to a high-fat secretion when the neonate begins to leave the pouch, a time that corresponds roughly to the birth of eutherians. The composition of milk remains relatively constant during mid-lactation but changes considerably in late lactation, reflecting the involution of the mammary gland tissue and the greater influx of blood constituents [1].

1.1.2. Cow milk proteins

The properties of milk and properties of the majority of dairy products are affected more by the proteins they contain than by any other constituent. The milk proteins also have many unique properties. Because of this and because of their technological importance, studies of milk proteins have been done intensively and consequently milk proteins are the best described food proteins.

Doing research on milk proteins started from the early nineteenth century. Pioneering work was reported by J. Berzelius in 1814, by H. Schubler in 1818 on the

physico-chemical status of milk proteins and by H. Braconnot in 1830 who published the first paper in which the word casein was used. A method for the preparation of protein from milk by acid precipitation was described in 1938 by J. G. Mulder, who coined the term "protein" The acid-precipitated protein was referred to as casein (some early authors called acid-precipitated milk protein caseinogen, which is converted by rennin to casein, that coagulates when Ca^{2+} exists; this situation is analogous to the conversion of fibrinogen in blood by thrombin to fibrin, which coagulates when Ca^{2+} is present). The term "casein" was universally adopted as the English word for the pH-4.6-insoluble proteins in milk about 70 years ago. The method of acid (isoelectric) precipitation of casein was refined by Hammarsten [19] and, consequently, isoelectric casein is frequently referred to as casein *nach* Hammarsten. A developed technique for separation of casein was published in 1918 by L. L. van Slyke and J. C. Baker.

α_{s1}-, α_{s2}-, β-and κ-caseins constitute whole casein in bovine milk with the percentage of about 38, 10, 35 and 12 %, respectively. However, SGE or PAGE indicate much greater heterogeneity due to small differences in one or more of the caseins, referred to as microheterogeneity, which arises from five factors.

The liquid whey remaining after isoelectric precipitation of casein from skim or whole milk is a dilute solution of proteins (whey or serum proteins; ≈ 0.7 % in bovine milk), lactose, organic and inorganic salts, vitamins and several constituents at trace amounts. The whey proteins were fractionated by J. Sebelein by salting-out with $MgSO_4$ in 1885, into soluble (albumin) and insoluble (globulin) fractions. According to McMeekin [20], in 1889 A.Wichmann crystallized a protein from the albumin fraction of whey by addition of $(NH_4)_2SO_4$ and acidification, a technique used to crystallize blood serum albumin and ovalbumin. Using the techniques available 100 years ago, the whey proteins were found to be similar to the corresponding fractions of blood proteins and were considered to have passed directly from blood to milk; consequently, the whey proteins attracted little research effort until the 1930s.

Besides caseins and whey proteins, milk also includes two other groups of proteinaceous materials—proteose peptones (PPs) and non-protein nitrogen (NPN), which were recognized in 1938 by S. J. Rowland who observed that, after heating milk fat at 95 °C for 10 min, the whey proteins co-precipitated with the caseins during acidification to pH 4.6. When the pH-4.6-soluble fraction of heated milk was added up to 12 % of trichloroacetic acid (TCA), some nitrogenous compounds precipitated and were designated " proteose peptone " nitrogenous compounds that remained soluble in 12 % TCA were designated NPN. A modified version of S. J. Rowland's scheme is still used to quantify the principal nitrogenous groups in milk.

Thus, by 1938, the complexity of the milk protein system had been described (i.e. caseins, lactalbumin (now known to consist mainly of α-lactalbumin, β-lactoglobulin and blood serum albumin), lactoglobulin, PPs and NPN, which represent approximately 78, 12, 5, 2 and 3 % respectively of the nitrogen in bovine milk). However, knowledge of the milk protein system was still rudimentary and vague at this stage. Knowledge on the chemistry of milk proteins has advanced steadily during the twentieth century, as can be followed through the progression of textbooks and reviews on dairy chemistry [21-23].

The principal, and many of the minor, milk proteins have been well characterized. The principal properties of the six milk-specific proteins are summarized in Table 1.1. A number of features warrant comment.

The six principal lactoproteins are small molecules, a feature that contributes to their stability. The primary structures of the principal lactoproteins of several species are known, as are the substitutions in the principal genetic variants.

The whey proteins are well structured, but the four caseins lack stable secondary structures; classical physical measurements indicate that the caseins are unstructured. However, theoretical considerations indicate that, rather than being unstructured; the caseins are very flexible molecules and have been referred to as rheomorphic [24-26].

The inability of the caseins to form stable structures is due mainly to their high content of the structure-breaking amino acid proline; β-casein is particularly rich in proline, with 35 of the 209 residues. The open, flexible structure of the caseins renders them very disposed to proteolysis, which facilitates them as a source of amino acids.

Table 1.1. Properties of the Principal Lactoproteins.

Properties	**Caseins**				**β -Lg**	**α -La**
	α_{s1}-	**α_{s2}-**	**β -**	**κ -**		
MW	23612	25228	23980	19005	18362	14174
Residues	199	207	209	169	162	123
Conc in milk (g/L)	12-15	3-4	9-11	2-4	3.0	0.7
Phosphate residues	8-9	10-13	4-5	1-2	0	0
½ Cystine	0	2	0	2	5	8
Sugars	0	0	0	Yes	0	0
Prolyl residues per molecule	17	10	35	20	8	2
A_{280}, 1% 1cm	10.1	11.1	4.6	9.6	9.4	20.1
Secondary structure	Low	Low	Low	Low	High	High
$H\Phi_{ave}$	4.89	4.64	5.58	5.12	5.03	4.68
pI	4.96	5.27	5.20	5.54	5.2	4.3
Partial specific volume (ml/g)	0.728	0.720	0.741	o.734	0.751	0.735

In contrast, the native whey proteins, especially β-lactoglobulin, are quite resistant to proteolysis, and at least some are excreted in the feces of infants. This feature is important because most of the whey proteins play a non-nutritional function in the intestine and, therefore, resistance to proteolysis is important.

The casein proteins are very hydrophobic molecules however, with the exception of β-casein, they are not exceptionally hydrophobic. The hydrophobic residues of bovine caseins are exposed and they have a high surface hydrophobicity and consequently they have not the stable secondary and tertiary structures.

One of the more notable features of the amino acid residues of the caseins is that the hydrophobic and hydrophilic residues are not distributed uniformly, thereby giving the caseins a distinctly amphipatic structure. This feature, coupled with their open flexible structure, gives the caseins good surface activity, and good foaming and emulsifying properties, making casein the functional protein of choice for many applications. Because of their hydrophobic sequences, the caseins have a propensity to yield bitter hydrolysates [1].

1.1.3. Whey proteins

Approximately 20 % of whole proteins in bovine milk are constituted from whey proteins (serum proteins). It was recognized early that acid whey proteins consist of two groups of proteins, lactoglobulins and immunoglobulins. Lactoglobulin proteins are salted out in 50 % saturated $(NH_4)_2SO_4$ or saturated $MgSO_4$ and contain mainly immunoglobulins. Lactalbumin proteins are soluble in 50 % saturated $(NH_4)_2SO_4$ or saturated $MgSO_4$. The lactalbumin proteins contain a couple of proteins, β-lactoglobulin and α-lactalbumin, and a number of minor proteins, involving blood serum albumin and lactoferrin, that have been isolated by various procedures and crystallized [22, 27].

There is considerable interest in the production of the major and many minor whey proteins on a commercial scale for nutritional, nutraceutical or functional applications. Several techniques are improved to produce several proteins from bovine whey in industrial-scales [28].

Approximately, there is 65 g dry matter, including 50 g lactose, 6 g protein, 6 g ash, 2 g non-protein nitrogen and 0.5 g fat, in 1 kilogram of the whey. β-lactoglobulin and α-lactalbumin compose 50 % and 25 % of whey proteins, respectively and the rest consists of several minor proteins involving immunoglobulins, proteose-peptone, bovine serum albumin and β-casein [29]. Table 1.2 shows the concentration of major proteins in bovine milk.

Table 1.2. Concentration of Major Proteins in Milk [30].

Proteins		Concentration (g/L)	Approximate % of Total Protein
Caseins	Alpha-casein	15-19	42
	Beta-casein	9-11	25
	Kappa-casein	3-4	9
	Gamma-casein	1-2	4
Whey proteins	Beta-lactoglobulin	2-4	9
	Alpha-lactalbumin	1-1.5	4
	Proteose peptones	0.6-1.8	4
	Serum albumin	0.1-0.4	1
	Immunoglobulins	0.6-1.0	2

Several productions can be achieved during the liquid whey processing in the industries including whey powder, concentrated whey, whey protein fractions, lactose, lactalbumin, and whey protein concentrates and isolates [1].

1.2. Bovine β-lactoglobulin

β-Lg is a bovine milk protein which contains 162 amino acid residues and its molecular weight is 18.3 kDa [31]. It belongs to lipocalin group (a mixture of the Greek *lipos*, "fat, grease" and *calyx*, "cup") [32, 33]. This proteins group has the ability to bind hydrophobic ligands inside their cavity. One could rationalize this effect and run an argument to the effect that β-Lg functions as a transport protein for hoydrophobic molecules like vitamin A and phospholipils [34].

Although β-Lg enjoys the richest whey protein in the milk of almost all mammals (about 10 % of total protein or about 50 % of whey protein), it does not exist in the milk of human beings, rodents or lagomorphs. α-lactalbumin is the most

aboundant whey protein in the human milk. Many researchers have investigated bovine β-Lg [1].

There exists ten well-known genetic variants of bovine β-Lg., however, the most abundant ones are β-Lg A and B [35] which differ only in two amino acid residues, Asp64Gly and Val118Ala respectively. It is believed that the quaternary structure of β-Lg differs among monomers, dimers or oligomers according to the medium such as temperature, pH, and ionic strength, yet note should be taken that the dimmer form is the dominant form in physiological conditions [36-38]. The various quaternary structures of β-Lg could contribute to a natural balance among hydrophobic, electrostatic and hydrogen-bond interactions [1, 39, 40].

1.2.1. Molecular structure of bovine β-Lg

β-Lg as other proteins in bovine milk, is produced under the control of prolactin hormone in the secreting epithelial cells of mammary gland [41]. Messenger RNA coding β-Lg is produced in the mammals and is translated into pre-β-Lg with 180 amino acid residues [42]. Out of these 180 amino acid residues in pre-β-Lg, the mature protein contains only 162 amino acids with the molecular weight of 18400 D and the rest constitutes a signal peptide with 18 amino acids [43]. Figure 1.1 displays the amino acid sequences of bovine β-Lg A acoording to PDB data [44]:

Since β-Lg is aboundant in bovine whey and it is relatively easy to purify and form crystals, it was an early object of X-ray diffraction studies of protein crystals. As far as the difficulties with analyzing β-Lg crystalography and the existence of multiple crystal forms are concerned, this was a very ambitious project [45]. Nonetheless, X-ray diffraction studies showed that β-Lg in its monomer form was roughly spherical with a block of electron density in a rod-like structure across one face.

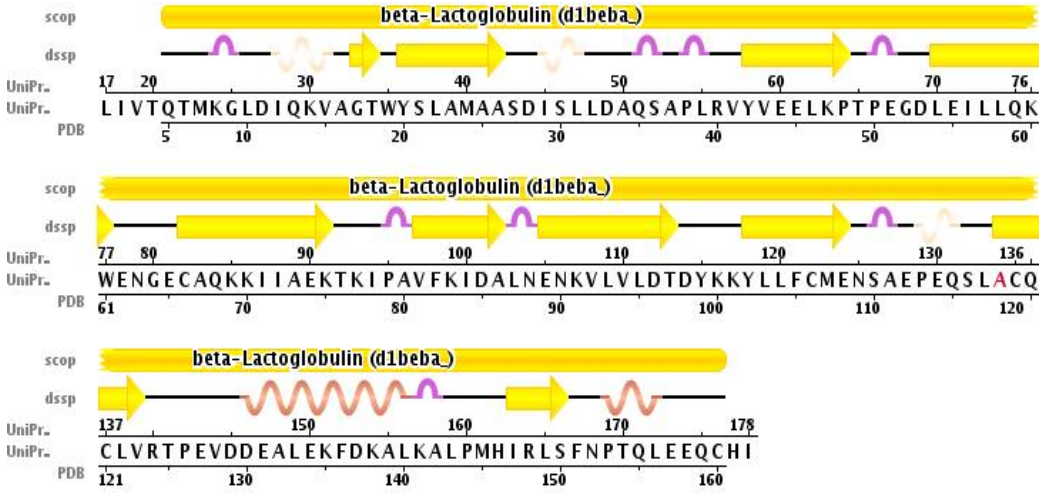


Figure 1.1. Amino acid residue details of bovine β-Lg A.

Another attempt in finding the structure was to approximate which amino acid residues may fold together to form the helices, strands and sheets. To do so, calculation using sequence data and structural probabilities were used [46]. It predicted that the secondary structure of β-Lg includes 50 % β-sheet, 15 % α-helix and 15–20 % reverses turn [46]. It should be mentioned that several residues which form the extended structures of the original β-Lg, had a natural tendency to form α-helical structures when the trifluoroethanol or amphiphiles were present [47-49].

In the late 1980s, the first β-Lg's structure with medium-resolution was determined [34]. The structural similarity between β-Lg and various kinds of proteins, including plasma retinol binding protein (RBP), has raised hypothesis for the function of bovine milk β-Lg. The recognizable eight-stranded β-barrel that form calyx, flanked by a three-turn α-helix was revealed in the higher resolution structures. At neutral pH, the greater part of the β-Lg dimer interface is its ninth strand [34, 50, 51]. The β-barrel is composed of two β-sheets, namely, strands A to D which make one sheet and strands E to H which forms the other. Two disulfide bonds are responsible for linking Cys66 on CD loop with Cys160 close to C-terminus and Cys106 on G strand with Cys119 on H

strand. the fifth cystein, Cys121, remains free the protein structure. In comparison to the loops which link BC, DE and FG strands, the loops which link AB, CD, EF and GH strands are longer and more flexible. Figure 1.2 indicates the structure of β-Lg in its mono- and dimmeric form.

The A and B variants are very similar in structures. , The Asp64Gly substitution leads to the adoption of different conformations on CD loop, though [52]. The Val118Ala substitution does not bring about any recognizable modification to the structures, however the isopropyl group in Val residue is bigger than methyl group in Ala residue so β-Lg variant B is less well packed and has less hydrophobicity than A variant. These lead to lower staibility for B variants during thermal denaturation [52].

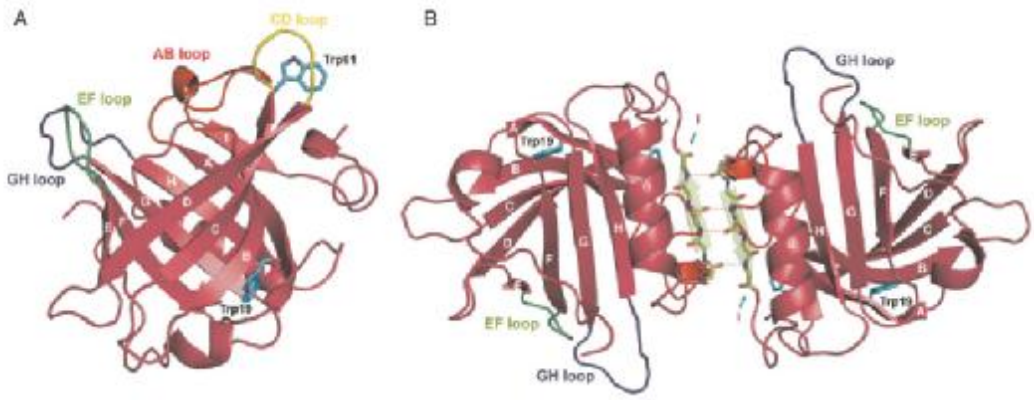


Figure 1.2. (A) Cartoon representation of bovine β-lactoglobulin unliganded form. Eight antiparallel β-strands (A–H) form central calyx surrounded by flexible loops: AB, CD, EF and GH. (B) Cartoon representation of β-lactoglobulin dimer. Monomers are associated by hydrogen bonds formed between residues 146–150.

In the late 1950s, very careful thermodynamic and titrimetric measurements [53, 54] showed the presence of a carboxylate residue which enjoyed a different pK_a value (about 7.3). This hangs on a pH-dependent conformational change which corresponds to the previous measurements of pH-dependent sedimentation coefficients [55] and

particular optical rotation data [56]. Much later, X-ray structure analysis [57] at pH values higher and lower the so-called Tanford transition showed that, at pH 6.2, the loop EF is close to the top of the barrel and burries Glu89 (the carboxylic acid with the anomalous pK_a) into the calyx. The EF loop is far from the barrel at pH 8.1 to the effect that the previously buried glutamic acid appears in the surface of the protein in the carboxylate form [57].

The former crystallography studies on the bovine β-Lg structure in the presence of retinol indicated that retinol binding place is in the surface of β-Lg [58], apparently later a body of fluorescence data confirmed it [59-61]. However, subsequent structural analysis has determined that all retinoid groups such as vitamin A, fatty acids and cholesterol such as vitamin D bind internally to the hydrophobic calyx [62]. The X-ray crystallographic results have been confirmed by Circular dichroism (CD) and NMR experiments. Ligand binding is elaborated comprehensively below, since it defines the potential physiological function of β-Lg in addition to its stability. Due to the ability of this protein to bind different ligand, it aroused considerable interest in the subject of protein–ligand interactions. Until now, almost all studies proved that the binding of fatty acids or retinoid species is inside the calyx. It is not beyond logic that nearly all ligands can bind inside the calyx of β-Lg at pH ≈ 7 except some bulky ligands (like serotonin and Vitamin D_2) [1].

As the ligand-binding modes and Tanford transition were clarified by high-resolution X-ray crystallography [57, 63], NMR studies launched into the to investgation of β-Lg structure in acidic pH solutions where β-Lg is in its monomeric form [64-67]. Although these NMR research proved that at pHs lower than pH 2 the tertiary structure of β-Lg continues to exist and have provided a lot of information about protein dynamics and the stability of its structure, standard X-ray crystallographic techniques can not provide these valuable information [1].

1.2.2. Structure of bovine β-Lg in aqueous solution

In order to investgate protein structures in solution, NMR spectroscopy can be utilized [68]. The method is suitable for proteins in monomeric form and with molecular weights below 25 kDa and generally needs recombinant single labled (^{15}N) or double labled ($^{15}N/^{13}C$) material for molecules with molecular weights more than about 8 kDa. Thus, almost all NMR research on bovine β-Lg have been done where the molecule is monomeric mean at pHs between 2 and 3. The recognizable eight-stranded β-barrel was revealed in early studies using recombinant wild type β-Lg B [65]. Nevertheless, the complete structure of β-Lg (variant A) [69, 70] needed the use of isotopically labeled recombinant β-Lg [71, 72]. As two groups from Tokyo and Edinburgh determined the full structure by NMR independently and near simultaneously, therefore, this has presented fruitful comparisons [73].

Kuwata *et al*. [69] and Uhrinova *et al*. [70] demonstrated the β-Lg structure in solution. The results were similar with the results of X-ray crystallography at pH 6.2 that were published earlier. It should be noted that for providing practical NMR spectra the drastically lower pH is required. At this pH the loop EF is clearly close to the open end of the β-barrel. In the X-ray structures, the side chain of the Glu89 residue "latch" is buried. The greatest difference compared to the Z lattice X-ray structure at natural pH [57] is that the three-turn α-helix is different in position regarding the β-barrel. This is probably attributable to the fact that as pH increases, the positive charge on the surface of protein increases as well [70]. At lower pH, there is a movement of about 3.5 Å on the conformation of the loop AB, which is substantial to disrupt the dimer interface.

At the N-terminal and C-terminal of β-Lg, more differences were diagnosed that could be explained in terms of the use of recombinant protein with a non-native N-terminal for the NMR structure studies and potential crystal-packing influences at the C-terminal for the X-ray structure studies. In the electron density maps on the

crystallographic studies, some amino acid residues in the C-terminal part of β-Lg (residues about 152 to 162) could not be observed [1].

1.2.3. Purification of β-Lg

Many methods have been developed for the purification of bovine β-Lg from bovine milk. For the first time, Palmer (1934) launched into the purification methods of β-Lg from bovine milk [74]. Many other purification methods have their roots in his method. Firstly, raw milk is Centrifuged to remove fats and secondly the solution is acidified to precitipate caseins from milk. Finally the remaining solution is referred to as whey. There are different methods to isolate bovine β-Lg from whey. These methods of β-Lg isolation have been shown in Table 1.3. Also some purification methods have been developed on the basis of the stability of β-Lg against proteolysis. Pepsin enzyme can hydrolyze all bovine whey protein except β-Lg, so that it can be be used for this purification method [75]. Precipitations followed by ion exchange are the most appropriate methods for large scaled purifications of bovine β-Lg [76, 77].

1.2.4. Stability of β-Lg in the digestive tract

The acidic conditions of the stomach have little effect on β-Lg other than to promote a reversible dissociation [78]. Additionally, Pepsin enzyme is not able to hydrolyze native β-Lg in acidic environment [79, 80]. The simulated gastric fluids studies have demonstrated the stability of β-Lg in the acidic conditions of the stomach [79, 81]. The existence of β-Lg in the small intestine is because of its resistance to degradation in the stomach conditions. However, finally the bulk of β-Lg digestion takes place in the small intestine [82].

Table 1.3. Purification Methods for β-Lg from Bovine Milk.

Method	Fat removal	Casein removal	Fractionation of the whey proteins	Final purification
1	---	Acid precipitation at pH 5.8	Na_2SO_4 precipitation, 30 ˚C	Dialysis Crystalization
2	Na_2SO_4 precipitation, 40 ˚C	Na_2SO_4 precipitation, 40 ˚C	Acid precipitation at pH 2.0	Dialysis Crystalization
3	$(NH_4)_2SO_4$ precipitation, 20 ˚C	$(NH_4)_2SO_4$ precipitation, 20 ˚C	Acid precipitation at pH 2.0	Dialysis Crystalization
4	Centrifugation	$CaCl_2$ precipitation at pH 6.6	Dialysis Anion exchange	Gel filtration Crystalization
5	Centrifugation	Acid precipitation at pH 4.6 Filtration, Dialysis	Gel filtration	
6		Acid precipitation at pH 4.6 Centrifugation	N-retinyl-celite affinity chromatography	
7		Acid precipitation at pH 4.6 Centrifugation Filtration	Bio affinity chromatography	
8	Centrifugation	Acid precipitation at pH 4.4-4.5	Base precipitation at pH 7.2 Centrifugation Anion exchange	Gel filtration

Although it seems that ingested native β-Lg in the small intestin of mothers could be to some degree secreted in its native form to their breast milk, there are contradictory results for the existence of bovine β-Lg in the breast milk of human beings. some studies have proved that β-Lg exists in the milk of mothers [83, 84]. Yet, in another study conducted by Conti et al. [85] the reliability of these results were questioned to the effect that they showed the existence of cross reactivity of bovine β-Lg with the C-terminus fragment of human β-casein, questioning immunological procedure of β-Lg recognition. Despite some healthy mothers' diet was rich in bovine

milk and dairy products, the peptides of bovine β-Lg with a molecular mass more than 10000 Da were never detected [1, 85].

Although native β-Lg highly resists peptic hydrolysis, heat denaturated β-Lg is not resistant. At temperatures above 80 °C, the structure of β-Lg changes irreversibly and pepsin digests it more easily [82, 86]. Therefore, it is possible that the greater part of heat denaturated β-Lg is digested in the acidic conditions of stomach, while *in vivo* research demonstrate that in the small intestine, native β-Lg can be found intact [87].

1.2.5. Effect of temperature on bovine β-Lg

Heat induced characteristics of different variants of bovine β-Lg is important in commercial aspects, by two reasons, firstly, because they have an important role to foul processing equipment and secondly, this fact that the heat induced aggregation of β-Lg can convey the functional qualities of dairy products. Therefore, heat induced characteristics of bovine β-Lg has been studied extensively by a lot of researchers using a lot of techniques.

Differential scanning calorimetry (DSC) shows the midpoint of the thermal unfolding transitions at neutral pH is ≈ 70 °C, [88], when the protein dimer dissociates and the constituent monomer β-Lg molecules starts to unfold. The free cysteine, Cys121 that is stated at the carboxylate end of strand H, and some hydrophobic amino acid residues that lead to the formation of aggregated protein by both hydrophobic and covalent intermolecular interactions, are the most important causes of β-Lg unfolding [89, 90]. A mixture of disulfide-bonded polymeric species can be formed by exchanging disulfide bonds during heat treatments.[91].

It has been showed that a mutant β-Lg with an additional cysteine can form another disulfide bond with free Cys121 (a third disulfide bond). It have been determined that blocking free thiol can postpone heat denaturation (by 8-10 °C) and therefore heat-induced aggregation of β-Lg [92]. In mixtures of α-lactalbumin (α-La),

bovine serum albumin (BSA) and bovine β-Lg, or bovine β-Lg and one or other of α-La and BSA at high temperatures and pH 6.8, hetero and homo polymeric disulfide bonded can be formed [93]. BSA and β-Lg can catalyse the creation of α-La–α-La disulfide connection (there is no free cysteine in α-La) [93]. At low pH, when the protein is monomeric, at temperatures below 70 °C denaturation is largely reversible [94]. The large aggregates of protein can be formed during heating more than 70 °C temperatures. These aggregates species are mainly formed by non-covalent bonds [95].

The exact denaturation procedure is a very complex process and a lot of factors including pH, genetic variant, ionic environment, protein concentration and presence or absence of ligands have an effect on it. Both adding calyx-bound ligands and lowering the pH [96-98], increase the resistance of the protein to heat induced denaturation. It has been showed that at pH 6.7, the stability of the genetic variant decreases in the order of C > A > B and β-Lg A has the smallest cooperative unfolding transition [99]. The resistance of protein to heat denaturation depends strongly on its concentration at pH 6.7-8 and the concentrations up and about. The protein is more susceptible for unfolding at concentration of about 1.4 mM [89]. At high protein concentration (≈ 6 mM), it is possible that, the protein loos directly its tertiary structure from its native dimer form [89].

There is some evidence that there is more than one step in heat induced unfolding. As a result of antibody binding affinities, Kaminogawa *et al*. showed that at least there should be two steps for heat induced denaturation of β-Lg A [100]. The first step starts by changing in protein conformation close to the N-terminal and subsequently the second step begins by changing some part of the three-turn α-helix. Additionally, Casal *et al*. have used Fourier-transform infrared (FT-IR) measurements for β-Lg B in 50 mM phosphate buffer, pH 7, and demonstrated a decrease of helical content near the beginning of unforlding [101]. Circular dichroism and FT-IR measurements have been applied by Qi *et al*. to propose that β-Lg A in temperatures

higher than 65 °C and 30-60 mM NaCl,at pH 6.5, creates a molten globule protein that its β structure has been reduced [102]. NMR studies of β-Lg A at pH 2-3, by exchanging hydrogen atom of the backbone amide protons with deuterium (H/D), have determined a stable calyx consisting of the strands H and FG. This stable core could be possibly stabilized with the disulfide bond Cys106-Cys119 flanked by F and G strands [94, 103]. A considerable secondary structure has been reported at high temperatures even at temperature 90 °C [1, 101-104].

1.2.6. Effect of pressure on bovine β-Lg

High-pressure induced changes of food decreases processing damages and because of growing consumer need for products which have been exposed to at least processing damage, this technique has commercial importance. Pressure treatment has the potential to be used in producing more functional and organoleptic dairy products in comparison with products that formed by mere thermal treatments.

Among the main whey proteins, β-Lg has the slightest resistance to pressure treatments [105]. Probably this is because of existence of the β-barrel which has big solvent-exposed hydrophobic pocket, and the fewer disulfide bonds in β-Lg (two disulfide bonds in comparison to four disulfide bonds in α-La, for example) that causes inefficient packing. At pressures valued below 10 MPa, it is possible that the calyx contracts, therefore, the molar volume of bovine β-Lg is reduced. Numerious investigations have indicated that β-Lg in exposed to the pressure becomes more susceptible to enzymatic cleavage, probably due to modifications in its structure caused by pressure. It has been demonstrated that free cysteine can be affected at between 50 and 100 MPa [106].

The pressures excess of ≈ 300 MPa, can irreversibly modify the tertiary and quaternary structure of β-Lg at natural pH. At neutral pH and pressures about 900 MPa, the results of fluorescence and circular dichroism spectroscopy of β-Lg showed that

pressure can induce aggregation of β-Lg monomers, and can irreversibly modify the tertiary structure of β-Lg. Still, of the latest studies of hydrolysis of protein in the presence of trypsine indicate that, at pressures as low as 150 MPa, no permanent effect on the conformation of β-Lg A was found but at pressures as high as 300 MPa, D and G strands of β-barrel in the protein are separated and forms disulfide bridged aggregates [107].

Bovine β-Lg in the presence of either α-La or BSA exposed to high pressures at pH 6.6. It has been found that aggregation happened only between β-Lg itself by the formation of intermolecular disulfide bonds. Yet β-Lg–α-La or β-Lg–BSA disulfide-bonded species were not observed [105], in contrast with heated mixtures where these species are detected [93].

To make the protein's primary sequence with the pressure-induced conformational changes correlated, NMR amide H/D exchange studies of β-Lgs variant A and B at neutral pH were done by Belloque *et al*. while the solutions were subject to pressures above 400 MPa [108]. At pressures as high as 100 MPa, small H/D exchange was observed which in turn suggest that any conformational modifications taken place is not as much as the ones at pressurs of up to 400 MPa. This effect could be explained in terms of less exposure of the most of amide protons to the solvent in comparison with their exposure in the native conformation at pressurs of up to 400 MPa. At 200 MPa and above a significant enhancement in the amount of H/D exchange indicated increased flexibility in the protein conformation. On the other hand the spectra of control samples obtained in H_2O compared to D_2O before and after pressure treatment were similar. This result demonstrated that generally pressure-induced structural changes were not irreversible at pressures higher than 400 MPa. It has been suggested that firstly the structural changes of β-Lg A is more susceptible than β-Lg B variant during pressure treatments and secondly in the β-barrel of β-Lg, the conformation of strands F, G and H

are the most stable changes in pressure. These results confirmed heat-induced structural changes results [94, 103].

The results of small-angle X-ray scattering and FT-IR studies proposed that, the unfolded state consists considerable secondary structure even at 1 GPa. Combination of pressurization and heat treatment results has indicated that there is a minor effect on the sensitivity of β-Lg to pressures about 200 MPa by changing the temperature range from 5 to 37 $^{\circ}$C [109]. However, circular dichroism spectroscopy results indicated that a molten globule protein with a structure of α-helix could be formed by applying both of pressure and moderate temperature at 294 MPa/62 $^{\circ}$C and 600 MPa/50 $^{\circ}$C [110].

Therefore, during investigation on the pressure-induced changes of the protein stability and conformation, it is necessary to consider that the probability of increasing temperature due to quick pressure treatment on the sample requires to be taken into consideration.

Enzymatic proteolysis shows that β-Lg conformation is more stable to pressure treatments at acidic pH than at neutral and basic pH [111]. Yet, conformational studies of monomeric β-Lg, at acidic pH exposed to pressures higher than 200 MPa, by NMR techniques indicated that there is a forming of two intermediates in an unfolded situation through an independently unfolding of the two β-sheets, which persumes to hold still considerable amounts of secondary structure in these two intermediates [112].

A three-step mechanism has been proposed for denaturation of β-Lg at neutral pH and ambient temperature, which includes the above observations. Pressure of 50 MPa partially collapses the hydrophobic cavity (which can reduce ligand binding capacity) together with exposure of Cys121. Pressure near to 200 MPa leads to the additional reversible interruption of the calyx structure that is accociated with decline in volume of the molecule. Pressures more than 200 MPa lead to aggregation reactions irreversibly including exchanges of disulfide-bonded [1, 113].

1.2.7. Effect of chemical denaturants on bovine β-Lg

Frequently, chemical denaturants are used to study proteins folding-unfolding processes and to distinguish mechanisms and transition situation of these phenomenons. Usually, denaturants mainly alcohols, 2,2,2-trifluoroethanol (TFE), guanidinium chloride (GdmCl) and urea are used.

The quantity of α-helix in the secondary structure of native β-Lg has been calculated in theoretical calculations considerably higher than is actually appeared [46]. The native conformation of β-Lg is due to the competition between β-sheet forming long-range interactions and α-helix-favoring local interactions. However, this balance can be disturbed by addition of alcohols such as TFE and consequently decreases of the hydrophobic interactions and increases of the helical affinity in the protein sequence have been observed. Tanford *et al*. used optical rotary dispersion measurements and demonstrated that the α-helix amount of bovine β-Lg is increased during adding alcohols such as ethanol, 2-chloroethanol, and 1-propanol to the protein solution [114].

Current experiments have a propensity to favor utilizing TFE, where the transition of β-sheet to α-helix in β-Lg is highly cooperative, occurring over the range of about 15–20 % v/v of co-solvent. The great amount of α-helix structure, that is named TFE-state, have been observed in half of the molecule in the N-terminus part [66]. It has been demonstrated, in magnetic relaxation dispersion experiments of the solvent nucleus, that this situation is an open, solvent-permeated structure (on the contrary of the molten globule in the collapsed situation) and that its structure is accompanied by an increasingly swelling of the protein through enhancing the concentration of TFE. Using high concentrations of protein (8 % v/w) and TFE (50 % v/v) can result in gel formation and fibrillar aggregation of bovine β-Lg at both neutral and acid pH [115].

The urea ability to induce protein unfolding probably is because of two resons, firstly, formation of hydrogen-bonds with the protein amino acid residues and secondly, decreases in the amount of the hydrophobic forces. Thus, unlike TFE, the hydrophobic

effect and the helical propensity together are decreased. A two-state transition has been firstly suggested for urea-induced unfolding of bovine whey β-Lg at pH 2. Subsequently, an investigation on bovine β-Lg B by NMR H/D exchange experiments at acidic pH also showed a two-state mechanism for urea-induced unfolding of protein between folded and unfolded state of β-Lg through a cooperative unfolding of the C-terminal of the major α–helix and β-barrel. However, recently Dar *et al*. have indicated that urea also causes unfolding via an intermediate, albeit with intermediate structural properties between those of the native and unfolded states. Sodium dodecyl sulfate (SDS), anionic amphiphiles or palmitate, can bind inside the hydrophobic cavity of β-Lg, as a result it has been showed that in the presence of these ligands β-Lg is resistance to urea-induced unfolding [116].

Frequently, in studies of protein stability, GdmCl is substituted to urea. In most researches on the stability of protein in the presence of chemical denaturants at acidic or neutral pH, GdmCl will not be stable and it will be entirely dissociated. At concentrations less than 1 M of GdmCl, the electrostatic repulsion between amino acid residues of the protein with positive charge has been screened by chloride ions. Consequently, in comparison with neutral urea molecule, GdmCl has further electrostatic interactions which can stabilize or destabilize protein structure and noticeably it depends on the concentration of GdmCl [117].

Circular dichroism, fluorescence and UV differential absorption spectroscopy have been used by D'Alfonso *et al*. to monitor the effect of some chemical denaturants including GdmCl and urea on the conformation of bovine β-Lg B between pH 2 and 8. Discrepancies between unfolding free energies obtained using urea and GdmCl should be idicated if it was assumed that only an intermediate transition exist for GdmCl-induced unfolding of protein. This intermadiate state has the same secondary structure with the native β-Lg, except more inflexibility that it has in the adjacent of the Trp amino acid residues. This depends on the screening of electrostatic repulsions among

charged amino acid residues [118]. It is reported that the intermediate-unfolded state of bovine β-Lg A induced by GdmCl at pH 2 has more α–helical structure [116].

It has been showed that porcine β-Lg also can unfold in the presence of GdmCl via an intermediate transition state. The bovine β-Lg was more stable than the porcine one and it was more α-helix structure in the intermediate state of porcine β-Lg than the bovine one. The majority of the hydrophobic–hydrophobic interactions have been preserved between the hydrophobic cavity of the native state of porcine β-Lg and bovine β-Lg. Nonetheless, four pairwise interactions have been lost in the porcine β-Lg in comparison with bovine β-Lg because the Phe105 amino acid residue in bovine β-Lg has been changed to Leu in porcine β-Lg. This result shows that this aromatic side chain can has a very significant function in the enhanced stability of the bovine β-Lg in comparison with porcine β-Lg [118]. It should be mentioned that Phe105 side chain has predominantly resistance to the exchange of H/D during heat denaturation of β-Lg [1, 94].

1.2.8. Characterization of heat-induced changes to proteins by polyacrylamide gel electrophoresis (PAGE)

It is evident that less commonly used polyacrylamide gel electrophoresis (PAGE) separation in different environments (e.g. sodium dodecyl sulfate [SDS] solution, in the presence or absence of a disulfide bond reducing agent) allows the separation and differentiation of the various polypeptide chains. SDS-PAGE dissociates the processing induced non-covalent bonds and leaves the covalent bonds intact, whereas native-or alkaline-PAGE separates the whey proteins with hydrophobic and disulfide bonds intact. Therefore, it is possible to show whether the induced β-Lg aggregates are hydrophobically linked aggregates, reducible disulfide cross-linked aggregates etc. in heated solutions of pure β-Lg or commercial whey protein concentrated (WPC) solutions [93]. The same techniques have been used successfully to

examine the pressure-induced aggregations of various dairy proteins, revealing the subtle and not-so-subtle differences [105, 119].

When heat-treated or pressure-treated samples were analyzed using various PAGE techniques, many changes in the PAGE patterns of these samples were noted [93, 97, 105, 113, 119, 120]. It became necessary to identify each of these changes and aggregates observed in different PAGE environments. For simplification, some specific nomenclature had to be formulated, e.g. “native monomer”, “native dimer”, “SDS monomer”, “SDS dimer”, etc. (see Table 1.4).

Two-dimensional (2D) PAGE (2D native-and then SDS-PAGE, and 2D SDS and then reduced SDS-PAGE) can be applied to further characterize various intermediate species of protein aggregates and the high-molecular-weight aggregates produced as a consequence of heat or pressure induced denaturation. A combination of two PAGE techniques can be an even more powerful technique; it can show the composition of the protein complexes (aggregates) and/or the types of bonds by which the protein aggregates are held together in the heat-treated [93, 120] or pressure-treated [105, 119] samples.

In the 2D native and then SDS-PAGE procedure, a sample containing protein complexes is analyzed using one-dimensional (1D) PAGE in a Tris HCl buffer at pH 8.7, called alkaline-or native-PAGE. The proteins or protein complexes separated on native-PAGE consist of all complexes including non-covalent (such as hydrophobic) and covalent (such as disulfide bonds)-bonded aggregates. The 1D native-PAGE gel strip with its separated protein bands is transferred to SDS-PAGE in the second dimension (i.e. transferred into a dissociating environment). Once the proteins in the strip are partially equilibrated with the SDS to form SDS-protein complexes, they are electrophoresed into a new (SDS) environment in a second dimension SDS-PAGE. SDS-PAGE in the second dimension dissociates non-covalent bonds (mainly

hydrophobic aggregates) from the non-reduced native-PAGE gel strip, whereas the covalent bonds (disulfide bonds) remain unaffected.

Table 1.4. Nomenclature of Different Forms of Proteins and Protein Aggregates Analyzed Using Various Electrophoretic Techniques.

Nomenclature	Applicable to	Description
Native	All	Has all the characteristics of native proteins
Native dimer	β-Lg	Normal state of native β-Lg between pH 4 and 7 (at 30 °C and 0.05 M NaCl)
Native-like	All	Behaves like native proteins on alkaline (native)-PAGE
Non-native momomer	All	Behaves like native proteins on SDS-PAGE, but not on alkaline-PAGE
SDS monomer	All	Has mobility on SDS-PAGE close to that expected from molecular monomer
SDS dimmer, trimer, etc.	All	Has mobility on SDS-PAGE close to that expected from molecular dimmer, trimer, etc.
Apo	α-La	Deficient in calcium (or other divalent cation)
Holo	α-La	Same as native α-La

The other type of 2D PAGE is SDS-and then reduced SDS-PAGE. The initial proteins mixture can be separated by this kind of 2D PAGE technique using SDS-PAGE with all the native and process-induced disulfide bonds. The separated proteins and protein aggregates are treated with a disulfide bond reducing agent, 2-mercaptothanol (2-ME), while still in the gel strip. It is possible to use this gel strip as a control (the sample source) for analysis of the second dimension PAGE. The proteins as the reduced SDS-protein species can be separated by the SDS-PAGE in the second dimension. Therefore the components of each of the different disulfide-bonded aggregates can be identified.

1.2.9. Ligand binding to β-Lg

A great number of hydrophobic molecules have an affinity to interact with β-Lg (see Sawyer *et al*. [121]). The binding site of these hydrophobic ligands remains to be controversial [59-61]. Nonetheless, nowdays, the results of NMR studies of β-Lg including chemical shift changes and relaxation times of protein amino acids have been utilized to follow the location of bound ligands on the protein.

Another technique, CD, presents dependable proof interacting with protein. In this technique, an achiral chromophore "lights up" in the CD spectrum and changes of CD signals aoccured during the interaction of protein with ligands. Circular Dichroism was used for bovine β-Lg to indicate clearly the competitiveness properties of retinol and fatty-acid binding to β-Lg. The interaction of these and such chirally active ligands as retinoic acid and *trans*-parinaric acid, was investigated within different pHs and within non-chromophoric fatty-acid ligands to achieve Tanford transition's parameters. More lately, this technique has been employed to investigate the interaction of these and other ligands, containing piperine, to β-Lg [122, 123].

A further technique, fluorescence spectroscopy, has also been widely used to study ligand binding, where, classically, the changes in the fluorescence intensity of tryptophan residues are scrutinized. The fluorescence intensity changes can be either positive or negative during the binding process of ligands with the protein. The bovine β-Lg has two tryptophans, namely, Trp19 and Trp61. the former is hidden inside the calyx and is in the beginning part of A strand (see Figure 1.3 b), while the other latter is mostly uncovered on the surface of the protein and is part of the loop CD. The fluorescence intensity of these Trps is susceptible to small changes in the locations of near quenchers such as close charged Arg124 residue near to Trp19 and the Cys66-Cys160 disulfide bridge near to Trp 61. The results of X-ray crystollagraphy, NMR and CD spectroscopy show that interpreting the fluorescence measurments as proof of ligand binding is not absolute.

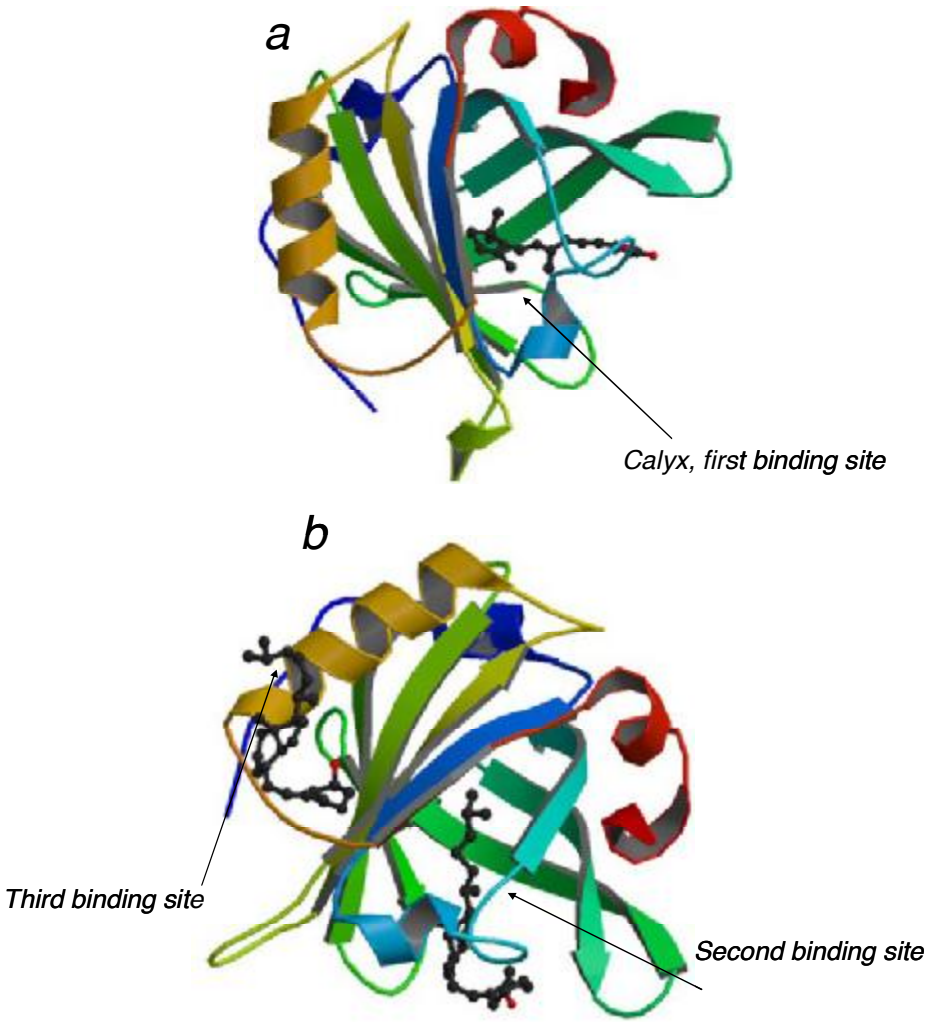


Figure 1.3. Ligand-binding sites on β-Lg as obtained from NMR experiments results of the binding of small hydrophobic molecules to β-Lg at low pH. (a) first binding site iside the calyx showing the primary binding site of fatty acids flavor components (b) Second binding site for flavor components at pH 2 that adjacent to the three-turn helix and strand G and the third binding site at N-terminal ends of A, B, C and D strands, and the C-terminal strand.

It has been suggested that the conformational changes of the loop EF is pH-dependent, caused by the protonation of Glu89 that is general in all variants of β–Lg, using a the results of NMR measurements, docking simulations and electrostatic calculations by Ragona *et al*. [124]. They have also found the ligand binding site of

palmitic acid by the opening of EF loop. They have determined in the former studies by using ^{13}C-labelled palmitic acid that as pH increases, the conformational changes of the ligand takes place.

Recently, it has been done an investigation on the binding of palmitic acid with Ala34Cys mutant β-Lg using NMR technique. They have found a very strong interaction between protein and ligand inside the calyx, but they have demonstrated that the binding site is more dynamic at the open end of the calyx [125]. The results were in a good agreement with the results obtained by Ragona *et al*. [124] and the X-ray crystallography results on the interaction of fatty acids to β-Lg [57, 126], which indicated that the hydrophobic tail was substantially more well ordered than carboxylate head group. The studies of the mutant β-Lg, Ala34Cys, showed that the plasticity of the D strand and the loops EF and GH let β-Lg to bind a large variety of ligands [125]. X-ray crystallography and NMR results illustrated that during the binding of ligands with β-Lg, the interior lipocalin structure remains unchange except some modifications in the conformations of Phe105 and Met107 amino acid residues.

It has been clearly demonstrated by X-ray crystallographic data that the binding site of both fatty acids and retinol is inside the calyx [57, 121, 126]. High ionic strength conditions have been used to study ligand biding to β-Lg by X-ray crystallographic experiments, while NMR and induced CD experiments, for studing ligand biding to β-Lg, have been done under low ionic strength conditions. The agreement of X-ray crystallographic results with NMR and induced CD results under different ionic strength showed that the X-ray crystallographic results are independent on ionic strength. It has been demonstrated that the conformation of β-Lg, particularly the hydrophobic calyx, in near-zero ionic strength and at the pI of the β-Lg (≈5.3) conditions is preserved. This result can be evidence for ligand binding site of β-Lg that is located inside the hydrophobic cavity at ≈ pH 7 [127].

Though investigation on the binding of some ligands such as palmitic acid to β-Lg showed that at pH 2 these ligands can not bind protein, NMR studies of the binding of the flavor compounds including γ-decalactone and β-ionone at acidic pH demonstrated the binding of these ligands to β-Lg [128]. Hence, three binding sites have been suggested for β-Lg.

1) The most important binding site in the interior of the hydrophobic cavity (calyx).

2) The second binding site including perturbations of Trp19, Tyr20, Tyr42, Glu44, Gln59, Gln68, Leu156, Glu157, Glu158 and His161 amino acid residues.

3) The third binding site including Tyr102, Leu104 and Asp129 amino acid residues.

Figure 1.3 shows three binding sites on β-Lg. Although it was initially considered that fatty acids can not bind to porcine β-Lg, it was recently demonstrated by NMR studies that in the presence of fatty acids, the pH for 50 % uptake of ligand has shifted from ≈ 5.8 for bovine β-Lg to 9.7 for porcine β-Lg. This result is in relation with structural changes of the EF loop [1, 124].

1.2.9.1. Drug delivery of β-Lg

A wide variety of ligands including fatty acids, retinol, aromatic compounds and phospholipids can bind to β-Lg [57, 63, 129, 130]. Binding to β-Lg provides protection, from heat, oxidation and irradiation degradations for some hydrophobic ligands such as retinol and β-carotene. So, it seems that β-Lg can be used as a adaptable transporter of a wide variety of hydrophobic ligands in applications of controlled delivery essentially in drug delivery.

The binding constants of β-Lg with different compounds vary extensively, from as small as 1.5×10^2 M^{-1} for 2-heptanone to as high as 6.8×10^5 M^{-1} for palmitate and 5×10^7 M^{-1} for retinol [121].

It may take place various interactions including hydrogen bonding, hydrophobic and electrostatic interactions, during the interaction of ligands with β-Lg. Additionally these interactions are the energetic forces for stabilization and for the conformational transitions of proteins. Consequently, amphiphilic and hydrophobic ligands may possibly have an effect on the conformation of the protein. Anionic phospholipids including dimyristoylphosphatidylglycerol, anionic surfactants such as sodium dodecyl sulfate, and cationic surfactants including dodecyltrimethylammonium chloride and dodecyldimethylammonium bromide can change the tertiary structure of β-Lg, inducing its refolding from a dominant β-sheet to a prevailing α-helical secondary structure [131, 132].

According to binding properties of β-Lg, it can bind a variety of ligands and also the interaction being thought important from the drug delivery point of view.

Serotonin (5-hydroxytryptamine, 5-HT, Figure 1.4 a) is a monoamine neurotransmitter. 5-HT and its derivatives have been extensively found in the animals' gastrointestinal tract. In the human body, from the whole 5-HT, approximately 80 to 90 % of serotonin has been found in the enterochromaffin cells in the gut (Kulchitsky cells). 5-HT in these cells can be used to normalize intestinal activities [133]. The rest of 5-HT and its derivatives have been produced in serotoninergic neurons in the central nervous system (CNS) and they have a variety of functions such as appetite, muscle contraction, sleep, the regulation of mood, and some cognitive functions such as learning and memory. 5-HT has been also found in fungi and plants, such as fruits and vegetables, as well as in animals [134].

Although serotonin was found out over 60 years ago, the studies of 5-HT and its 5-HT receptors continue to yield novel proof of its physiological importance in almost every main system and organ, such as pulmonary, genitourinary, gastrointestinal, and the cardiovascular systems in addition to the central nervous system [133].

5-HT is best known as a neurotransmitter modulating the activities of an extensive neuropsychological processes range. Although, drugs targeting 5-HT receptors are widely utilized in psychiatry and neurology, most 5-HT is discovered in the exterior of the CNS, and essentially all of the known fifteen 5-HT receptors are stated inside in addition to outside the brain [133]. 5-HT modulates many human behavioral processes. This compound can regulate mammary gland homeostasis and the lactation-to-involution switch. One of the 5-HT targets is the mammary epithelial tight junctions, and the interruption of these tight junctions marks a premature step of mammary gland involution [135]. 5-HT is also very important as a gastrointestinal signaling compound. 5-HT is a paracrine messenger as sensory transducers used by enterochromaffin cells (EC). 5-HT is an information transmitter to the CNS and it can initiate peristaltic and secretory reflexes by activating intrinsic and extrinsic primary afferent neurons, respectively [136]. Abnormalities in 5-HT signaling are putatively implicated as causes of bowel diseases.

Arachidonyl serotonin (AA-5HT, Figure 1.4 b) is a molecule that can inhibit the activity of fatty acid amide hydrolase (FAAH) enzyme. FAAH function is to inhibit the activity of anandamide and other endogenous cannabinoids. The IC_{50} value of 12 μM is obtained for the inhibition of the FAAH activity, isolated from mouse neuroblastoma cells, by AA-5HT. Both the K_m (Michaelis-Menten constant that is defined as the concentration of substrate that leads to half-maximal velocity) and the V_{max} (the maximum catalytic rate that can be achieved by a particular enzyme) of the enzyme are affected by AA-5HT, indicating that this ligand is a very strongly binding, competitive inhibitor of FAAH. AA-5HT does not inhibit $cPLA_2$ (cytosolic phospholipase A_2) and is basically devoid of cannabimimetic activity.

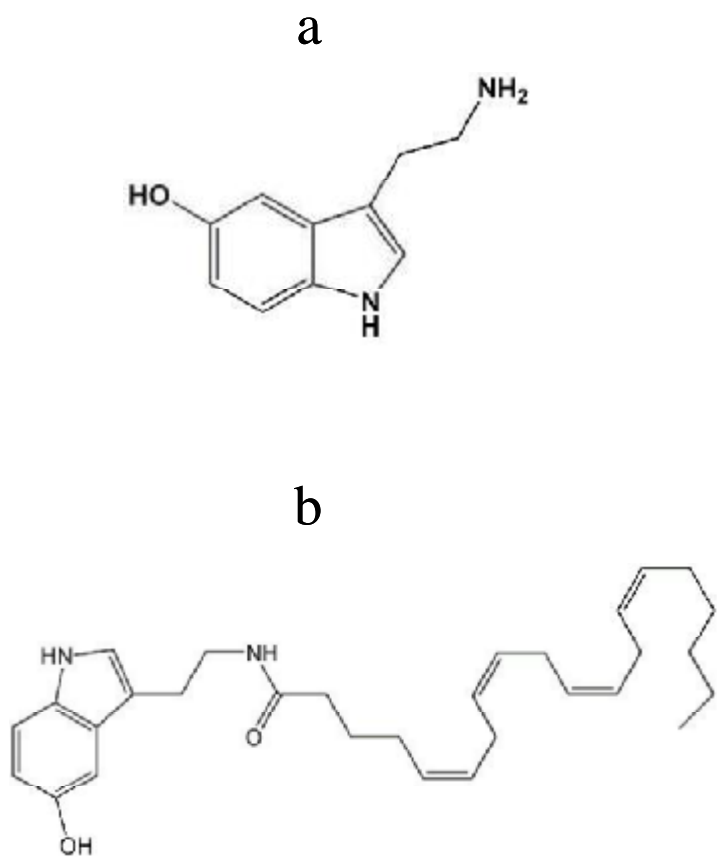


Figure 1.4. Structures of (a) serotonin and (b) arachidonyl serotonin.

This compound has been chosen as a lipophilic analog of 5-HT with promising applications in biochemistry and drug design. It modulates the activity of the enteric nervous system, influencing gastric and intestinal clearance. It affects the regions of central activities, including motor regulation, pain reduction, memory / learning, and reward. Finally, the endocannabinoid system's role in milk ingestion and the survival of the newborn and, maybe more noticeably, its critical relation in appetite stimulation in the adult organism, may not only proceed our understanding of the physiology of food intake and growth, but may also lead to infant's "failure to thrive" and therapeutic applications in wasting diseases [137, 138].

1.2.10. Role of free cysteine (Cys121) on β-Lg structure

Through heating β-Lg at neutral pH to above about 60 °C, it is irreversibly denatured, due to its polymerisation through inter-and intra-molecular disulphide bond formation and exchange promoted by Cys121 [139, 140]. The molecular details of the heat denaturaed β-Lg and polymerisation procedure of the protein have not been fully elucidated yet, but they clearly involve several successive reactions and intermediates [120, 141] leading to irreversible modifications of β-Lg structure at different levels [90, 142]. Cys121 has an important function in this heat polymerization process of β-Lg: chemical blocking of the free cysteine prevents it from aggregation and renders its denaturation reversible [139, 140, 143].

It has been also demonstrated that blocking chemically the free thiol group can exert a destabilising effect. The secondary and tertiary structures of the reacted β-Lg have been found to be modified [143]. In some cases, the balance between the monomer and dimer structures of the modified β-Lg has been observed to be shifted towards the monomer. Finally, some chemically blocked β-Lg has been found to be less resistant to denaturants, heating, or their affinity for fatty acids was reduced. In all these studies, it has been suggested that Cys121 plays a crucial role in stabilizing the β-Lg structure [61, 143].

However, because Cys121 has a rather low accessibility, partial denaturation is required in order to expose this residue to thiophilic reagents. Such denaturation can be, in some cases, partially irreversible. For example, when studying β-Lg with its thiol groups blocked by iodoacetamide, McKenzie et al. [144] have suggested the presence of equimolar amounts of free Cys119 and Cys121 in the native protein. This result arose from the harsh conditions used during the alkaline treatment, which allowed disulphide bond exchange to occur between Cys106, Cys119 and Cys121 [145, 146]. Moreover, chemical modifications are never 100 % effective, and the blocking groups can react with other residues than Cys121. Thus, the destabilizing effect of thiol blocking may be

due to the modifying groups introduced and/or to the stress caused by the chemical treatments rather than to the simple elimination of the free thiol group.

1.3. Allergy

An international define for allergy is that allergy is "*a hypersensitivity reaction initiated by specific immunologic mechanisms*". It is separated mostly in two categories, cell-mediated and antibody-mediated allergies.

Over 40 years ago, hypersensitivity reactions have been classically devided into four types by Gell and Coombs and their classification is still used [147]. These kinds of hypersensitivity reactions are anaphylaxis, antibody-mediated cytotoxic reactions, immune complex-mediated reactions, and delayed type hypersensitivity that have been established as Type I, Type II, Type III and Type IV, respectively. In this classification, type I is best understood, from the four main allergic reaction types, and it is submitted into the classical immuno-reactivity reactions. It is shown that hypersensitivity reactions in anaphylaxis reactions (Type I), are reffered as immediate anaphylactic or allergic reactions that are marked through one or more than one clinical reactions including, angiodema, urticaria, rhinitis or conjunctivitis, asthma and cardio-respiratory (anaphylactic) shock, [148]. The second type of hypersensitivity reactions (Type II) is antibody mediated reactions. In this kind of allergic reactions, mostly IgM and IgG as cytotoxic antibodies, make the allergic reactions. Two special mechanisms have been proposed for damaging cells caused by these cytotoxic antibodies. In the primary mechanism, the straight act of neutrophils, eosinophils, and macrophages, that are related to immunoglobulin-coated target can damage cells. The next mechanism can be stimulated by antibody-mediated reactions (Type II) and consequence in cell lysis [148]. Tissue injury mediated by immune complexes has been seen in the hypersensitivity reactions Type III. The secondary damage of the cells is as a reason of microprecipitates

produced in small vessels. These microprecipitates are created during forming immune complexes, when precipitating antibodies (mainly IgM) recognize antigens. Finally, the Type IV of hypersensitivity reactions is mediated by means of T-lymphocytes cells and generally marked as skin eruptions [148].

1.3.1. Induction of IgE production

Clinically, the most important and most prevalent type of hypersensitivity reactions is atopic allergy or IgE mediated immediate. B-cell and T-cell epitopes together can be found in allergens. B-cell epitopes are introduced as some area on the protein surface that soluble membrane bound antibody molecules are able to recognize them. The small peptides bound to major histocompatibility complexes (MHC), that T-cells can recognize them, are named T-cell epitopes.

For eliciting the production of IgE antibody, at first B-cells should recognize structural epitopes on the allergen's surface. Moreover, this requires Th2 type cells to stimulate conformational epitopes for transforming to IgE secreting plasma cells. These type cells (Th2 type) are responsible for recognizing allergens as processed linear peptides. Therefore T-cells cannot recognize non-processed allergenic proteins, and consequently allergens initially must be processed by APC's and then they shold be accessible on their surface while they are in conjunction with a group of molecules named MHC II type molecules[149].

It has been proposed that the responsibility to induce response of Th2 type cells requires a lot of factors. One of these factors that can support the shift to Th2 type responses is genetic factors background of the host [149]. There is a transcription factor named GATA3 that is responsible for regulating the equilibrium between the response of Th1 and Th2 cells by inducing creation of IL-4. GATA3 can inhibit Th1 and promote the Th2 response. Some ecological factors such as reduced exposure to natural

infections, changes of the commensal flora, and the reduced size of the family are able to promot the shift [149].

A lot of reasons have been suggested for the allergenicity properties of proteins. An ovoid shape, repetitive motifs in the amino acid sequence, heat stability, and resistance to gastrointestinal degradation are some suggestion as to why protein might be allergens [79]. Allergenicity properties of proteins can be increased by enzymatic activity. Some allergens can enhance their own permeability in the bronchial epithelium, and decrease the proliferation of Th1 cells by means of their activity in proteolysis consequently biasing the immune-response towards the Th2 type [150]. The allergy mechanism is shown in Figure 1.5.

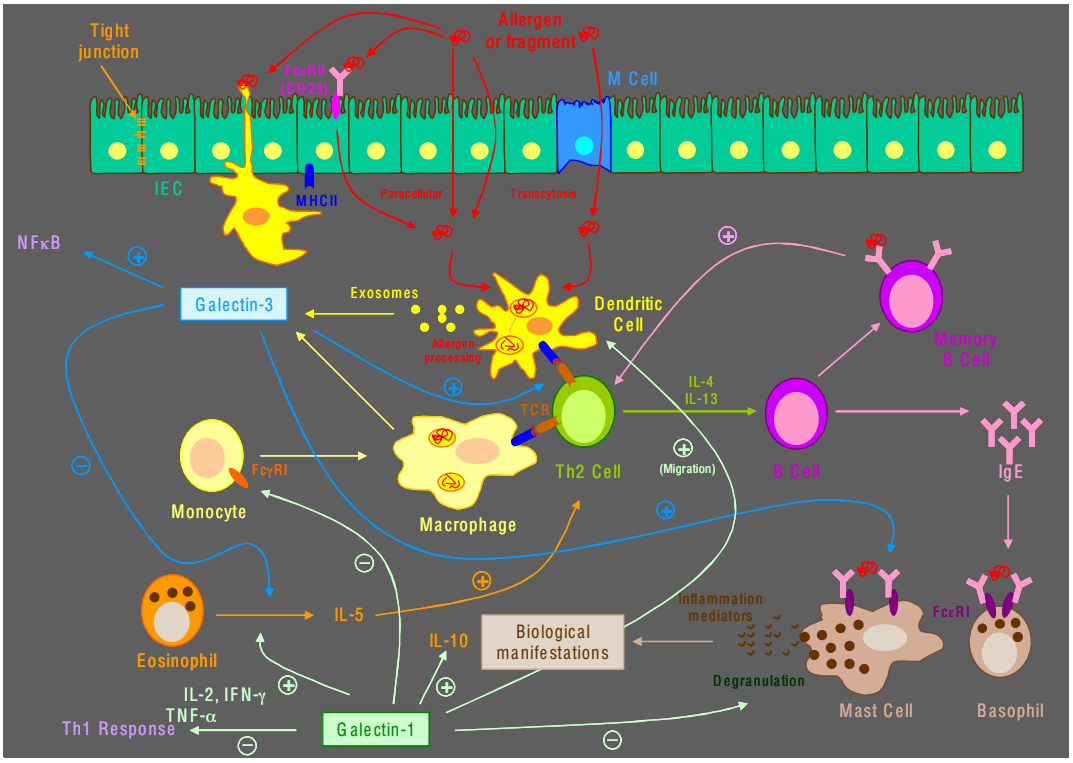


Figure 1.5. Schematic of the allergy mechanism in the human body.

1.3.2. Food allergy

Food hypersensitivity has been defined by European Academy of Allergy and Clinical Immunology (EAACI) as every unfavorable reaction due to food ingestion. This can be divided in two classes, non-allergic food hypersensitivity and a food allergy including an immunologic response [151]. Only during demonstrating immunologic reactions, the phrase "*food allergy"* is suitable to use. When IgE takes part to food in hypersensitivity reactions, the phrase "*IgE-mediated food allergy"* is appropriate to use [151].

In Europ and American countries, food allergy is known as a common problem. Food hypersensitivity is increasing like other atopic disorders [152]. Because the first area to face up to a great variety of food allergens is the gastrointestinal tract, it should be expected that an extensive variety of gastrointestinal allergic reaction disorders has been developed [152]. Hypersensitivity reactions to foord have been reported for about 20 % of people in industrialized countries. However, the incidence of confirmed allergy is much lower and about 6-9 % of child and about 1-2 % of adults have food allergy problem. [152].

1.3.3. Food allergens

While human use a wide variety of foods, but only a small number of foods make hypersensitivity reactions in human body [152]. Substances that react with IgE antibodies and induce allergic sensitization or induce hypersensitivity reactions have been defined as food allergens. It can not be found any conformational features to predict strong food allergen from the weaker one. An afficial list of common food allergens have been shown in Table 1.5.

1.3.4. Cow milk allergy and β-Lg

Both children and adults have been affected from the hypersensitivity reactions to some foods or food ingredients. Food allergies in the children, particularly infants and babies are more important because of the influence on their growth. Milk is used as the first food for infants. A hypersensitivity reaction against cow milk proteins has been defined cow milk allergy (CMA) that is an important problem in pediatrics [152].

Cow milk allergy (CMA) is in the third position among the six most important food hypersensitivity reactions, so that abou 2.4% of newborns have been affected from hypersensitivity reactions against cow milk proteins (CMP). Therefore, childhood cow milk allergy is the third most common allergies to food in France, with approximately 9 % of the total allergies diagnosed [153]. The most important nutrition source in babies is breast milk. After weaning, breast milk is usually replaced with cow milk products. Therefore cow milk allergy is one of the most important kinds of food hypersensitivity reactions in infants.

Table 1.5. Some of the Most Common Food Allergens (Official List of Allergens IUIS, International Union of Immunological Societies, Allergen Nomenclature Subcommittee).

Food	Allergen	Trivial name
Cow's milk	Bos d5	β-Lg
	Bos d4	α-La
	Bos d8	Caseins
Egg	Gal d1	Ovomucoid
	Gal d2	Ovalbumin
	Gal d3	Ovotranferrin
Fish	Gad c1	Parvalbumin
Potato	Sola t1	Patatin
Shrimp	Pen a1	Tropomyosin
Peanut	Ara 1	Vicilin
	Ara2	Conglutin

α_{S1}-, α_{S2}-, β-, and κ-caseins, α-lactalbumin, and β-lactoglobulin (β-Lg) are the most abundant proteins in bovine milk. The most important allergens in bovine milk are caseins, β-Lg, and α-lactalbumin [154-156]. Other proteins present in milk in lower amounts including BSA, lactotoferrin, and IgG-heavy chain are also recognized by CMA patients [155, 156]. Natale *et al*. showed that about 45% of patients that have hypersensitivity responses to bovine milk in SPT, had IgE antibodies specific to β-Lg [155]. Infant formulas generally include β-Lg that is frequently the first foreign antigen for babies. Therefore β-Lg can be an important antigen in the progress of more prevalent food allergies. Initially, in 1960's β-Lg has been found as an important antigen. Some studies demonstrated that an intact tertiary structure of β-Lg is required for the immunoreactivity of this protein [157].

Investigation on β-Lg's B-cell epitopes have been done by means of β-Lg peptides produced during trypsinolysis or cyanogen bromide treatment of β-Lg; also synthetic peptides have been also used. Although the functional capacity of these epitopes was not investigated, but the results showed that the main epitopes of β-Lg are amino acid residues of 21-40, 41-60, 102-145, and 148-168. In mast cells, for inducing a release of immune mediators, allergens must be bivalent and contain two epitopes. One bivalent B-cell epitope including amino acids 149-162 have been identified at the C terminus of β-Lg in an investigation utilizing trypsinolysis and synthesized peptides [158, 159].

A study using five patients with cow milk allergy has been done and three types of T-cell epitopes of human beings, including amino acid sequences of 30-47, 97-117, and 142-162, have been identified for cow β-Lg [158]. These epitopes were in the β-sheet structure of the protein. The peptide containing residues of 97-117 was the main epitope. This peptide was one of the three human T-cell epitopes that have been identified before. Amino acids 101-112 were the core peptide recognized most

effectively. This sequential epitope is hidden inside the protein with an orientated edge in the direction of the β-Lg surface [158]. Mice models have been used to study murine T-cell epitopes. The most powerful T-cell reactions in mice Balb/c have been proposed for peptides including sequences 67-75, 71-79 and 80-88 on β-Lg molecule [160].

1.3.5. Significance of milk processing in the pathogenesis of CMA

Nowadays, milk is quite exceptionally consumed in its raw state. Heating (pasteurization), one of the most commonly applied treatments of milk, induces sometimes important protein structural changes. Heating also induces chemical modifications of proteins, which can influence IgE binding and the allergenicity [161].

An important issue which can change the immuno-reactivity charachteristics of milk proteins is thermal treatments that are used in processed liquid milk products. During heat denaturation of proteins, the natural tertiary structure of them is frequently damaged. Therefore during heating of proteins, a lot of of native protein epitopes recognized by antibodies are damaged as well [161, 162]. Heat treatments on proteins have reduced the immuno-reactivity properties of a protein in many cases. ELISA inhibition studies have been demonstrated that heat-induced denaturation of β-Lg decreases the protein IgE reactivity, however it can not be eliminated totally [163]. In the opposite side, unfolding and heat denaturation of the β-Lg may possibly create various novel allergen epitopes which could not be found or could not be available in the native structure of bovine β-Lg [161, 162].

Heat treatment on milk during industrial processing can modify the digestibility of milk. The degradation of β-Lg by enzyme proteolysis is more susceptible when heat-induced denaturation of protein is occured [80]. The effect of this digestibility changes on the immuno-reactivity properties of β-Lg is not identified. Some studies showed that heat processing of whey can influence its ability to induce a humoral response. Although heat processing of milk will not produce new antibodies on whey proteins, but

native whey can induce an IgG antibody reaction against whey proteins mainly against β-Lg. Heat processing of whey proteins did not influence on the ability of whey proteins for inducing oral tolerance. In reality, oral tolerance can be induced by both native and unfolded whey proteins.

Because milk contains significant amounts of lactose and lower quantities of other reducing sugars, milk proteins are modified by so-called Maillard reaction or glycation during heating [164]. Glycation is one of the most frequent chemical modifications during industrial processing. It occurs in the presence of reducing sugars during the heating of proteins. This is a complex reaction, leading to the production of advanced Maillard reaction products (AMPs) or advanced glycated end products (AGEs). In this reaction, the first step consists of the creation of Schiff type adducts of reducing sugars with primary amino groups of mainly lysyl residues of the proteins, leading to the formation of Amadori and Heyns products. The reaction does not stop there; additional reactions involving polymerization and condensation reactions, causing to the production of brown pigments called melanoidins. In milk, Maillard reaction mainly leads to the Amadori products. The extent of glycation in commercial foods has been affected by many factors including the heating temperature, the duration of thermal process, and the concentrations of reducing sugars. The amount of lysine blockage of bovine whey proteins is the same as in other food systems and is well-documented [164]. Typical AGEs such as pyrraline or carboxymethyllysine do not play a major role in heated milk when compared to the Amadori product lactuloselysine. Additionally, the conditions of glycation used were specifically set to reduce the amount of AGE or to eliminate them altogether.

Several studies attempted to evaluate the ability of the recognition of allergens, modified by the Maillard reaction, by IgE [165-170]. In some cases, glycation-induced structural changes on allergens can increase their recognition by IgE [165, 168], whereas in other cases, glycation reduces IgE binding [166, 169] or has no effect [166,

167]. No general effect was observed; the effect seems to be allergen-and sugar-dependent.

1.4. Objective of the thesis

β-Lactoglobulin (β-Lg) is a bovine milk protein which enjoys the richest whey protein in the milk of almost all mammals and it belongs to the lipocalin group. Nonetheless, it does not exist in human milk and milk of other primates. Although the biological function of this lipocalin protein is not well undertood, but it has been suggested that it may carry fatty acids through the digestive tract. A great number of hydrophobic molecules have an affinity to interact with β-Lg including retinol, palmitic acid, some lipids and phospholipids. β-Lg is also the most allergenic protein in the mammalian milk. Heating can alter the structure of β-Lg and modify the sensitivity of this protein to proteolytic degradation, but there is not a lot of information about the effects of heat-induced denaturation of β-Lg on its immuno-reactivity properties.

Evaluation of the effect of heat-induced denaturation and the moderate glycation of bovine milk β-Lg on its allergenicity was the main aim of the first part of this doctoral thesis. The structural characterization of these modified protein has been carried out with different technique such as CD and electrophoresis and the results obtained thank to application of these methods prove that all these structural modifications were inducing changes in the antigenic character or immuno-reactivity of this important food protein and changed it's allergenicity properties.

In the second part of this thesis, in order to avoid the side effects of blocking the thiol group of β-Lg in partially irreversible denaturing conditions, it is of great interest to carry out studies on a genetic mutant of bovine β-Lg, in which Cys121 is substituted by an amino acid with approximately the same side chain characteristics. The aim of this part was to produce in the yeast *Pichia pastoris* a wild-type (WT) and a mutant β-

Lg, that the free Cys at position 121 was mutated into Ser. The structural characterization of this Cys121Ser mutant has been carried out with different technique and it ensures that all these structural modifications induceing changes in the antigenic character or immuno-reactivity of this important protein.

Finally, a further aim of this doctoral thesis was the study of interactions of some drugs such as 5-HT and AA-5HT with β-Lg, which were investigated using fluorescence intensity and circular dichroism (CD) spectroscopy to describe the potential of β-Lg to bind such important regulators of lactation and intestinal transit as 5-HT and AA-5HT executing regulatory functions. The binding constants and binding sites of 5-HT and AA-5HT on β-Lg were identified, and the effects of the ligand-protein interactions on the structure of β-Lg were discussed.

The detailed aims are shown below:

1. Purification of β-Lg in its native structure from cow milk and characterization of this protein.

2. Preparation and charecterization of heated and glycated β-Lg.

3. Evaluation of the effect of heat-induced denaturation of bovine β-Lg on binding of IgE from CMA patients.

4. Determination of the effect of moderate glycation on the degree of recognition by IgE.

5. Preparation and charecterization of wild type recombinant β-Lg and mutant recombinant β-Lg.

6. Evaluation of the allergenicity of wild type and mutant recombinant β-Lg.

7. Study on the interactions of some drugs such as 5-HT and AA-5HT with β-Lg

Materials and Methods

2.1. Materials

All the materials used for experiments were obtained from Sigma-Aldrich and Merck Chemical Co. All the used reagents were of highest available degree of purity. For preparing all proteins and doing all allergenicity experiments, all of the solutions were prepared using double distilled water and were used fresh after preparation. The concentrations of β-Lg were determined from the optical density of prepared solutions using the extinction coefficient at 280 nm of 17600 M^{-1} cm^{-1} [59]. In the case of glycated β-Lg, the concentrations of protein were adjusted by BCA assay kit (Sigma, St Quentin-Fallavier, France).

In binding experiments, 5-HT and AA-5HT stock solutions were prepared by dissolving them at a concentration of 15 mM in 75 % ethanol and then diluting to a final concentration of 1 mM with 10 mM phosphate buffer, pH 7.4, or 10 mM glycine buffer, pH 2.0. Samples were prepared by mixing β-Lg and 5-HT or AA-5HT stock solutions in varying proportions. The highest resulting ethanol concentration in solutions was less than 4 %, which had no major effect on protein structure. The samples were incubated for 2 h at room temperature prior to analysis.

2.1.1. Preparation of β-lactoglobulin

β-Lactoglobulin variant A (β-Lg A) was isolated in Nantes INRA laboratory from the milk of a homozygous cow by a salting out/in method according to Maillard and Ribadeau-Dumas precipitating the majority of whey protein when conserving uniquely β-Lg in solution [171]. Homogeneity of the protein preparation was assessed by high performance gel permeation chromatography and SDS gel electrophoresis. The obtained preparations of β-lactoglobulin were over 98 % pure.

2.1.2. Preparation of heated proteins

Lyophilized purified native bovine β-Lg (variant A) obtained in our laboratory in Nantes INRA was diluted in 100 mM PBS buffer pH 7.4 and its concentration was adjusted to 2.7 mg/mL, corresponding to its concentration in milk, after verification of its content with the BCA assay kit (Sigma, St Quentin-Fallavier, France). β-Lg was heated with a Touchgene gradient thermocycler (Techne Inc., Princeton, NJ, USA) at the rate of 1 °C/min and incubated for 20 min at different temperatures, 65, 75, 85 and 95 °C. After heating, protein was rapidly cooled (about 1 °C/sec) to 4 °C and used immediately for any studies, specially of its recognition by IgE.

2.1.3. Preparation of glycated proteins

Purified native bovine β-Lg (variant A) was glycated with lactose (β-Lg/Lac), ribose (β-Lg/Rib), glucose (β-Lg/Glu), galactose (β-Lg/Gal), arabinose (β-Lg/Ara) and rhamnose (β-Lg/Rha).

A concentration of 0.217 mM of β-Lg and 0.217 M of sugars were prepared in 0.1 M phosphate buffer, pH 6.5. All the solutions were filtrated on 0.22 μm acetate cellulose filters (Millipore, Bedford, MA, USA). After filtration, the protein and sugar solution were mixed and put in well-capped flasks. To produce glycated proteins, the mixed solution were heated at 60 °C for 72 h in a water bath. in this mild heat treatment, self-aggregation of β-Lg could not be occured. Strictly anaerobic and sterile conditions is needed for this reaction, so that, all experiments were carried out under this condition the experiment environment were purged and saturated with N_2. After finishing glycation experiment, the dialysis of various fractions of glycated proteins were done against distilled water for 24 h and consequently all fractions were freeze-dried in lyophilizer, and lyophilized proteins were put at -20 °C to use them for a long time. β-Lg heated at 60 °C for 72 h without sugar (β-Lg/60 °C) was used as a control. The number of accessible amino side chains and the progress of the glycation were demostrated by

the adapted ortho-phthaldialdehyde (OPA) technique [172]. A mixture of 40 mg of OPA, which is dissolved in 1 mL of methanol, 100 mg of N-dimethyl-2-mercaptoethylammonium chloride (DMMAC), 50 mL of 0.1 M sodium borate buffer, pH 9.2, and 1.25 mL of 20 % w/w SDS in water was used for preparing OPA solution. After that, 50 µL of the protein solution with a concentration of 2 g/L in 50 mM sodium phosphate buffer, pH 7.8 was mixed with 1 mL of OPA solution. The absorbence of mixed solution after a minimal delay of 5 min was checked at 340 nm. the standard solutions of L-leucine with a concentration range of 0.25-2.00 mM were used to obtain calibration curve. The degree of modification of the proteins was as follows: β-Lg/60°C, 6.2 %; β-Lg/Lac, 34.4 %; β-Lg/Rha, 40.6 %; β-Lg/Glu, 41.2 %; β-Lg/Gal, 41.9 %; β-Lg/Ara, 55.0 % and β-Lg/Rib, 69.4 %. Lyophilized glycated proteins were diluted in PBS before use (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, pH 7.4) and their concentrations were adjusted to 1 mg mL^{-1} with the BCA assay kit (Sigma, St Quentin-Fallavier, France).

2.1.4. Preparation of mutant proteins

In order to avoid the side effects of blocking the chemically thiol group of β-Lg in partially irreversible denaturing conditions, it is of great interest to carry out studies on a genetic mutant of bovine β-Lg, in which Cys121 is substituted by an amino acid with roughly the same side chain characteristics. We expressed in the yeast *Pichia pastoris* a wild-type (WT) and a mutant β-Lg, in which the free Cys at position 121 was changed into Ser using the plasmids prepared by Jayat et al. [140]. The procedure of preparation and expression of recombinant proteins by Jayat et al. is described below.

2.1.4.1. Mutant design and cloning

The WT recombinant β-Lg expression system previously constructed by Wilson et al. [173] was used as a template. In this system, the complementary DNA (cDNA) of bovine β-Lg coding for WT (variant A) was inserted into the *XhoI*/*XbaI* site in the multiple cloning site of the expression vector pGAPZαA (Invitrogen, Groeningen, The Netherlands). This plasmid of 3147 base pairs can express the bovine β-Lg in the yeast Pichia pastoris. The gene of interest is placed under the control of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter, allowing constitutive expression of the recombinant protein. pGAPZαA also contains the α-factor signal peptide from *Saccharomyces cerevisiae*, which allows secretion of the protein into the culture medium. This sequence contains an additional multiple cloning site, rich sequence cleavage sites of restriction enzymes. β-Lg cDNA was inserted between XhoI and XbaI sites.

A gene conferring resistance to Zeocin™, an antibiotic, was used as a selection marker. The Cys121Ser mutation was performed according to the Quick Change protocol from Stratagene (La Jolla, CA) with the following primers: 5' primer: 5'-GC-CTG-GTC-TGC-CAG-AGT-CTG-GTC-AGG-ACC-CC-3'; 3' primer: 5'-GG-GGT-CCT-GAC-CAG-ACT-CTG-GCA-GAC-CAG-GC-3' (mismatched bases are underlined). Amplified DNA was transformed into Epicurian *E. coli* XL1-blue cells (Stratagene) and plated for selection on Luria-Broth (LB)-agar plates described below supplemented with Zeocin™ (25 μg/mL). Plasmids from positive clones were purified with the Miniprep Kit from Qiagen (Venlo, The Netherlands) and sequenced to confirm the correct introduction of the mutation (MWG Biotech, Ebersberg, Germany).

The positive plasmid was linearised with BspH1 and electroporated into *P. pastoris* according to the EasySelect™ *Pichia* Expression Manual from Invitrogen. For linearization, the vector/ β-Lg pGAPZ contains an origin of replication allowing it

to be stored as a circular episome (not integrated in the chromosome) and to be replicated in E. coli. However, it contains no yeast origin of replication, making it unable to maintain it episomal form: it must be inserted into the genome by recombination. The plasmids were linearized before being introduced into *P. pastoris* to increase the efficiency of this recombination. The plasmids were hydrolyzed by the enzyme B*sp*HI (New England Biolabs), which cleaves the plasmid. The reaction was performed for 2 h at 37 °C and then the plasmids were purified by gel extraction kit (Qiagen) and resuspended in 15 µL of ultra-pure water. The hydrolysis was monitored on a 0.8 % agarose gel and DNA concentration was measured.

The plasmids were inserted into *P. pastoris* by electroporation. In this process, subjecting cells to a strong electric field causes a temporary permeabilization of their membranes. The transformation was carried out following the protocol "Easy Select Pichia Expression" (Invitrogen):

A fresh colony of yeast has been cultivated at 30 °C in 2 mL of YPD medium (1 % yeast extract, 2 % peptone, 2 % glucose). This pre-culture was used to inoculate a 100 mL culture medium YPD. When theoptical density of 600 nm was between 1 and 5, the culture was stopped. Preparation of yeast was continued at 4 °C using sterile solutions. All centrifugation steps were 5 min at 3000 g.

The cells were centrifuged and washed successively with 1 volume (compared to the volume of culture) and ½ volume of water and 4 mL of 1 M sorbitol. The pellet was resuspended in 200 µL of 1 M sorbitol. 80 µL of cells were then mixed with 5-10 µg of DNA. After 1 min, the electric shock was performed with a Gene Pulser II electroporator (Bio-Rad, Hercules, CA). After the shock, the cells were immediately resuspended in 1 mL of 1 M sorbitol and incubated for 1 h at 30 °C without stirring so that they are rebuilding their membrane without loss of intracellular material.

Selection for multi-copy transformants was carried out by plating clones on increasing levels (100 to 800 µg/mL) of Zeocin™. The best producing clones were

selected by culture for 48-72 h in YPD medium (1 % yeast extract, 2 % peptone, 2 % glucose) and protein expression was assayed by SDS-PAGE.

2.1.4.2. Protein expression in *P. pastoris*

Yeasts produce recombinant proteins in varying amounts depending on how many copies they have integrated the plasmid into their genome and location of insertion events. To select a clone producing β-Lg in large quantities (tens of mg/L) expression tests were performed on the colonies obtained after electroporation. These were grown overnight at 30 °C in YPD medium under shaking (300 rpm). 200 mL of each culture were then collected, centrifuged for 5 min at 5000 g, and 10 μL of the supernatant were subjected to polyacrylamide gel electrophoresis (SDS-PAGE) to quantify the protein. In parallel, the OD_{600} culture was measured for normalizing the amount of protein present in quantities of cells.

By selecting the best colony for producing recombinant protein, a first pre-culture of this fresh colony of *P. pastoris* has been conducted in 2 mL YPD supplemented by zeocin (100 µg mL^{-1}) at 30 °C under shaking (200 rpm) overnight. This pre-culture was used to inoculate 2 L culture flasks containing 250 mL of buffered glycerol complex medium (BMGY) supplemented by glucose (1 % yeast extract, 2 % peptone, 1.34 % yeast nitrogen base, 0.04 % biotin, 2 % glucose, 100 mM potassium phosphate, pH 7.0) supplemented with Zeocin™ (100 μg/mL), each. Constitutive expression of the protein was performed at 30 °C under shaking (200 rpm) during 3 days with occasional pH readjustment with 1 M phosphate buffer, pH 7.0 and glucose addition. The protein expression during the culture was followed by SDS-PAGE each day. After 72 h, the supernatant was collected by centrifugation (45 min, 10000 g) for further purification of the protein.

2.1.5. Protein purification

In the culture conditions used, β-Lg was the main protein present in the supernatant. The culture medium was centrifuged (45 min at 10000 rpm) and then filtered under vacuum (0.4 μm filter) to remove all cells and cellular debris. The ultrafiltration steps of the supernatant were performed with cassette Vivaspin 20 (cutoff 10 kDa, Vivascience) to concentrate our production and to remove small compounds, salts, peptides, and sugars. A two-step purification chromatography has been easily implemented.

2.1.5.1. Ion exchange chromatography

All preparative chromatography steps were carried out at 20 C on an Äkta Purifier System and followed with the Unicorn Control software (Amersham Biosciences, Piscataway, NJ). The ultrafiltration retentate was loaded onto an anion exchange chromatography. The β-Lg is negatively charged at neutral pH (pI=5.2), it can be purified by an anion exchange column. This step separates the β-Lg compounds carry a positive or zero. It also helps to concentrate the sample and reduce its volume to a few hundred mL. The ion-exchange chromatography 26 mm) in Tris-HCl was performed on a column Q-Sepharose XL Streamline, (200 x 26 mm; Pharmacia, Peapack, NJ). 20 mM phosphate buffer pH 7.0 was used as buffer A and 20 mM phosphate buffer pH 7.0 with 1M NaCl was used as buffer B. The protein was eluted at a flow-rate of 5 mL/min with 20 mM phosphate buffer, pH 7.0 by increasing steps of NaCl concentration (0.1 / 0.3 / 0.5 / 1 M). The protein content of each fraction was checked by SDS-PAGE. The β-Lg was mainly eluted at 0.3 M NaCl. Before further purification, these fractions were concentrated by ultrafiltration (Vivaspin 20, 10 kDa MWCO, Vivascience) and a new concentration was achieved by ultrafiltration until a maximum volume of 15 mL.

2.1.5.2. Size exclusion chromatography

The size exclusion chromatography separates compounds according to their size and bulk. The different compounds interact with a matrix of porous resin beads: small compounds inter the pores, which retards compared to larger. The size exclusion chromatography was performed on a column XK26 (700 x 26 mm, Pharmacia) equilibrated with 50 mM sodium phosphate, 0.1 M NaCl, pH 7. The sample was injected on the column with aliquots of 2 to 5 ml with a flow rate of elution of 3 mL/min. The eluted samples were collected in the fractions of 5 mL and the purity of each fractions was checked by liquid chromatography reversed phase (RP-HPLC) and SDS-PAGE. The fractions containing the pure protein were pooled, concentrated by ultrafiltration, dialysed against distilled water and lyophilised for storage at 4 $^{\circ}$C.

2.1.6. Peptide digestion

Protein samples were prepared at 2 mg/mL in 20 mM citrate-phosphate, pH 2.8. Enzyme was porcine pepsin from Sigma (St-Louis, MO). Digestion was performed at constant temperature 37 $^{\circ}$C with 2 % enzyme / substrate ratio (w/w). Aliquots were taken after 24 h and the reaction was stopped by addition of 0.2 M Tris-HCl, pH 8.0 (125 μL/100 μL reaction). The extent of digestion was then determined by RP-HPLC.

2.2. Methods

The focus of our research effort is to evaluate the effect of heat denaturation and the moderate glycation of bovine milk β-Lg on its allergenicity. We developed and used a number of structural, biochemical and biophysical techniques, particularly circular dichroism (CD), fluorescence, electrophoresis, HPLC, ELISA and etc. to characterize structural changes of protein during some modifications and prove that all these structural modifications were possible to induce changes in the antigenic character or immuno-reactivity of this significant protein. We have used also molecular biology

methods to express wild type and mutant proteins by the procedure of Jayat et al. as described before. And finally several methods were used to discriminate binding properties of this imperative protein. All the methods we have used in our study effort are described below.

2.2.1. Electrophoresis experiments

Proteins were analyzed by 12 % SDS-PAGE. Briefly, proteins were diluted in loading buffer (50 mM Tris-HCl, pH 6.8, 4 % (w/v) SDS, 10 % (v/v) glycerol, 0.025 % (w/v) bromophenol blue, 2-mercaptoethanol ± 3 %) and and boiled for 3 min. Samples were loaded on 12 % polyacrylamide gel prepared according to Table 2.1. Migration was performed for about 1 h in migration buffer (Table 2.1).

Table 2.1. Composition of the Gel for 12% SDS-PAGE Electrophoresis.

Solution	Stacking gel Vol. (mL)	Resolving gel Vol. (mL)
Tris 2.0 M pH 8.8	---	0.665
Tris 0.5 M pH 6.8	0.3	---
Acrylamide 40%	0.2	1.2
Distilled water	2.0	2.135
SDS 10%	0.025	0.04
APS10%	0.020	0.04
TEMED	0.004	0.008
Sample buffer: SDS 4%, glycerol 10%, Tris HCl 50 mM pH 6.8, 0.025% (w/v) bromophenol blue, ± 2-mercaptoethanol 3%.		
Migration buffer: Tris 50 mM (12.0 g), glycine 0.384 M (57.6 g), SDS 0.1% (2.0 g) (dissolve in 2L distilled water).		
Colorant solution: ethanol 30%, acetic acid 5%, H_2O 65%, Coomassie blue R250 0.2%.		
Decolorant solution: ethanol 30%, acetic acid 5%, H_2O 65%.		

The electrophoresis was performed at 10 mA (stacking gel) and 20 mA (running gel) for each gel. Gels were stained with an aqueous solution containing 30 % (v/v) ethanol, 5 % (v/v) glacial acetic acid and 0.25 % (w/v) Coomassie brilliant blue and distained with a solution containing 30 % (v/v) ethanol, 5 % (v/v) glacial acetic acid. After destaining, plates were scanned and intensities of bands were quantified using Quantity One software (Bio-Rad, Hercules, CA).

SDS-PAGE electrophoresis was performed under reducing and non-reducing conditions. In reducing conditions the sample buffer was containing 3 % reducing agent 2-mercaptoethanol (β-ME).

Table 2.2. Composition of the Gel for 10% Native-PAGE Electrophoresis.

Solution	Stacking gel Vol. (mL)	Resolving gel Vol. (mL)
Tris-glycine concentrate	---	0.80
Tris 0.5 M pH 6.8	0.360	---
Acrylamide 40%	0.315	1.0
Distilled water	2.325	2.20
APS10%	0.025	0.04
TEMED	0.005	0.008
Sample buffer: Tris HCl 50 mM pH 6.8, glycerol 20%, 0.025% (w/v) bromophenol blue.		
Tris-glycine concentrate: Tris 0.25 M (30.0 g), glycine 1.92M (144 g).		
Migration buffer: Dilute 5 times Tris-glycine concentrate with distilled water.		
Colorant solution: ethanol 30%, acetic acid 5%, H_2O 65%, Coomassie blue R250 0.2%.		
Decolorant solution: ethanol 30%, acetic acid 5%, H_2O 65%.		

Alkaline (native)-PAGE electrophoresis was performed on a 10 % acrylamide gel according to Table 2.2. Samples were mixed with loading buffer (50 mM Tris-HCl,

pH 6.8, 20 % glycerol, bromophenol blue traces) and without boiling were injected to the wells. The electrophoresis buffer was 50 mM Tris-HCl, 0.384 M glycine. Migration was performed for about 1 h in migration buffer (Table 2.2). The electrophoresis was performed at 10 mA (stacking gel) and 20 mA (running gel) for each gel. Gels were stained with an aqueous solution containing 30 % (v/v) ethanol, 5 % (v/v) glacial acetic acid and 0.25 % (w/v) Coomassie brilliant blue and distained with a solution containing 30 % (v/v) ethanol, 5 % (v/v) glacial acetic acid. After destaining, plates were scanned and intensities of bands were quantified using Quantity One software (Bio-Rad, Hercules, CA).

2.2.2. Circular dichroism measurements

Circular Dichroism (CD) spectra were recorded on a CD6 dichrograph (Jobin Yvon, Longjumeau, France), using cells of the appropriate lengths and a scan time of 2 nm/s, 0.2 nm resolution, 10 accumulations, and 1.0 nm bandwidth. All measurements were carried out at 20 $^{\circ}$C with thermostatically controlled cell holders. The instruments were calibrated with ammonium d-10-camphorsulfonic acid. The data were expressed as molar residue ellipticity $[\theta]$, which is defined as $[\theta] = 100\ \theta_{obs}/cl$, where θ_{obs} is the observed ellipticity in degrees, c is the concentration in residuemol/cm^3, and l is the length of the light path in cm.

For CD spectra in the far-UV region meant for allergy experiments, the samples concentration was 1 mg/mL in 100 mM PBS buffer, pH 7.4. The spectra were recorded between 190 nm and 260 nm using a quartz cuvette with 0.1 mm path length. Each spectrum was the accumulation of 10 successive measurements and baseline was corrected by subtracting buffer spectrum. Chou and Fasman method was used for deconvolution of the CD spectra and calculation of α-helix and β-structure using Dichro 2 program.

A protein concentration of 0.5 mg/mL was used for near-UV CD spectra and the spectra were recorded between 260 and 350 nm using quartz cuvette with 1 cm path length. 15 scans were averaged per spectrum. The baseline was corrected by subtracting the corresponding solvent spectrum from sample spectrum. For all experiments, the β-Lg concentration was determined spectrophotometrically assuming $\varepsilon_{278} = 17600\ M^{-1}\ cm^{-1}$.

2.2.3. HPLC experiments

Chromatography was carried out at 20 °C on a 2695 Separation Module with a 996 Photodiode Array Detector and followed with the Millenium™ software, all from Waters (Milford, MA). Protein samples were prepared at 1 mg/mL.

GP-HPLC (gel filtration chromatography) was run on a TSK Gel 3000 SWXL column (300 x 7.8 mm i.d.; TosoHaas, Montgomeryville, PA) equilibrated with 50 mM sodium phosphate, 0.1 M NaCl, pH 7.0 or 10 mM glycine-HCl, pH 2.0. Elution was performed at a flow-rate of 0.8 mL/min and peak detection was monitored at 220 nm. The column was calibrated with six protein standards covering a molecular mass range from 13.7 to 150 kDa.

RP-HPLC (reverse phase chromatography) was run on a Nucleosil™ column (C_{18}, 300 Å, 10 μm, 250 x 3 mm i.d.) from Macherey-Nagel (Hoerdt, France). The column was equilibrated with solvent A (5 % aqueous acetonitrile, 0.1 % trifluoroacetic acid, TFA) and elution was performed at a flow-rate of 1 mL/min with a linear gradient from 0 to 100 % solvent B (70 % aqueous acetonitrile, 0.09 % TFA) over 25 min. The constant temperature of 30°C was utilized for the column and solvents used in RP-HPLC. UV-Vis spectrophotometer at 214 nm was employed to detect the eluted peaks. It was calculated the peak area relating to the non-hydrolyzed structure of the protein.

This peak area (the non-hydrolyzed protein's peak area) was used as a standard to report the hydrolysis results according to this peak, which represents 100 %.

2.2.4. Mass spectroscopy experiments

Electro-Spray Ionisation Mass Spectrometry (ESI-MS) experiments were performed on a Thermo Finnigan LCQ Advantage (San Jose, CA) by infusion at a flow-rate of 2.5 μL/min. Samples were at 5 pmol/μL in H_2O-acetonitrile (1/1, v/v) + 0.5 % formic acid.

2.2.5. Preparation of sera

A series of 47 French sera and 119 Iranian sera from CMA patients presenting various symptoms were used. Total milk proteins-and β-Lg-specific IgE concentrations were determined with the Phadia ImmunoCAP System®. All the sera had total milk proteins-specific IgE concentrations from <0.35 kAU/L to 57.8 kAU/L (arbitrary Abbott unit). Thirteen French sera and 87 Iranian sera had β-Lg-specific IgE values from 0.36 kAU/L to 26.3 kAU/L and four French sera and 32 Iranian sera had β-Lg-specific IgE concentration < 0.35 kAU/L. The use of the sera was approved by the Ethical Committee of the Angers Academic Hospital, France and Ethical Committee of the Imam-Khomeini Hospital in Tehran, Iran.

2.2.6. Colorimetric ELISA assay (C-ELISA)

Maxisorp™ bottom flat transparent 96 micro titration plates (Nunc™, Roskilde, Denmark) were coated overnight with 100 μL per well native, heated, sugar modified, recombinant, and mutant β-Lg diluted to 5 μg mL^{-1} in PBS, with native β-Lg diluted to 5 μg mL^{-1} in 100 mM carbonate buffer pH 9.6 or with PBS in wells used as negative controls. After coating they were washed 3 times with PBS containing 0.1 % (v/v)

Tween-20 (PBS/T) and saturated for 2 h with 250 μL of a solution of PBS/T containing 1 % (w/v) polyvinyl alcohol (Sigma) (PBS/T/PVA). Plates were washed 3 times with PBS/T and incubated for 90 min at room temperature with 100 μL of mouse monoclonal anti-β-Lg antibody (IgG) diluted to 1:100 in PBS/T/PVA from hybridoma supernatant. Plates were washed 3 times with PBS/T and incubated for 1 h at room temperature with a peroxidase-conjugated anti-mouse IgG (Bio-Rad Laboratories, Hercules, CA, USA) diluted 1:3000 in PBS/T/PVA. The secondary antibody binding was revealed after 3 washes with PBS/T by addition of 100 μL of ortho-phenylene diamine (OPD) (Sigma, 0.4 mg mL^{-1} in 50 mM citrate buffer, pH 5.5). The staining reaction was stopped after 20 min incubation at room temperature by addition of 100 μL of 2 M H_2SO_4. Absorbance was measured at 492 nm with the microplate absorbance reader EL_X800 (BioTek Instruments, Inc., Winooski, VT, USA).

2.2.7. Fluorescent ELISA assay (F-ELISA)

Maxisorp™ bottom flat white 96 micro titration plates (Nunc™) were coated overnight with either 100 μL per well of a mouse monoclonal anti-human IgE antibody (IgG2β) (Fitzgerald, Concord, USA) diluted to 1:2500 (1.6 μg mL^{-1}) in PBS or with native, heated and sugar modified, recombinant wild type and mutant Cys121Ser β-Lg diluted to 5 μg mL^{-1} in PBS, or with native β-Lg diluted to 5 μg mL^{-1} in 100 mM carbonate buffer pH 9.6. After coating they were washed 3 times with PBS/T and saturated for 2 h with 250 μl of a solution of PBS/T/PVA. Plates were washed 3 times with PBS/T. Wells coated with the anti-human IgE antibody were incubated with 100 μL of serial dilutions to ½ from 160 ng mL^{-1} to 0.08 ng mL^{-1}, plus one dilution at 1 ng mL^{-1}, of the 2nd WHO international reference preparation of human IgE (prepared in PBS/T/PVA). In the wells coated with allergens and in those coated with PBS as negative control, 100 μL of 1:25 dilutions of sera from patients were added. After an

overnight incubation at 4 °C , the plates were washed 3 times with PBS/T and incubated for 2 h at room temperature with an alkaline-phosphatase conjugated polyclonal anti-human IgE (Sigma) diluted 1:1000 in PBS/PVA. The secondary antibody binding was revealed, after 3 washings with PBS/T, by addition of 4-methylumbelliferyl phosphate (4-MUP) substrate diluted 1:5 in 1 M Tris-HCl, pH 9.8. The fluorescence emission was measured after 90 min incubation at temperature 37 °C with the microplate fuorescence reader FL_X800 plate reader (BioTek Instruments) fitted with a 360 nm excitation filter and a 440 nm emission filter. The reading parameters were adjusted to 45 in sensitivity for a reading from the top.

Controls included secondary antibody on capture antibody (no IgE), secondary antibody on IgE (no capture antibody), secondary antibody on allergens (no patient serum), and secondary antibody on serum (no allergen).

To relate fluorescence intensity to IgE concentrations, a sigmoid 4-parameters model [$y = d + (a-d)/1+(x/c)^b$)] was adjusted to standard curve data by non-linear regression using the Solver option from Microsoft® Excel 2000 by minimizing the sum of the squared differences. Limits of detection (mean + 3 SD) and quantification (mean + 10 SD) were calculated from fluorescence data of the 8 wells with no IgE. Fluorescence data measured for each antigen and serum were corrected by subtracting the fluorescence of the control with no antigen and corresponding specific IgE concentrations were calculated from the adjusted standard curve provided that the fluorescence exceeded the quantification limit.

2.2.8. Competitive ELISA

Maxisorp™ bottom flat white 96 micro titration plates (Nunc™) were coated overnight with either 100 μL per well of a mouse monoclonal anti-human IgE antibody (IgG2β) (Fitzgerald, Concord, USA) diluted to 1:2500 (1.6 μg mL^{-1}) in PBS or with

native β-Lg diluted to 5 μg mL^{-1} in PBS buffer. After coating they were washed 3 times with PBS/T and saturated for 2 h with 250 μl of a solution of PBS/T/PVA. Plates were washed 3 times with PBS/T. Wells coated with the anti-human IgE antibody were incubated with 100 μL of serial dilutions to ½ from 160 ng mL^{-1} to 0.08 ng mL^{-1}, plus one dilution at 1 ng mL^{-1}, of the 2nd WHO international reference preparation of human IgE (prepared in PBS/T/PVA). In the wells coated with native β-Lg and in those coated with PBS as negative control, 100 μL of a pool of sera from patients pre-incubated for 2 h at 37 $^{\circ}$C in the presence of increasing concentrations of competitor (native, heated and sugar modified, recombinant wild type and mutant Cys121Ser β-Lg) were added. The final dilution of the serum was 1:20, and the final concentrations of the inhibitors ranged from 0 to 100 µg mL^{-1} (1:10 serum pool dilution and inhibitor solution were mixed v/v to a final volume of 100 µL). After an overnight incubation at 4 $^{\circ}$C , the plates were washed 3 times with PBS/T and incubated for 2 h at room temperature with an alkaline-phosphatase conjugated polyclonal anti-human IgE (Sigma) diluted 1:1000 in PBS/PVA. The secondary antibody binding was revealed, after 3 washings with PBS/T, by addition of 4-methylumbelliferyl phosphate (4-MUP) substrate diluted 1:5 in 1 M Tris-HCl, pH 9.8. The fluorescence emission was measured after 90 min incubation at temperature 37 $^{\circ}$C with the FLX_{800} plate reader (BioTek Instruments) fitted with a 360 nm excitation filter and a 440 nm emission filter. The reading parameters were adjusted to 45 in sensitivity for a reading from the top.

Controls included secondary antibody on capture antibody (no IgE), secondary antibody on IgE (no capture antibody), secondary antibody on allergens (no patient serum), and secondary antibody on serum (no allergen). Because of the low amount of serum available, the experiment was realized once in triplicate.

The concentration of protein (inhibitor) needed to inhibit 50 % of IgE binding (IC_{50}) was calculated from the inhibition curves by relating fluorescence intensity to

inhibitor concentrations. A sigmoid 4-parameters model [$y = d + (a-d)/1+(x/c)^b$)] was adjusted to standard curve data by non-linear regression using the Solver option from Microsoft® Excel 2000 by minimizing the sum of the squared differences. Limits of detection (mean + 3 SD) and quantification (mean + 10 SD) were calculated from fluorescence data of the 8 wells with no IgE. Fluorescence data were corrected by subtracting the fluorescence of the control with no antigen.

2.3. Ligand binding experiments

As we declared before, β-Lg has been found in complexes with a wild variety of ligands and lipids such as butyric and oleic acids. In this part of our research, the interaction of serotonin and one of its derivatives, arachidonyl serotonin (AA-5HT), with β-Lg was investigated using various methods such as CD and fluorescence intensity measurements. Their binding by β-Lg may be one of the peripheral mechanisms of the regulation of the content of serotonin and its derivatives in the bowel of milk-fed animals. The methods we have used in this regard are summarized as following.

2.3.1. Steady-state fluorescence measurements

Fluorescence measurements were performed using an RF-5000 Shimadzu spectrofluorimeter with a cell compartment thermostated at 293 K. Protein intrinsic fluorescence experiments were performed at constant β-Lg concentration (5 μM) titrated with different 5-HT or AA-5HT concentrations, in order to have at least a molar *ratio* of ligand/protein equal to 20. Emission spectra were recorded from 300 to 450 nm with an excitation wavelength of 280 nm. The results of all measurements of fluorescence were corrected by the results of the same titrations of medium without

protein. Spectral resolution was 5 nm for both excitation and emission in these experiments.

The fluorescence of 5-HT or AA-5HT was measured at each of the above concentrations and at 10 μM or 40 μM in the presence of various concentrations of β-Lg used for reverse titrations. These spectra were recorded from 310 to 450 nm with an excitation wavelength of 300 nm. All fluorescence measurements were corrected by the same titration of medium without ligand. Spectral resolution for 5-HT and AA-5HT was 2.5 nm and 5 nm, respectively, for both excitation and emission. The observed fluorescence intensities were corrected for dilution in all fluorescence experiments.

Competitive experiments were performed to highlight the binding site of serotonin and its derivative. In these experiments, retinol which binds tightly to the internal cavity of the protein was used. In the first set of competitive displacement titration experiments, protein intrinsic fluorescence experiments were performed at constant β-LG/serotonin concentration (5 μM to produce a molar ratio of 1) titrated with different retinol concentrations, in order to attain at least a molar β-LG/retinol ratio of 20. The reverse titration experiments were performed at a constant molar ratio of β-LG/retinol equal to 1, titrated with different serotonin concentrations, in order to have at least a molar β-LG/serotonin ratio of 20. Emission spectra were recorded from 300 to 450 nm with an excitation wavelength of 280 nm and scan rate of 60 nm/min. The results of all measurements of fluorescence were corrected by the results of the same titrations of medium without β-LG. Spectral resolution was 5 nm for both excitation and emission in these experiments.

2.3.2. Ultrafiltration experiments

Ultrafiltration offers a convenient and rapid technique with numerous advantages over alternative methods such as conventional gel filtration chromatography. We used

Centricon cartridges for ultrafiltration to confirm serotonin binding by β-LG. When ultrafiltration was performed with a Centricon ultrafiltration device (Vivaspin 15, 10 kDa MWCO VS1501), free ligands were efficiently separated from the β-LG and β-LG/serotonin complexes. 2 mL solutions of β-LG alone, serotonin alone, β-LG/serotonin with molar ratios of 0.5, 1.0, and 2.0, and also unspecific casein/serotonin mixtures at molar ratio of 2.0 used as a control, were prepared. Absorbencies of each solution were measured before ultrafiltration at 280 nm. The solutions were ultrafiltered for 30 min at 1000 rpm and absorbencies of each solution for both filtrate and concentrate solution were checked again after adjusting to the initial volume 2 mL.

2.3.3. Circular dichroism spectroscopy

CD spectra were recorded on a CD6 dichrograph (Jobin Yvon, Longjumeau, France), using a scan time of 1 nm/s, 0.2 nm resolution, 10 accumulations, and 1.0 nm bandwidth. All measurements were carried out at 293 K with thermostatically controlled cell holders. The instruments were calibrated with ammonium d-10-camphorsulfonic acid. The data were expressed as molar ellipticity $[\theta]$ (with units of degrees cm^2 $decimole^{-1}$), which is defined as $[\theta] = 100\ \theta_{obs}/cl$, where θ_{obs} is the observed ellipticity in degrees, c is the molar concentration (mole L^{-1}), and l is the pathlength of the light in cm.

Far-UV (190-260 nm) and near-UV (250-320 nm) CD spectra were obtained at a constant protein concentration of 10 μM and 20 μM, respectively, titrated with 5-HT or AA-5HT reaching concentrations of 0, 5, 10, 20, 40, and 80 μM in 10 mM phosphate buffer, pH 7.4, or 10 mM glycine buffer, pH 2.0. Pathlengths were 0.05 cm and 1 cm for the far-UV and near-UV region, respectively. The blank background was subtracted from the raw spectra for each spectrum.

2.3.4. Determination of the binding affinity constants

In general, the complexes involving non-covalent bonds (and many others) are reversible. For example, the binding of retinol to retinol-binding protein is a function of the dynamic equilibrium between retinol and retinol-protein complexes [174]. Likewise, there may be equilibrium between free and β-Lg-bound 5-HT and AA-5HT. In the case of equimolar (1:1) complexes, the 5-HT and AA-5HT fluorescence titration data can be analyzed by the following equation [175, 176]:

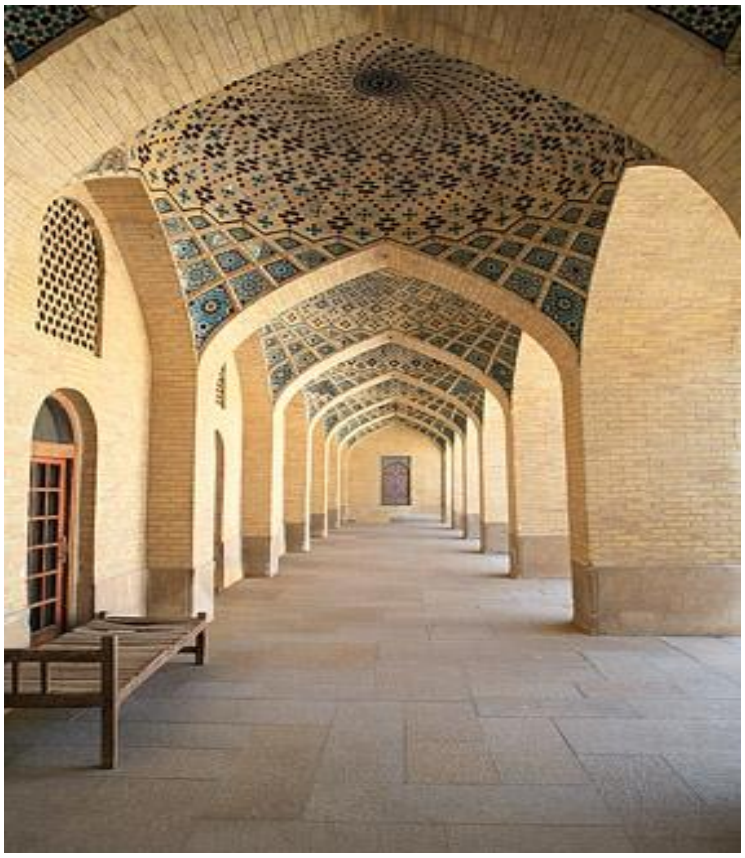
$$1/\Delta F = 1/\Delta F_{\max} + 1/(K_a \times \Delta F_{\max} \times [\beta - Lg] \quad (2\text{-}1)$$

where ΔF is the change in 5-HT or AA-5HT fluorescence intensity in the presence and absence of β-Lg, $\Delta F_{\max}$ is the maximal change in fluorescence intensity, K_a is the binding affinity constant, and $[\beta\text{-}Lg]$ is the concentration of β-Lg. According to this equation, the plot of $1/\Delta F$ as a function of $1/[\beta\text{-}Lg]$ will be linear. The intercept of this plot corresponds to $1/\Delta F_{\max}$ while the slope gives the estimate of the binding affinity constant, K_a. Otherwise, the binding constant and the binding number can be calculated according to the following equation [177, 178]:

$$\log[(F_0 - F)/F] = \log K_s + n \log[Ligand] \quad (2\text{-}2)$$

where F_0 and F are the fluorescence emission intensities of protein without and with ligand, respectively (after subtracting the fluorescence intensity of ligand alone in buffer solution). [*Ligand*] is ligand concentration, K_s is the binding constant, and n is the binding number. The plot of log $[(F_0\text{-}F)/F]$ as a function of log [*Ligand*] will be a linear plot with an intercept of log K_s and a slope of n.

Results and Discussion



3.1. Preparation of glycated β-Lg from bovine milk

We isolated β-Lactoglobulin variant A (β-Lg A) from the milk of a homozygous cow (for β-Lg A) by a salting out/in method according to Maillard and Ribadeau-Dumas precipitating the majority of whey protein and conserving uniquely β-Lg in solution [171]. After lyophilization, homogeneity of the protein preparation was assessed by high performance gel permeation chromatography (Figure 3.1). As shown in this Figure, the obtained preparations of β-lactoglobulin were over 98 % pure.

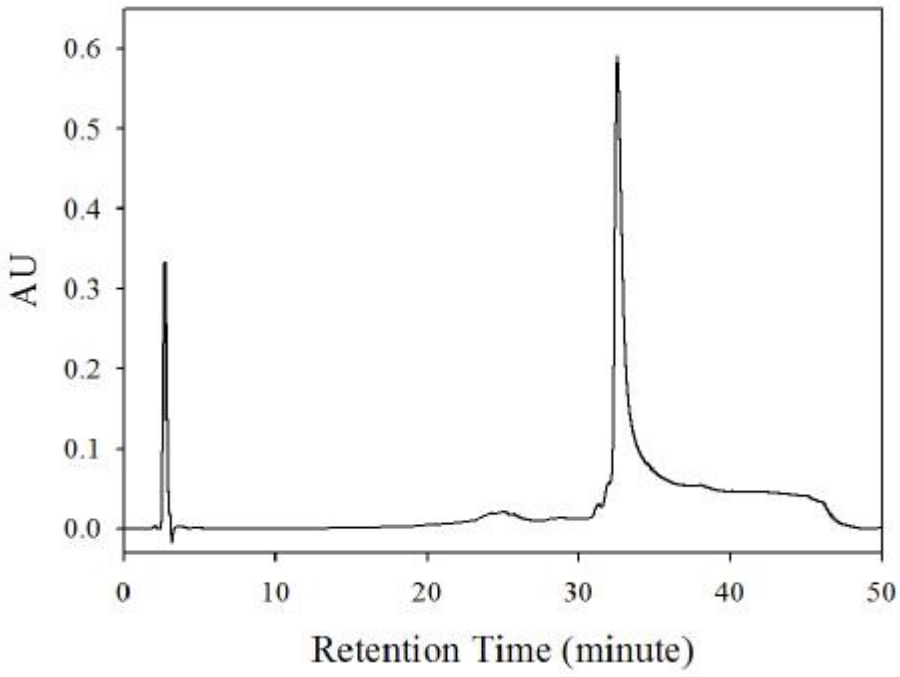


Figure 3.1. High performance gel permeation chromatography of β-Lg A isolated from the milk of a homozygous cow by a salting out/in method.

I used the obtained protein for preparation of glycated β-Lg according to the process described in experimental section (see part 2.1.3). This experiment was carried out in the presence of sugars which are present (or maybe present) in the cow milk in

various quantities. The used sugars in glycation reaction were lactose, ribose, glucose, galactose, arabinose, rhamnose that are shown in Figure 3.2.

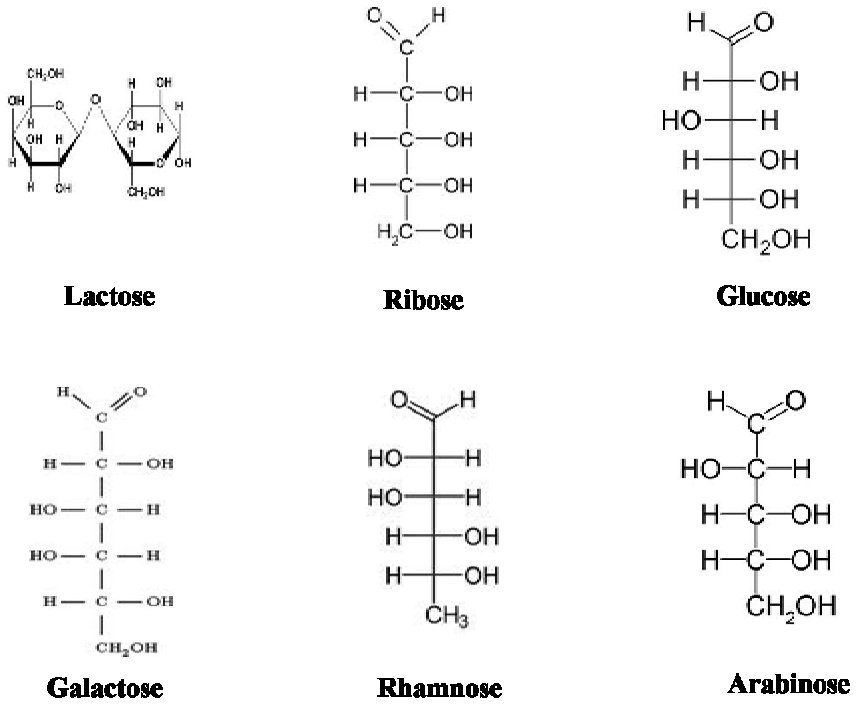


Figure 3.2. Molecular structures of six sugars used for the glycation experiments.

The Maillard reaction is not a simple unique reaction, but a complex series of reactions between amino acids and reducing sugars, usually quicker at increased temperatures. It is leading to the formation of advanced Maillard reaction products (AMPs) or advanced glycated end products (AGEs). In milk, Maillard reaction leads mainly to the Amadori products. In this reaction, the amino group of the basic amino acids reacts with reducing sugars and by loosing one H_2O molecule, produces Amadori product. The larger the sugar, the slower it will react with amino acids. The pentose sugars (5 carbon atoms), such as ribose, will react faster as hexose sugars (glucose) and disaccharides (lactose). From the amino acids, lysine, with two amino groups, reacts the fastest.

3.1.1. Determination of the modification degree and yield of glycation

A mild heating of the mixed solutions (protein and different sugars) at constant temperature of 60 °C continued for about 72 h. During this time, the mixed solutions in the flasks had a progressively intense to change to yellow color. This changing in the color of solutions was more obvious in the flasks containing β-Lg with arabinose or ribose, demonstarting the creation of more Advanced Glycation End products including melanoidins in comparison with other sugars. The control β-Lg, protein solution in the absence of any sugar, had a progressively intense to change to white opaque color (unclear solution). This changing in the color of control β-Lg solution was because of aggregation of β-Lg monomers.

The β-Lg protein has 15 ε-NH_2 and 1 α-NH_2 of lysine residues that are the most reactive amino groups in Maillard reaction. The OPA method has been utilized to deduce the number of modified amino groups and the degree of. This method needs a standard curve that was obtaind using L-leucine with a concentration range of 0.25-2.00 mM (Figure 3.3).

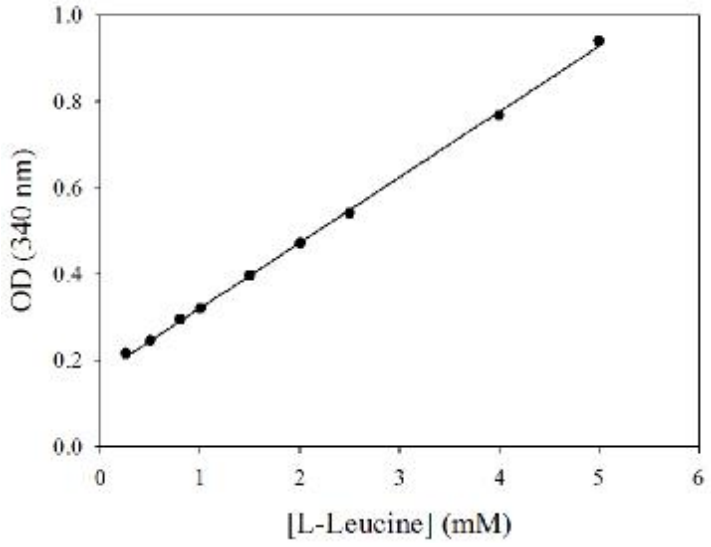


Figure 3.3. Standard curve obtained by using 0.25-2.00 mM L-leucine for OPA method.

According to this standard curve, the quantity of modified amino groups with the OPA method were obtained that are presented in Table 3.1.

There were not any modified amino groups on native β-Lg, hence, native β-Lg has been used as a reference to report all results relative to 100% of its amino groups. It is appeared that in control β-Lg, the protein solution without sugar, one lysyl group is modified (with glycation degree of 6.2 %). This results could be clarified with a conformational modification on the protein such as polymerization that could have a masking effect on one amino group. Approximately, the quantity of modified amino groups for glycated β-Lg in the presence of lactose, rhamnose, glucose, galactose, arabinose, and ribose were 5.5, 6.5, 6.6, 6.7, 8.8 and 11 amino groups, respectively. Therefore, a classification of sugars from fastest reactant to slowest one in glycation reaction on of β-Lg are as follows:

Ribose > arabinose > galactose ~ glucose ~ rhamnose > lactose

Table 3.1. Determination of the Glycation Degree of β-Lg as Determined by OPA Method.

Samples	Modified amino groups	Glycation degree (%)
Native β-Lg	0	0
Heated β-Lg	1.0 (±0.1)	6.2 (±1.0)
β-Lg/Lac	5.5 (±0.4)	34.4 (±2.5)
β-Lg/Rha	6.5 (±0.5)	40.6 (±3.1)
β-Lg/Glu	6.6 (±0.4)	41.2 (±2.5)
β-Lg/Gal	6.7 (±0.3)	41.9 (±1.9)
β-Lg/Ara	8.8 (±0.6)	55.0 (±3.7)
β-Lg/Rib	11.1 (±0.5)	69.4 (±3.1)

According to previous results, the modification degree in glycation reaction depende directly on the size of the sugar, as estimated [179, 180]. The sugars with shorter carbonic chain will react faster with amino groups of proteins in glycation

reaction because the sugars with shorter carbonic chain have released structure that can react faster with proteins.

3.2. Preparation of recombinant wild type and Cys121Ser mutant β-Lg

The expression of each recombinant β-Lg was tested in a culture of several clones in liquid medium followed by the quantification of the protein in the supernatant SDS-PAGE (Figure 3.4). The best producer of each protein was selected for large quantity production.

The recombinant proteins were produced in flasks. The use of flasks has the advantage of simplicity but it only allows limited control culture conditions (pH, oxygen and nutrients). Culture tests of P. pastoris in flasks showed that yeast grew very quickly (OD_{600} = 30 after 24 h). After 3 days, high concentrations in yeast, about 60 units of OD_{600} were obtained. This was accompanied by significant expression proteins.

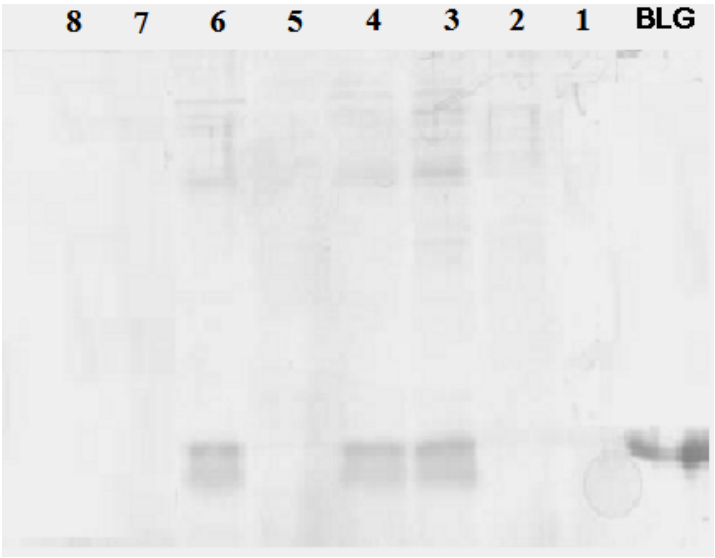


Figure 3.4. SDS-PAGE of culture for recombinant β-Lg in the presence of 2-mercaptoethanol for selecting best colonies. In this figure the best colonies were selected from numbers 3, 4 and 6.

3.2.1. Purification of recombinant proteins

The BMGY medium was used as a culture for production of proteins by yeast during 3 days. In the used culture conditions, β-Lg was the main protein present in the supernatant. The recombinant β-Lg was purified from the culture supernatant separated from yeast cells by centrifugation. Then the culture medium was filtered to remove all remaining cells. The ultrafiltration steps of the supernatant were performed to concentrate our production and to remove small compounds, salts, peptides, and sugars. The supernatant was dialyzed overnight and then purified by ion exchange chromatography.

3.2.1.1. Ion-exchange chromatography

The β-Lg is negatively charged at neutral pH (pI = 5.2), it can be purified by an anion exchange column. Preliminary tests showed that in our conditions (see Materials and Methods) β-Lg was eluted at NaCl concentration of 0.25 M, approximately. The conductivity of the supernatant after dialysis was generally between 20 and 30 mS cm^{-1}, corresponding to NaCl concentration of 0.2 to 0.3 M. In order to retain β-Lg on the resin, the supernatant was diluted to a conductivity of less than 10 mS cm^{-1} and then loaded onto the column. The elution was performed with concentrations of NaCl Growing: 0.1-0.3-0.5 to 1 M (see Figure 3.5).

The analysis of different fractions by SDS-PAGE confirmed the presence of recombinant β-Lg in eluted fractions at NaCl concentration of about 0.3 M (see Figure 3.6). These fractions also contained small amounts of compounds of high molecular masses. It was concentrated before further purification by size exclusion chromatography. After concentration, the resulting solution was strongly colored (brown).

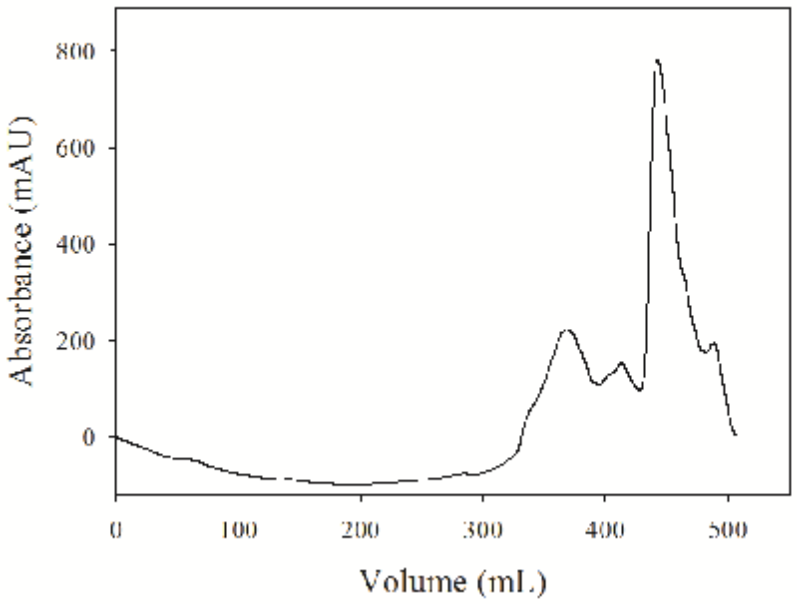


Figure 3.5. Elution profile of culture supernatant of recombinant β-Lg by anion exchange chromatography. Different fractions corresponding to each peak were analyzed by SDS-PAGE electrophoresis.

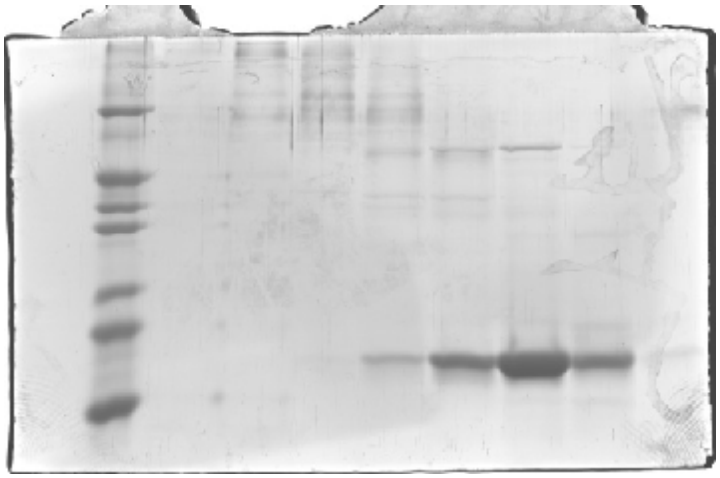


Figure 3.6. SDS-PAGE analysis of different fractions of recombinant β-Lg after anion exchange chromatography. Further purification of protein is needed corresponds to this figure. The first line is molecular mass marker.

3.2.1.2. Size exclusion chromatography

The concentrated fraction after the ion exchange chromatography was subjected to size exclusion chromatography. The elution profile consisted of 3 zones (Figure 3.7). Their analysis by SDS-PAGE (Figure 3.8) revealed that the first area was mostly composed of high molecular weight aggregates of β-Lg; the second zone contained the purified β-Lg; and samples eluted in the last zone, strongly colored, contained non-proteic compounds because they have a strong absorption in the UV but their spectrum is not typical of that of a protein and they do not appear on SDS-PAGE.

A study of these compounds by Denton et al. [72] showed that they are probably of carbohydrate nature.

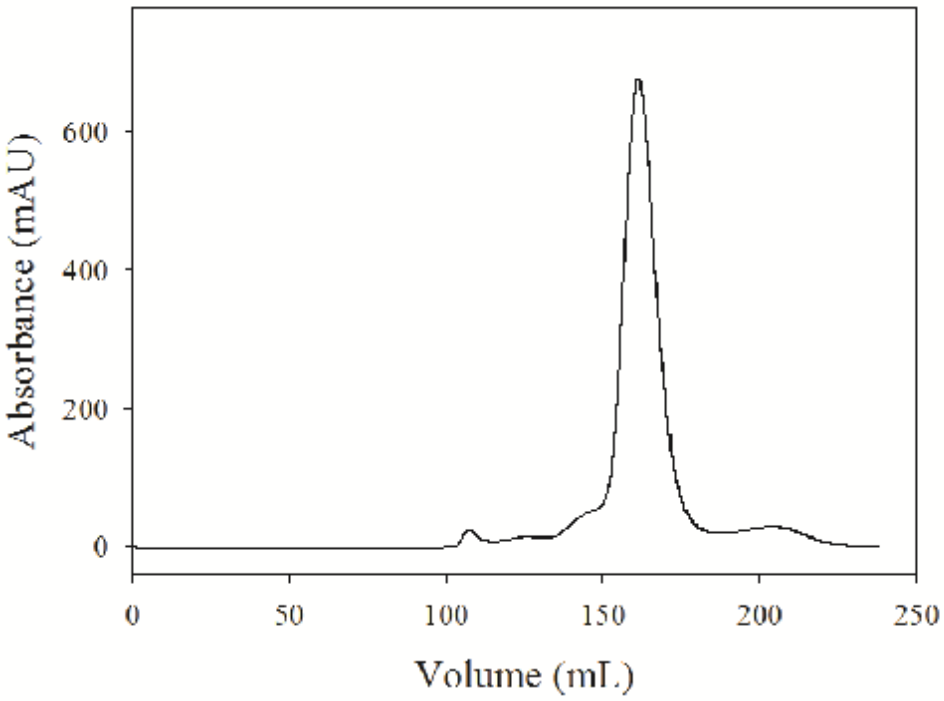


Figure 3.7. Elution profile of concentrated fractions of recombinant β-Lg after anion exchange chromatography by size exclusion chromatography. Different fractions corresponding to each peak were analyzed by SDS-PAGE electrophoresis.

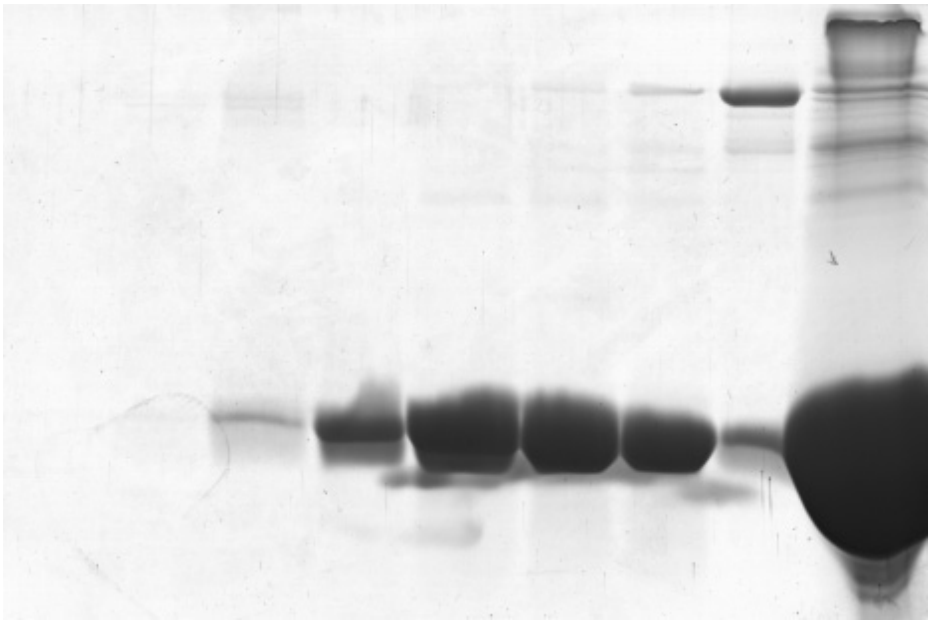


Figure 3.8. SDS-PAGE analysis of different fractions of recombinant β-Lg after size exclusion chromatography. Purified proteins are exist in lines 4, 5, 6 and 7. The first line is control concentrated protein after anion exchange chromatography.

The second fraction containing the β-Lg with a degree of purity (> 95 %), was dialyzed against ultrapure water and lyophilized. Our protocol allowed the proteins to be expressed at a concentration of about 70 mg/L of culture medium, what is in agreement with previously described yields for recombinant β-Lg production in shake flasks [71, 173]. The yield of purified protein was 50 mg/L of culture medium. All proteins were stored at-20 $^{\circ}$C for further experiments.

3.3. Characterization of heated and glycated β-Lg by electrophoresis

We have done polyacrylamide gel electrophoresis under Native-PAGE and SDS-PAGE (reducing and non-reducing) conditions to recognize the structural changes of glycated proteins and heated proteins during heat stress at different temperatures 65, 75, 85 and 95 $^{\circ}$C.

3.3.1. Native-PAGE results of heated β-Lg

The alkaline (native)-PAGE patterns of β-Lg forms when they were heated at temperatures 65, 75, 85 and 95 °C are shown in Figure 3.9. In the alkaline-PAGE system, the heated protein samples separated into a series of bands and some less well-defined areas of stained protein. The most mobile band in the gel lane was indistinguishable from that of the unheated native protein and can be termed "nativelike". The aggregations pattern is clear in the alkaline-PAGE that is belong dimer, trimer, and etc. protein. In the other hand, when β-Lg solutions were heated, non-native monomer β-Lg that were less mobile in alkaline-PAGE than monomer but more mobile than dimer were formed. These non-native monomer proteins were more common by increasing temperature and the overall formation of large aggregates was greatest for protein heated at 95 °C and least for β-Lg native.

3.3.2. SDS-PAGE results of heated β-Lg

SDS-PAGE stands for sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) and is a method used to separate proteins according to their size. Since different proteins with similar molecular weights may migrate differently due to their differences in secondary, tertiary or quaternary structure, SDS, an anionic detergent, is used in SDS-PAGE to reduce proteins to their primary (linearized) structure and coat them with uniform negative charges. Besides the addition of SDS, proteins may optionally be briefly heated to near boiling in the presence of a reducing agent such as 2–mercaptoethanol, which further denatures the proteins by reducing disulfide linkages, thus overcoming some forms of tertiary protein folding, and breaking up quaternary protein structure (oligomeric subunits). This is known as reducing SDS-PAGE, and is most commonly used. Non-reducing SDS-PAGE (no reducing agent) may be used when native structure is important in further analysis. It has been demonstrated that in SDS-PAGE under non-reducing conditions, just hydrophobic

interactions were disappeared, while in reducing conditions, the disappearance of hydrophobic interactions and disulfide bonds damaging togather has been observed. Comparison between SDS-PAGE results under reducing and non-reducing conditions allows us to establish the various types of bonds stabilizing aggregated proteins.

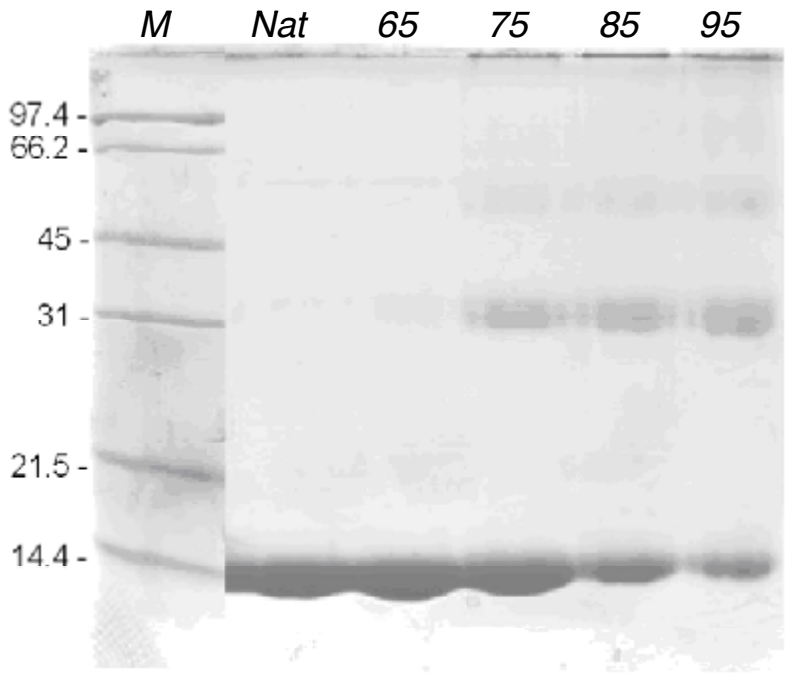


Figure 3.9. Native-PAGE patterns of β-Lg A that had been heated at different temperatures in 100 mM PBS buffer pH 7.4. Native β-Lg (line Nat) and β-Lg heated for 20 min at 65, 75, 85, and 95 °C (lines 65-95) in PBS buffer were analyzed on native-PAGE. M, molecular mass marker (kDa).

When heated β-Lg samples in non-reducing conditions (without 2−mercaptoethanol) were analyzed by 12 % SDS-PAGE (Figure 3.10), there was a band corresponding to the monomer (on the basis of the molecular weight standards) and a number of bands in a series of regions corresponding to decreases in mobility and increases in apparent molecular weight. Analysis of this SDS-PAGE results showed that

until 65 °C, β-Lg is present mostly in a monomeric form in PBS solution at pH 7.4 (band at about 14.4 kDa on the basis of the standards). A small proportion of β-Lg was also dimeric (band slightly higher than 31 kDa on the basis of the standards). After heating at higher temperatures the proportion of dimers increased and additional bands corresponding to larger polymers (trimers, tetramers, …) appeared. Although there were sharp bands at higher temperatures, they were high molecular weight aggregates that could not enter the resolving and stacking gels.

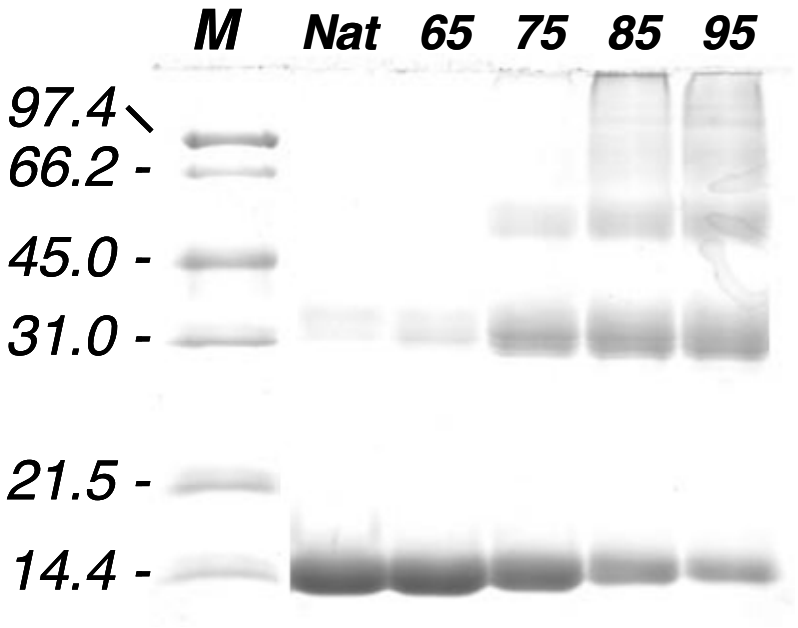


Figure 3.10. Heat-induced polymerization of β-Lg A that had been heated at different temperatures in 100 mM PBS buffer pH 7.4. Native β-Lg (line Nat) and β-Lg heated for 20 min at 65, 75, 85, and 95 °C (lines 65-95) in PBS buffer were analyzed on 12 % SDS-PAGE in non-reducing condition. M, molecular mass marker (kDa).

When the heated samples were reduced with excess 2–mercaptoethanol prior to electrophoresis (reducing condition) the only bands present were those corresponding to unfolded monomer protein (Figure 3.11), which migrated slightly more slowly than the

most mobile band in the unreduced protein samples. This indicated that the only heat-induced covalent bonds present were reducible by 2–mercaptoethanol and consequently were probably disulfide bonds between Cys residues. Furthermore, as it shown in Figure 3.11, under reducing conditions β-Lg migrated as a unique band indicating that heat-induced polymerization involves disulfide bond formation. These data are in agreement with what is already well known. Heat-induced denaturation and aggregation of β-Lg by the formation of disulfide bonds occur when it is heated at temperatures higher than 70-72°C (transition temperature) [142]. However, this allowed checking that in the samples used for ELISA, β-Lg structure has been effectively changed.

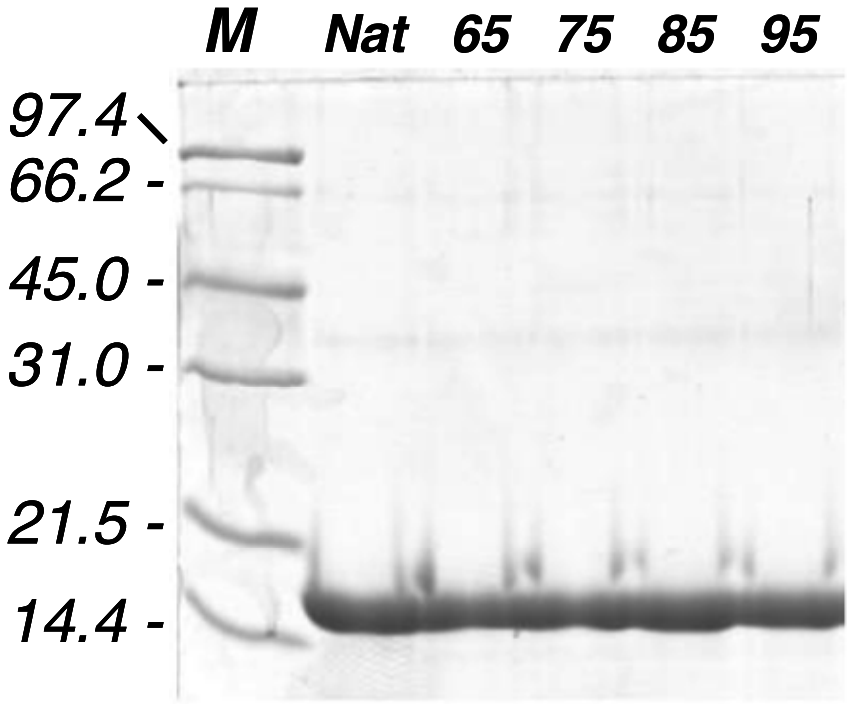


Figure 3.11. Heat-induced polymerization of β-Lg A that had been heated at different temperatures in 100 mM PBS buffer pH 7.4. Native β-Lg (line Nat) and β-Lg heated for 20 min at 65, 75, 85, and 95 °C (lines 65-95) in PBS buffer were analyzed on 12 % SDS-PAGE in reducing condition. M, molecular mass marker (kDa).

3.3.3. SDS-PAGE results of glycated β-Lg

SDS-PAGE electrophoresis was utilized to follow changes in the molecular masses of β-Lg during glycation experiments in the presence of various sugars. Figures 3.12 and 3.13 illustrate the strong effect of glycation on β-Lg. On SDS-PAGE, in non-reducing conditions (Figure 3.12), a band at about 31 kDa corresponding to β-Lg dimers was present. In reducing conditions (Figure 3.13), this band disappeared for non-glycated β-Lg but persisted in the case of glycated β-Lg. Consequently, in comparison with native β-Lg, glycated β-Lg is also able to form covalent dimers that do not involve disulfide bond formation.

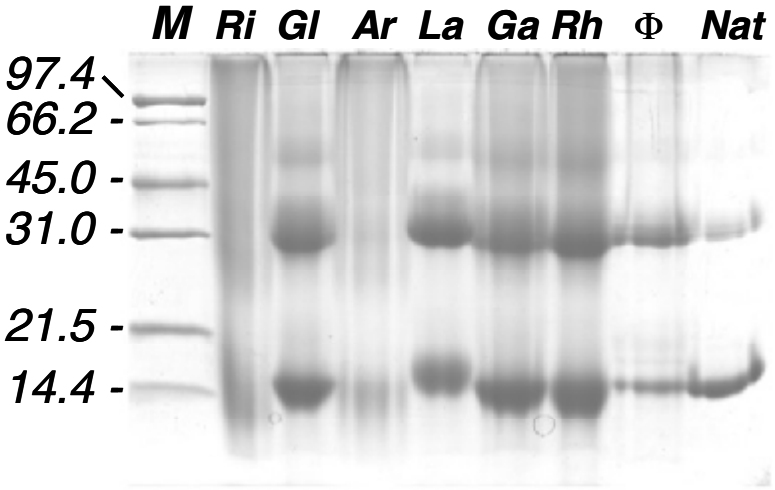


Figure 3.12. Polymerization of glycated β-Lg A in 100 mM PBS buffer pH 7.4. β-Lg glycated with ribose (Ri), glucose (Gl), arabinose (Ar), lactose (La), galactose (Ga), and rhamnose (Rh) as compared with native β-Lg (Nat) and β-Lg heated for 72 hours at 60 °C in the absence of sugar (Φ) were analyzed on 12 % SDS-PAGE in non-reducing condition. M, molecular mass marker (kDa).

During heat treatments on β-Lg in constant temperature of 60 °C in the presence of different sugars, a number of bands matching to 18, 36, 67 kDa and higher molecular masses were found. Yet, the corresponding bands started to decrease with increasing the

degree of glycation, demonstrated that the heterogeneity of the molecular masses for glycated proteins was apparently enhanced.

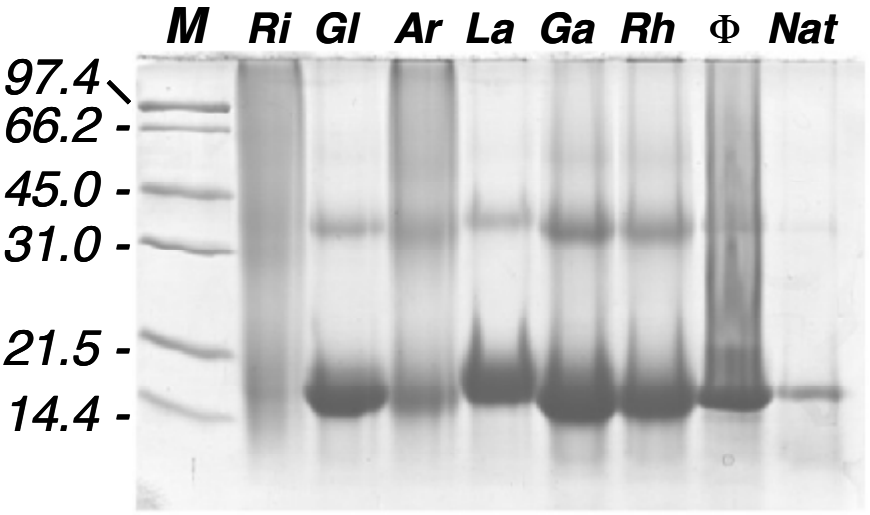


Figure 3.13. Polymerization of glycated β-Lg A in 100 mM PBS buffer pH 7.4. β-Lg glycated with ribose (Ri), glucose (Gl), arabinose (Ar), lactose (La), galactose (Ga), and rhamnose (Rh) as compared with native β-Lg (Nat) and β-Lg heated for 72 hours at 60 °C in the absence of sugar (Φ) were analyzed on 12 % SDS-PAGE in reducing condition. M, molecular mass marker (kDa).

In the case of substitution by ribose and arabinose (sugars inducing the highest degree of modification), a smear was observed in both conditions, indicating that substituted proteins form polymers stabilized by sugar induced covalent bonds. Consequently, it could be declared that in glycation experiments, all the formed glycated proteins were not stabilized only via disulfide bridges. It should be considered also covalent bonds including sugar cross-links in the stabilization of glycated proteins. Full modifications induced by glycation, including conformational changes, have been analyzed in detail by Chevalier et al. [181, 182].

3.4. Characterization of heated and glycated β-Lg by circular dichroism

A more accurate characterization of β-Lg samples was achieved by circular dichroism spectra measurements, which allowed monitoring of the folding changes of modified β-Lg.

The differences between the absorption of left-handed circularly polarised light (LCPL) and right-handed circularly polarised light (RCPL) is known as circular dichroism. It takes place in molecules with one or several chiral chromophores (that can absorb light). Therefore:

Circular dichroism = $\Delta A(\lambda) = A(\lambda)_{LCPL} - A(\lambda)_{RCPL}$,

where λ indicated the wavelength [183].

CD spectroscopy is a method of measuring the Circular dichroism of molecules in a range of wavelengths of 185-350 nm. this technique is utilized widely to do investigation on all types and sizes of chiral molecules. But the most important application of CD is to study large physiological macromolecules. The main application of this technique is in analyzing the secondary structure constituent of macromolecules, predominantly proteins. A lot of factors can affect the secondary structure of macromolecules including environmental conditions e.g. temperature and pH. In order to find the changes in the secondary structure of macromolecules due to changes in the environmental conditions or interaction of macromolecules with ligands, circular dichroism could be utilized. Additional informations including structural, kinetic and thermodynamic informations about macromolecules could be obtained from circular dichroism spectroscopy [183].

The electronic transitions could be monitored on the bases of experiments occurred in the visible and ultra-violet area of the electromagnetic spectrum. A chiral chromophore on the studied molecule can absorb one CPL state more than the other one and therefore the CD signal will be produced over the corresponding wavelengths. The

CD signal can be positive, if LCPL is absorbed more than RCPL, or negative, if LCPL is absorbed more than RCPL [183].

3.4.1. Far-UV circular dichroism results of heated β-Lg

The secondary structure of the proteins could be reflected principally by far-UV CD spectrum. Far-UV CD signal is derived and take places from the absorption of peptide bonds and the inherent chirality of the polypeptide chain [184, 185], and an analysis of the obtained spectra can give relative proportions of helical, sheet, etc. structure. However, Trp side chains and disulfide bonds can also contribute to the far-UV spectra [185]. Earlier analyses [102, 186] consistently indicate that the native β-Lg protein has ~ 40 % β-sheet and ~ 10 % α-helix; these values are supported by X-ray crystal structures [51, 52, 57, 63].

Far-UV CD spectra have been used to follow the evolution of the secondary structure of β-Lg when submitted to heat treatment at pH 7.4. The effect of heat treatment of β-Lg on the far-UV CD spectra is shown in Figure 3.14. The trough, with a minimum at 216 nm, gradually broadened and deepened so that the minimum shifted to lower wavelengths, indicating the loss of secondary structure between 65 and 75 °C, as already observed [102, 187]. The results of deconvolution of the CD spectra and calculation of α-helix and β-structure using Chou and Fasman method was judged to give the most close assessment of the secondary structure. Contents of α-helix and β-sheet elements in β-Lg during heating processes evaluated by deconvolution of their CD spectra are presented in Table 3.2. As it shown in this table and Figure 3.14, the α-helix and β-sheet structures were progressively converted to aperiodic structure.

Griffin *et al*. [186] interpreted their results to indicate that the β-sheet content of β-Lg A was essentially unaltered by heat treatment. Qi *et al*. [102], using CD results extending to 170 nm together with Fourier transform infrared results, concluded that the

α-helix content decreased from ~ 11 to ~ 2 % (with the midpoint transition at ~ 60 °C) and the β-sheet content decreased from 50 to 42 % (with the midpoint temperature at about 50 °C) as consequences of the increased temperature of the β-Lg A solution. In contrast, Prabakaran and Damodaran [187], who measured β-Lg far-UV CD at temperatures between 26 and 81 °C to obtain spectra that were qualitatively similar to those reported by Qi et al. [102], concluded that the α –helix content was ~ 19 % for β-Lg A and B solutions and was unaffected by temperatures between 26 and 81 °C and that the β-sheet content of β-Lg A decreased from 60 to 20 % at 81 °C, whereas the decrease for β-Lg B was significantly smaller. Comparison of the difference between the 46 and the 86 °C spectra for β-Lg A with those reported for β-Lg A by Prabakaran and Damodaran (1997) shows the same trend, with a more pronounced trough at ~ 207 nm [187].

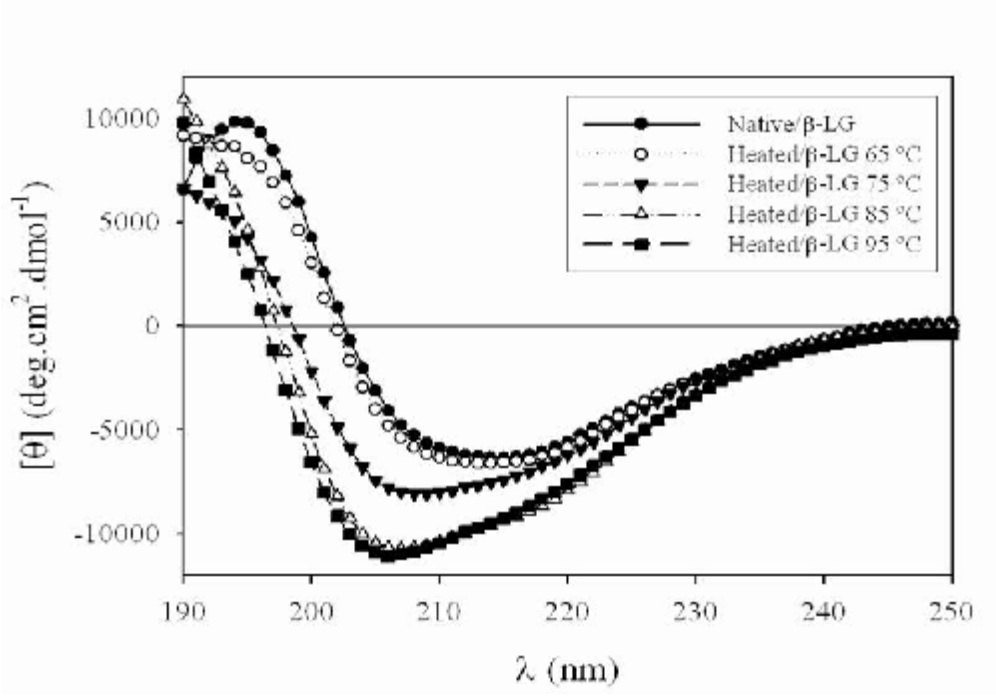


Figure 3.14. Far-UV circular dichroism spectra of native and heated β-Lg.

It is quite possible that the sheet content of β-Lg decreases when the protein (native or denatured) is at a temperature > 45 °C and increases again when the temperature is lowered; this would be in accord with the suggestions [102, 188] that β-Lg at high temperature may have characteristics with some similarity to those of the molten globule conformation. Alternatively, this effect could be a consequence of the decreased ratio of native dimers to monomers in the mixture and the loss of H-bonding involving the I-strands of β-Lg. Addition of chaotropic solutes, such as urea or guanidinium salts, diminishes the broad trough centered on 216 nm [189] as a consequence of the loss of most of the helical and sheet secondary structure. Consequently, there must be a considerable quantity of secondary structure present in the heat-treated protein samples.

Table 3.2. Native and Heated β-Lg Sequence Secondary Structure Based on Chou and Fasman Method (Dicroprot 2000).

Protein	Helix	Sheet	Other
Native	14	51	35
65 °C	13	48	39
75 °C	16	37	47
85 °C	18	26	56
95 °C	17	24	59

Previously, Belloque and Smith [103] demonstrated by NMR spectroscopy that the amide protons on Ile^{56}, Leu^{57}, Leu^{103}, Leu^{104}, Phe^{105}, Cys^{106}, Met^{107}, and Val^{123} did not exchange with deuterium atoms of D_2O (that was in the solvent) at 75 °C at pH 2, indicating the continued H-bonding of the β-strands β-C and β-G to the adjacent strands and that these two pieces of the β-sheet structure remain intact during the heat treatment

rather than coming apart, even transiently, at the higher temperatures and reforming when the solutions are cooled.

3.4.2. Near-UV circular dichroism results of heated β-Lg

Near-UV CD arises from the chirality of the environments of the side chains of the amino acid residues that have absorption bands in the near-UV, namely, the aromatic residues Trp, Tyr, and Phe and disulfide bonds [185, 190, 191]. The two small troughs at about 262 and 269 nm originated from the various Phe residues, and the two deep troughs at 286 and 293 nm are believed to arise from the ^1L_b transitions from one or both of the Trp residues [190].

The main chromophores in the near-UV CD are tryptophan, tyrosine, and phenylalanine [190]. Near-UV CD spectra allow us to characterize the tertiary structure of proteins mainly due to the asymmetries in the environment of the aromatic amino acids and to characterize the stability of their conformers. β-Lg contains two tryptophans (Trp 19 and Trp 61). It is known that Trp 61 is on the surface of the protein and has considerable rotational freedom, which together with Trp19 is the source of the near-UV CD signals at 286 and 293 nm [52, 57, 63].

Figure 3.15 demonstrates that heat treatment of β-Lg induces intensity decreasing of the deep troughs observed at 286 and 293 nm, confirming other recent findings [90, 192]. The changes in [θ] at 286 and 293 nm that were most probably due to the changes in the environment of Trp 19, were likely to reflect irreversible structural changes that occurred within the calyx of the β-Lg molecule as a result of heat treatment. However, even after heating at the highest temperatures shown in Figure 3.15, [θ] between 260 and 320 nm was greater than that of β-Lg in 7.3 M urea solution at pH 6.7 [189] or in 4 M guanidine hydrochloride solution at pH 3.2 [193], indicating that the chromophores were in chiral environments and suggesting that some ordered tertiary structure was present in the proteins after heat treatment.

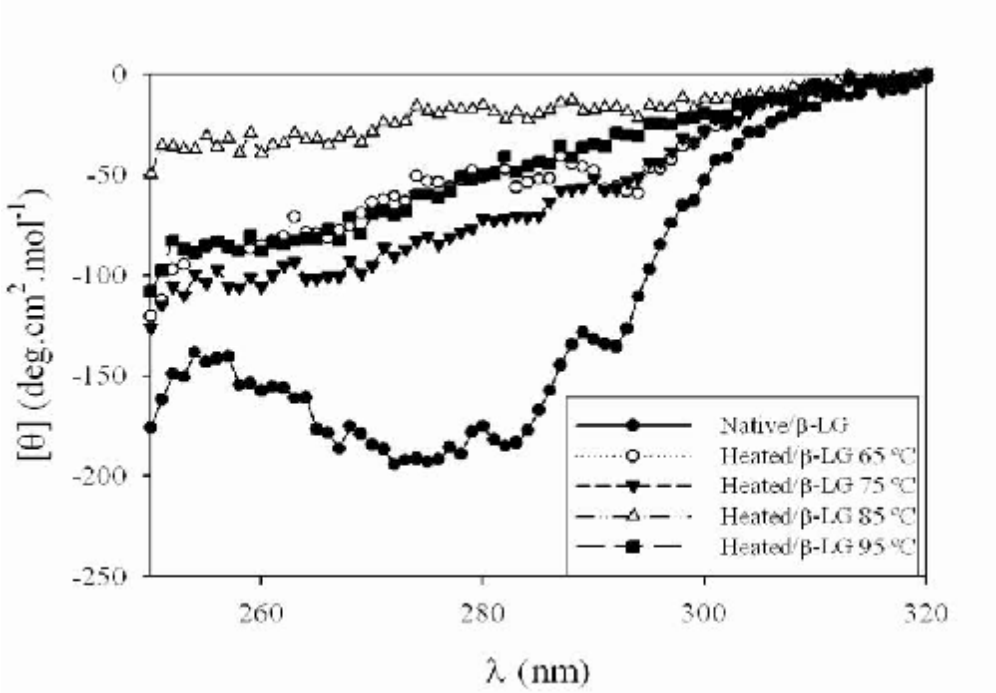


Figure 3.15. Near-UV circular dichroism spectra of native and heated β-Lg.

To conclude, these results confirm that the tertiary and secondary structures of heated β-Lg used for assaying IgE binding were progressively altered by heating.

3.4.3. Far-UV circular dichroism results of glycated β-Lg

The source of far-UV CD signal is from the absorption of peptide bonds and the secondary structure of proteins can be indicated from this signal. Provencher and Glockner showed that [194], the secondary structure of native β-Lg (Figure 3.16) contains ~ 47 % of β-sheet and ~ 14 % of α-helical structure. Aalthough, in comparison with native β-Lg, the intensity of the signal was decreased slightly in case of heated protein, it could not be observed a global secondary structure disorganization, indicating that the secondary and tertiary structure of β-Lg is still retained after heat treatments.

In the case of native and heated β-Lg, a minimum could be observed at wavelength of 216 nm that transferred to lower wavelengths in the case of glycated samples. This shift is more obvious at wavelength of about 207 nm, particularly in the

case of arabinosylated, galactosylated, glucosylated, and ribosylated β-Lg. On the contrary, the comparison between native β-Lg and β-Lg glycated with lactose and rhamnose illustrated the similar profile in the far-UV spectra (Figure 3.16). A comparison of CD spectra of native and heated β-Lg with glylated proteins proved that ribosylated β-Lg was the most modified protein, indicating important changes on the secondary structure of β-Lg during glycation with ribose. No near-UV CD experiments could be done for glycated proteins because during Maillard reactions a lot of chromophores could be produced.

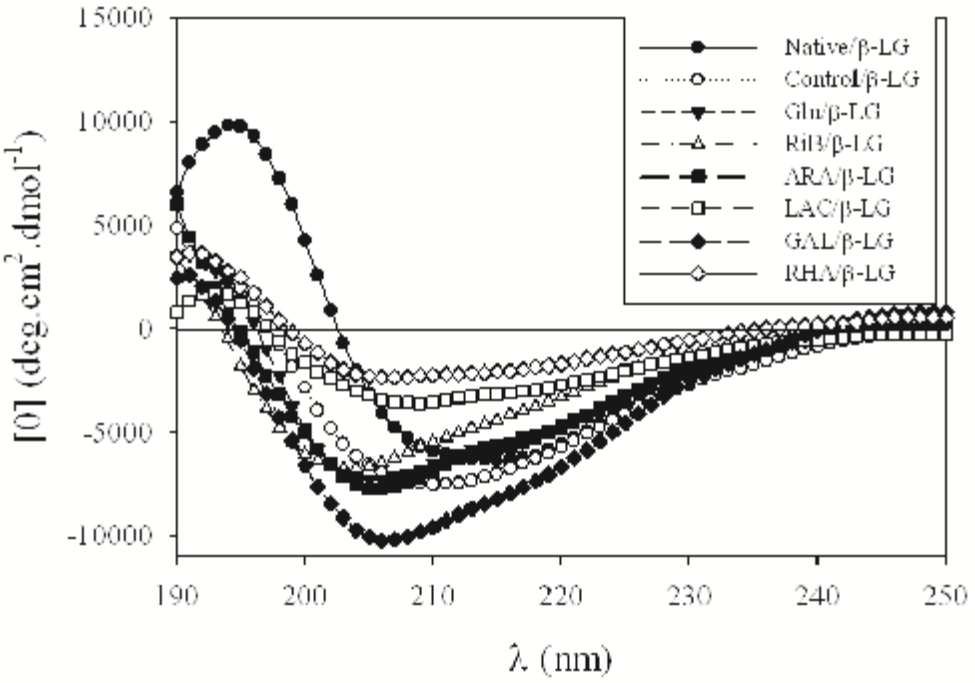


Figure 3.16. Far-UV circular dichroism spectra of native and glycated β-Lg. β-Lg glycated with ribose (Rib), glucose (Glu), arabinose (Ara), lactose (Lac), galactose (Gal), and rhamnose (Rha) were compared with native β-Lg and β-Lg heated for 72 hours at 60 °C in the absence of sugar (control).

The results of deconvolution of the CD spectra and calculation of α-helix and β-sheet structure using Chou and Fasman method was judged to give the closest

assessment of the secondary structure. Contents of α-helix and β-sheet elements in β-Lg during heating processes evaluated by deconvolution of their CD spectra are presented in Table 3.3. The results presented in this table confirmed the results obtained in Figure 3.16 that the most modified protein was ribosylated-β-Lg in comparison with native and heated β-Lg spectra, indicating a very significant modification on the secondary structure of this modified protein.

Table 3.3. Native and Glycated β-Lg Sequence Secondary Structure Based on Chou and Fasman Method (Dicroprot 2000).

Protein	Helix	Sheet	Other
Native β-Lg	14	51	35
β-Lg/Lac	17	44	39
β-Lg/Rha	13	46	41
β-Lg/Glu	23	32	45
β-Lg/Gal	26	35	39
β-Lg/Ara	32	11	57
β-Lg/Rib	39	3	58

3.5. Characterization of recombinant wild type and mutant β-Lg

3.5.1. SDS-PAGE and HPLC results of recombinant β-Lg

The purity of recombinant proteins was monitored by reverse phase HPLC and by SDS-PAGE. According to the integration of chromatographic peaks, all proteins were obtained with purity above 95 % (Figure 3.17). The electrophoresis profile of the wild type and the Cys121Ser mutant recombinant proteins in reducing conditions showed the existence of a single band corresponding to the β-Lg monomer and confirmed the purity of the samples (Figure 3. 18).

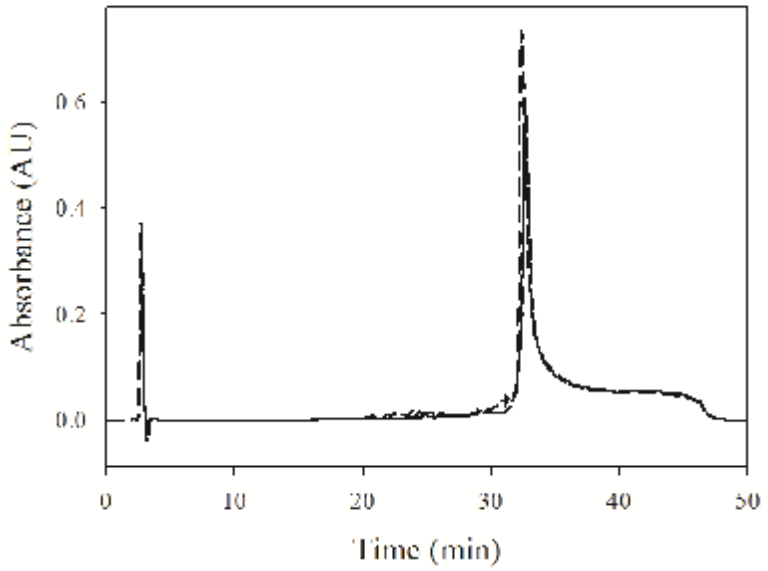


Figure 3.17. Elution profile in reverse phase HPLC of purified β-Lg A, recombinant WT and Cys121Ser mutant β-Lg. The retention time of 3 proteins is the same and all proteins were obtained with the purity of over 95 %.

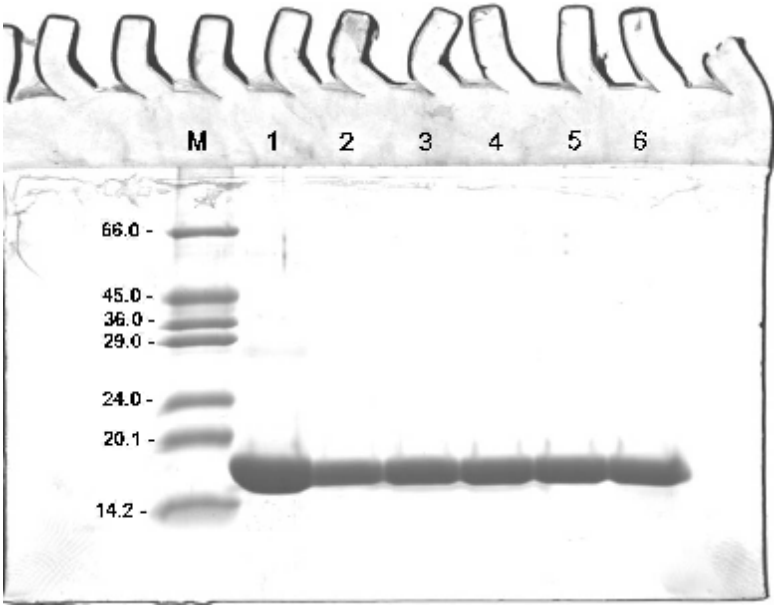


Figure 3.18. The electrophoresis profile of the recombinant proteins in reducing conditions confirmed the purity of the samples. Native β-Lg (lines 1 and 2), WT/β-Lg (lines 3 and 4) and Cys121Ser mutant/β-Lg (lines 5 and 6) were analyzed on SDS-PAGE. M, molecular mass marker (kDa).

3.5.2. Mass spectroscopy results of recombinant β-Lg

The identity of the proteins was confirmed by mass spectrometry. The following calculation method has been used to calculate molecular weight of proteins by mass spectroscopy:

Consider that:

$$m_1 = (M + Z) / Z \quad (3\text{-}1)$$

$$m_2 = (M + Z + 1) / (Z + 1) \quad (3\text{-}2)$$

which m_1, m_2, M and Z are the molecular weight corresponding to the first fragment (peak), the molecular weight corresponding to the next fragment after first one, the molecular weight of protein and the charge of each fragment, respectively. Combining equations 3-1 and 3-2 gives:

$$Z = (m_2 - m_1)/(m_1 - m_2) \quad (3\text{-}3)$$

$$M = (m_1.Z) - Z \quad (3\text{-}4)$$

That allowed us to calculate the molecular weight of protein. For example in the case of native β-Lg in figure 3.19, we consider m_1 and m_2 are 1529.9 and 1412.3, respectively. Using equation 3-3 gives:

$$Z = (1412.3 - 1) / (1529.9 - 1412.3) = 12$$

So the Z corresponding to fragment 1529.9 is 12 and for other fragments 1668.7, 1835.1, 1412.3 and 1311.9 is 11, 10, 13 and 14 respectively. By calculating Z, it is possible to find M using equation 3-4:

$$M = (1529.9 \times 12) - 12 = 18346.6 \text{ Da}$$

The same calculation method has been used for recombinant WT and Cys121Ser mutant β-Lgs and the results for M was 18353.5 Da and 18333.7 Da for WT and mutant β-Lgs, respectively. So, analysis of recombinant proteins by mass spectrometry (Figure 3.19) has confirmed their purity, matching the calculated molecular mass with their mass theoretical, and the lack of post-translational modifications.

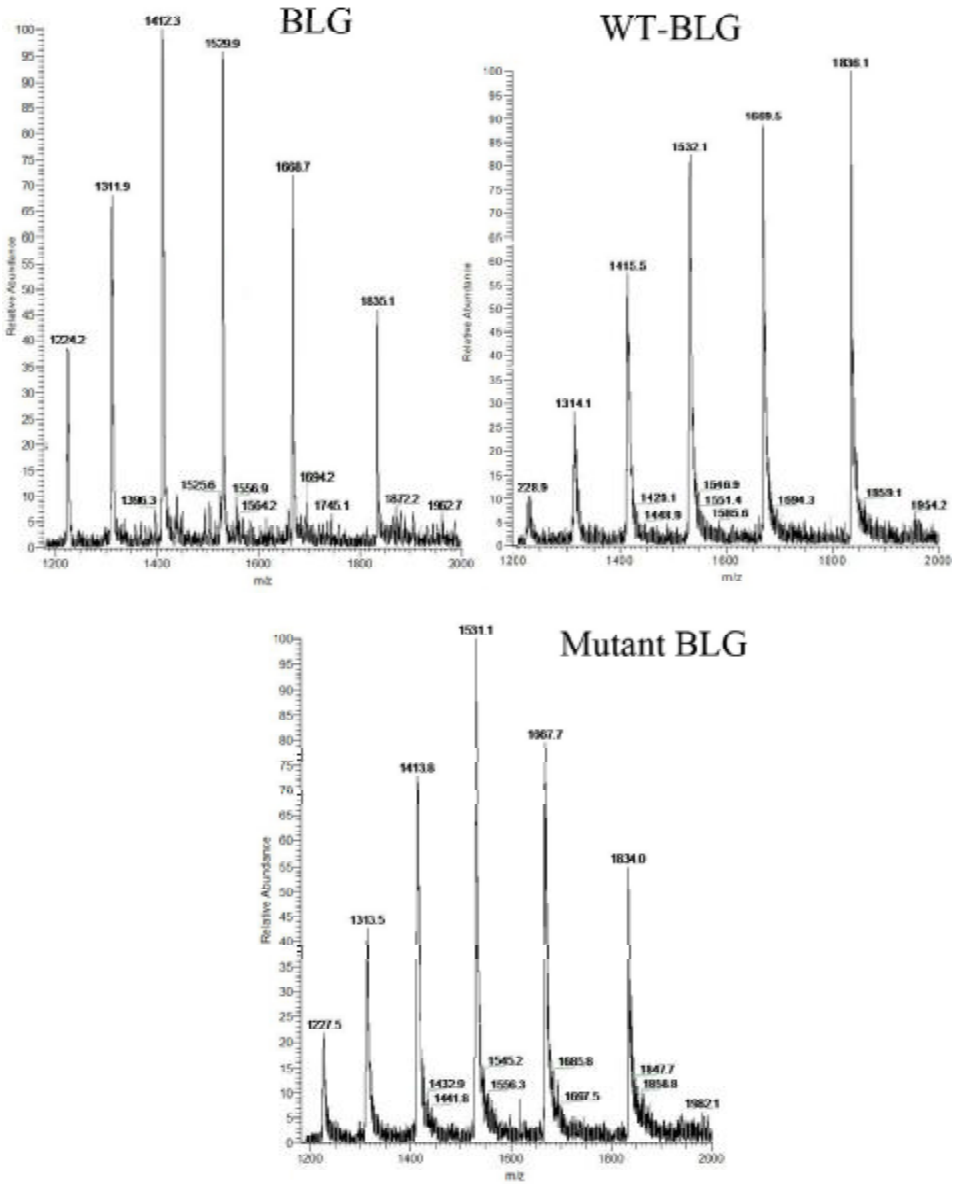


Figure 3.19. Mass spectroscopy results of native β-Lg, WT recombinant β-Lg and Cys121Ser mutant recombinant β-Lg. This figure has confirmed the purity of obtained proteins, matching the calculated molecular mass with their mass theoretical.

3.5.3. Circular dichroism results of recombinant β-Lg

The secondary structure of the recombinant β-Lgs was analyzed by far-UV CD. The spectra of WT and Cys121Ser were identical to that of the standard (Figure 3. 20 A), showing that the recombinant β-Lgs had the same secondary structure as native β-Lg.

The near-UV CD spectrum of WT was also very similar to that of the standard (Figure 3.20 B), indicating a similar tertiary structure. The Cys121Ser mutant spectrum showed only small differences compared to the WT spectrum in the 260-280 nm region, showing that the mutation did not affect the β-Lg tertiary structure to any large extent.

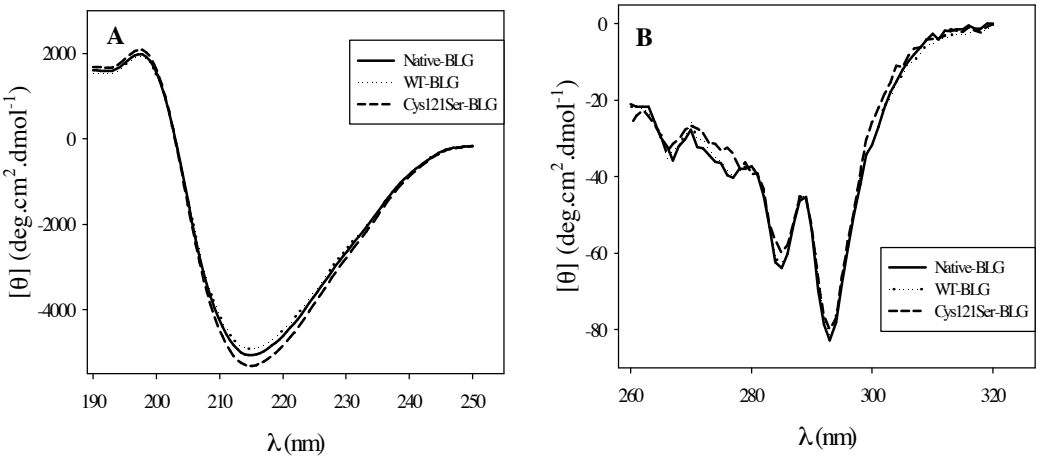


Figure 3.20. Circular dichroism of recombinant β-Lgs in 10 mM sodium phosphate buffer pH 6.7, in the far (A) and near (B) UV-CD spectroscopy. Solid line, native β-Lg; Thick line, WT/β-Lg; Dashed line, Cys121Ser mutant/β-Lg.

3.5.4. Effect of peptic digestion on recombinant β-Lg

Due to its stable structure at acid pH, β-Lg is resistant to pepsin, a protease secreted in the stomach and active at pH 2 [86]. The recombinant β-Lgs were therefore submitted to pepsinolysis, and the extent of reaction was followed by RP-HPLC (Figure 3.21). Both the native and the WT β-Lg are resistant to pepsin: more than 80 %

of the protein is undigested after 6 h incubation (Figure 3.21). In contrast, only 35 % of the Cys121Ser mutant β-Lg is still undigested after 2 h and the protein has almost completely disappeared after 6 h (Figure 3.21). This clearly shows that despite maintaining its structure at acid pH, the Cys121Ser mutant β-Lg is highly susceptible to pepsinolysis.

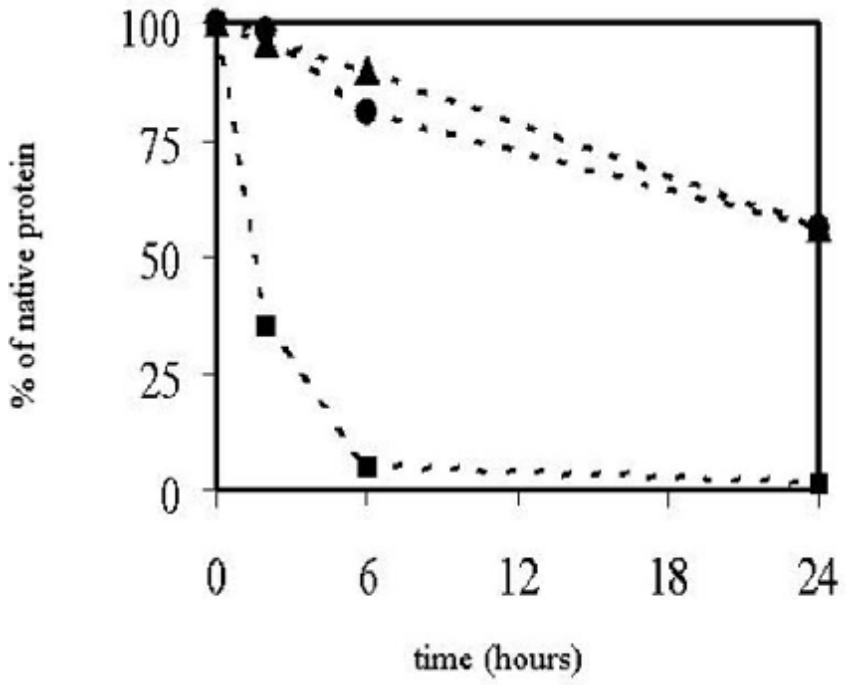


Figure 3.21. Disappearance in time of standard and recombinant β-Lg during proteolysis with pepsin. Triangles, standard β-Lg (Native β-Lg) ; circles, WT/β-Lg; squares, Cys121Ser mutant/β-Lg.

3.6. ELISA results of proteins

3.6.1. Response of patient sera to heated β-Lg

The binding of IgE from patients having cow milk allergy (CMA) on native and heated β-Lg was studied by F-ELISA. Because heating of β-Lg causes its polymerization and aggregation, it was essential to check that heated β-Lg was able to

coat the wells as well as native β-Lg. Consequently, in parallel to F-ELISA, the adsorption of heated β-Lg on microtitration plates was checked by C-ELISA using two β-Lg specific monoclonal antibodies mAb37 and mAb96, which bind β-Lg on two different epitopes independently of secondary and tertiary structures of β-Lg [195]. The result showed that coating of microtitration plate wells with heated β-Lg, whatever the temperatures, was identical to what was observed with native β-Lg (Figure 3.22).

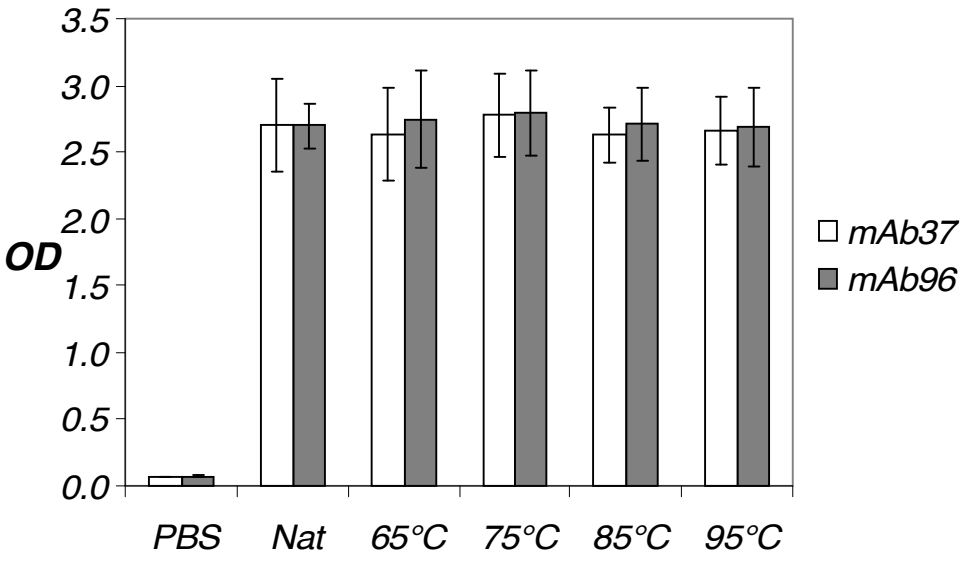


Figure 3.22. Colorimetric ELISA results of heated β-Lg, adsorption of heated β-Lg on ELISA plate. Signal from the binding (OD) of two anti-β-Lg monoclonal antibodies in wells coated with native (Nat) and β-Lg heated at 65, 75, 85, and 95 °C.

For each patient, the specific IgE titer was determined for native and heated β-Lg. The experiment was repeated three times. Three of the patients with [IgE] > 0.35 $kAUL^{-1}$ generated values above the detection limit but below the quantification limit. Consequently, they were eliminated from the study. In contrast, three out of the four patients with [IgE] < 0.35 $kAUL^{-1}$ generated well quantifiable IgE concentration values. For each treatment, specific IgE concentration values were

compared to that obtained with native β-Lg. The results are expressed as a percentage of the native β-Lg specific IgE titer (Figure 3.23). Significance of differences between medians was tested by the Kruskal-Wallis test. The result indicated that the differences of means observed between native and β-Lg heated at 65 °C (mean 106 %) and β-Lg heated at 75, 85, or 95 °C (means of 73, 55 and 64 %, respectively) were significant ($\alpha = 0.05$). At the opposite, the slight differences observed between means obtained with β-Lg heated at 75, 85, and 95 °C were insignificant.

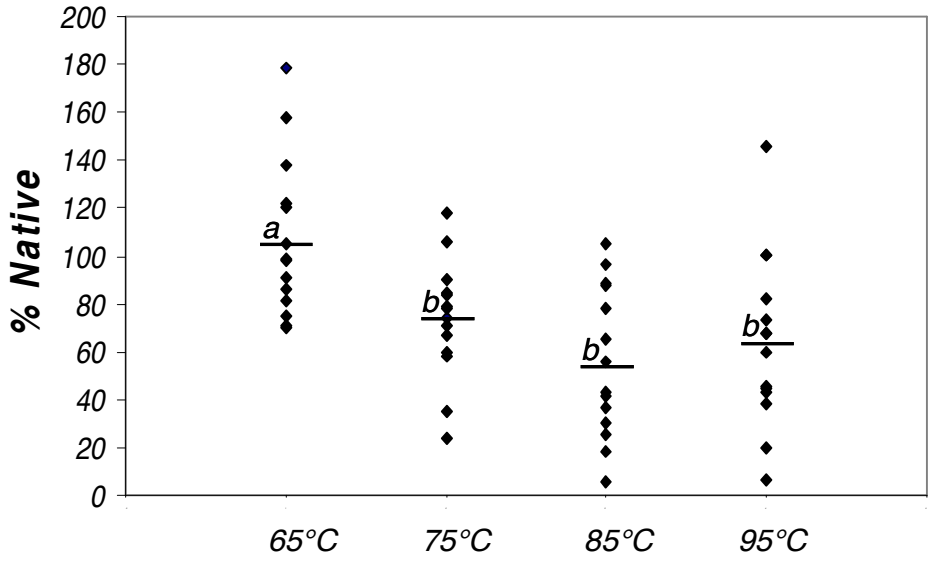


Figure 3.23. Global IgE binding on heated β-Lg; averages of the binding of IgE from 17 CMA patients on native (Nat) and β-Lg heated at 65, 75, 85, and 95 °C. Results are expressed as the percentage of the signal obtained by ELISA on native β-Lg. The mean is indicated by an horizontal black bar. Identical letters indicate that means are not significantly different at a level of 5 %.

A relative heterogeneity of patient responses was observed. This heterogeneity is illustrated in Figure 3.24, which shows the different individual responses of three patients. Patient M95 reacted as the average. Patient M86 had a β-Lg specific IgE

response strongly affected by β-Lg heating. In contrast, the binding of IgE from patient M88 was greatly reduced on β-Lg heated to temperatures higher than 65 °C. Ten out of fourteen patients (70 %) had a similar response to patient M95, 6/14 patients (43 %) had a similar response to patient M89, and only patientM88 (7 %) had a strong decrease in IgE binding to heated β-Lg.

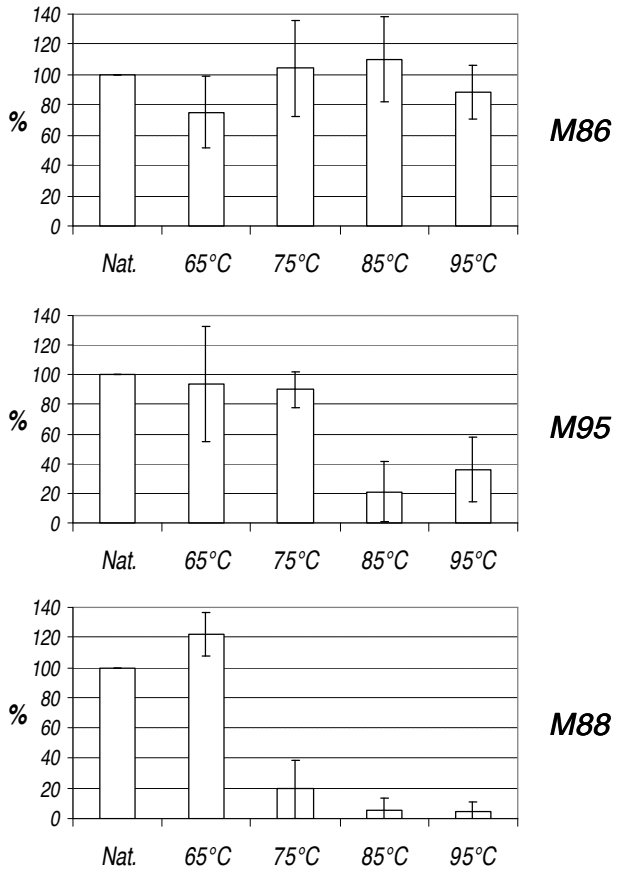


Figure 3.24. Individual IgE response of CMA patients to heated β-Lg; average of the binding of IgE from 17 CMA patients on native (Nat) and β-Lg heated at 65, 75, 85, and 95 °C. Results are expressed as the percentage of the signal obtained by ELISA on native β-Lg.

Lower binding of β-Lg specific IgE to heated β-Lg was confirmed by IgE binding inhibition experiments using a pool of sera constituted by the 17 tested sera (Figure 3.25). The standard deviation was not reported on the graph because it was always <10 %. The calculated IC_{50} value was 0.34 μg mL^{-1} for native β-Lg and increased progressively with the heating to reach a maximum of 11.74 and 11.63 μgmL^{-1} for 85 and 95 °C heated β-Lg, respectively (Table 3.4), indicating that heated β-Lg is less recognized than native β-Lg by IgE from CMA patients and that the effect increases progressively with temperature.

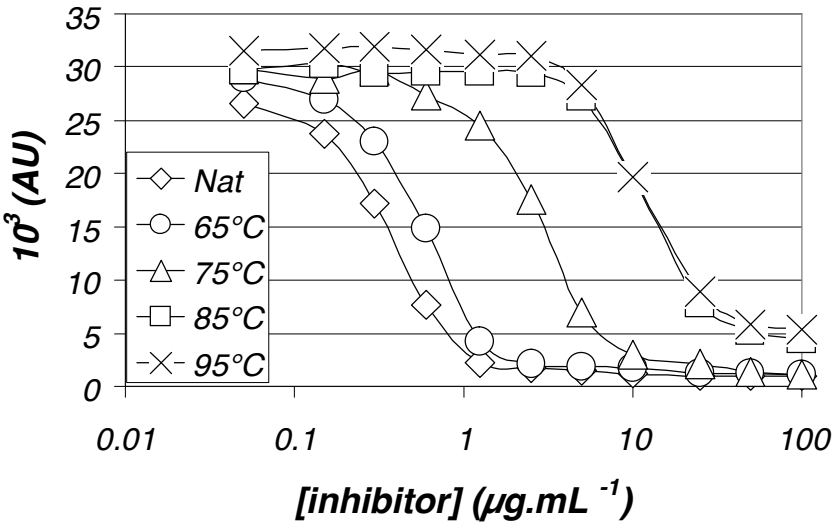


Figure 3.25. Inhibition of anti-β-Lg IgE in serum pool. Comparison between native (Nat) and heat-treated β-Lg at 65, 75, 85, and 95 °C. The signal (AU) resulting from the binding of IgE on native β-Lg coated plates is shown.

Table 3.4. Inhibition of IgE Binding to Native β-Lg by Native and Heated β-Lg. IC_{50} Values are Presented.

	β-Lg treatment				
	Native	**65 °C**	**75 °C**	**85 °C**	**95 °C**
IC_{50} (μg/mL)	0.34	0.52	2.44	11.74	11.63

3.6.2. Response of patient sera to glycated β-Lg

As well as in the case of heated β-Lg, the global adsorption of glycated β-Lg on ELISA plates was checked using monoclonal antibodies mAb37 and mAb96. The results showed that coating of microtitration plate wells was identical with either glycated β-Lg or with β-Lg incubated at 60 °C for 72 h in the absence of sugars (Figure 3.26).

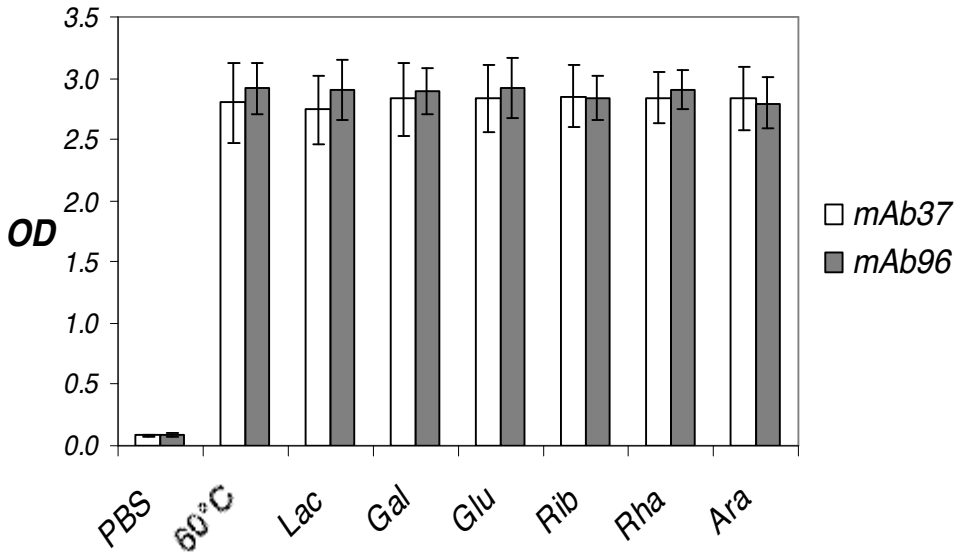


Figure 3.26. Colorimetric ELISA results of glycated β-Lg, adsorption of glycated β-Lg on ELISA plate. Signal from the binding (OD) of two anti-β-Lg monoclonal antibodies in wells coated with β-Lg heated for 72 h at 60 °C and β-Lg glycated with lactose (Lac), galactose (Gal), glucose (Glu), ribose (Rib), rhamnose (Rha), and arabinose (Ara).

To evaluate the effect of glycation of β-Lg on IgE binding, the mean IgE binding to glycated β-Lg was compared with IgE binding to β-Lg incubated at 60 °C for 72 h in the absence of sugars (no glycation) (Figure 3.27). The significance of differences between medians was tested by the Kruskal-Wallis test ($\alpha = 0.05$). The binding of

patient's IgE on β-Lg/Lac, β-Lg/Gal, β-Lg/Glu, and β-Lg/Rha (means of 99, 98, 95, and 74 %, respectively) was not significantly different from the IgE binding to β-Lg heated at 60 °C for 72 h. IgE binding to β-Lg/Ara and β-Lg/Rib was significantly lower. The obtained values represent 50 and 24 %, respectively, of the value obtained with control β-Lg. The difference between these two values was statistically irrelevant.

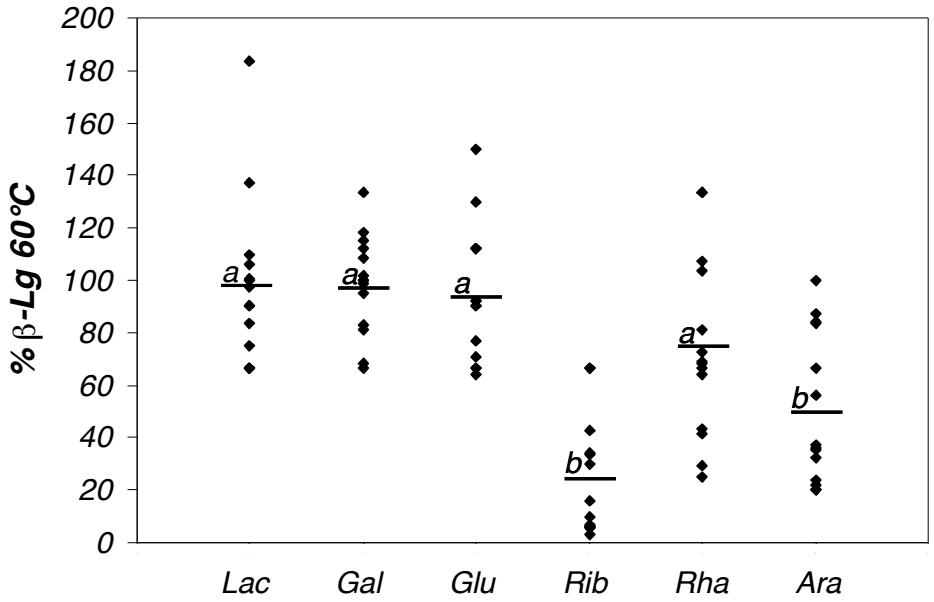


Figure 3.27. Global IgE binding on glycated β-Lg; average of the binding of IgE from 14 CMA patients on β-Lg glycated with lactose (Lac), galactose (Gal), glucose (Glu), ribose (Rib), rhamnose (Rha), and arabinose (Ara). Results are expressed as the percentage of the signal obtained by ELISA on β-Lg heated for 72 h at 60 °C in the absence of sugar (control). The mean is indicated by an horizontal black bar. Identical letters indicate that means are not significantly different at a level of 5 %.

Results obtained by indirect ELISA were confirmed by IgE binding inhibition experiments using the pool of sera previously described (Figure 3.28). The standard deviation was not reported on the graph because it was always < 10 %. The calculated IC_{50} value was 2.12 μg mL^{-1} for β-Lg heated for 72 h at 60 °C and 2.50 μg mL^{-1} for lactosylated β-Lg (Table 3.5). This difference was not significant. A considerable

increase of the IC_{50} (~ 100 µg/mL) was observed in the case of ribosylated β-Lg, indicating that the recognition of β-Lg by IgE from CMA patients is strongly impaired by a high degree of glycation, especially with ribose. Because of the too low amount of available sera, the other glycated β-Lgs were not assayed.

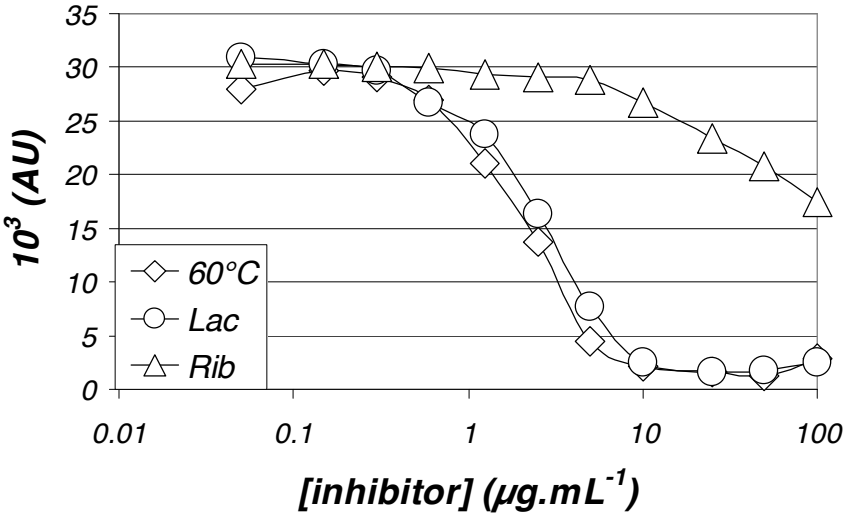


Figure 3.28. Inhibition of anti-β-Lg IgE in serum pool. Comparison between β-Lg heated for 72 h at 60 °C in the absence of sugar and in the presence of lactose (Lac) or ribose (Rib). The signal (AU) resulting from the binding of IgE on native β-Lg coated plates is shown.

Table 3.5. Inhibition of IgE Binding to Native β-Lg by β-Lg Heated for 72 h at 60 °C without the Presence of Sugar or with Lactose or Ribose. IC_{50} Values are Presented.

	β-Lg treatment		
	60 °C	**Lactose**	**Ribose**
IC_{50} (µg/mL)	2.12	2.50	~ 100

The results presented in this study show that low glycation of β-Lg has no effect on its recognition by IgE, whereas the strongest substitution rates are associated with a decreased recognition of β-Lg by IgE (Figures 3.27 and 3.28 and Table 3.6). Modification of β-Lg occurs mainly on lysyl residues. It is clear that substitution of lysyl residues contained in the epitopes weakens or prevents IgE binding. When β-Lg is substituted at a low rate, binding to epitopes is not much affected since remaining in the standard error range. Hence, they are hardly detectable (Table 3.6). However, when β-Lg is highly substituted (with important percentages of lysyl ε-amino groups glycated) IgE encounters less unhindered epitopes, and their binding to the protein is either decreased or nonexistent. In the early 1980s, Otani and Tokita [196] found that antigenicity (IgG binding) of heated β-Lg decreased when the temperature increased and that this effect was less in the presence of lactose, suggesting that lactosylation by the Maillard reaction increased the antigenicity of denatured β-Lg. They also showed that the sugar moiety linked to β-Lg can act as a neo-epitope generated during the browning reaction [197].

Table 3.6. Comparison Between the Glycation Degree of β-Lg and the IgE Binding. One Hundred percent of Binding Corresponds to the IgE Binding on β-Lg Heated for 72 h at 60 ˚C in the Absence of Sugar.

Sugar	Glycation degree (%)	Modification amino groups	IgE binding (%)
No	6.2 (±1.0)	1.0 (±0.1)	100
Lactose	34.4 (±2.5)	5.5 (±0.4)	98
Rhamnose	40.6 (±3.1)	6.5 (±0.5)	74
Glucose	41.2 (±2.5)	6.6 (±0.4)	95
Galactose	41.9 (±1.9)	6.7 (±0.3)	99
Arabinose	55.0 (±3.7)	8.8 (±0.6)	50
Ribose	69.4 (±3.1)	11.1 (±0.5)	24

The fact that glycated β-Lg did not show any increase of IgE binding as compared to the control β-Lg indicates that, in the conditions used, the glycation does not lead to the formation of neo-epitopes even stronger recognized by the studied IgE. This apparent discrepancy can be explained by the fact that Otani and Tokita in the early 1980s used to work with immunized animal, whereas we worked with human sera. This induced two kinds of important sources of variability:

(1) Otani and Tokita studied only β-Lg specific IgG epitopes, whereas we studied IgE epitopes, which are different, and their differences in the case of β-Lg are well-known [198];

(2) Additionally, the epitopes recognized by animal immune systems are often different from those recognized by the humans.

Moreover, the experimental conditions used by Otani and Tokita [197] for glycosylation lead to a more AMPs since they refer to browning products. In the present study, lactosylated β-Lg was not brown. There are several investigations of the effect of Maillard reaction on protein allergenicity. The effects of allergen glycation are variable according to the allergen tested, the sugars used and the stage of the Maillard reaction. No general effect on IgE binding could be seen. However, only scarce data about the effect of Maillard reaction are available. Consequently, it is not surprising that glycated β-Lg shows a lower allergenicity than nonglycated β-Lg as it was observed in the case of Pru av 1, the major allergen from cherry [166] and squid tropomyosin [169].

3.6.3. Response of patient sera to recombinant wild type and Cys121Ser mutant β-Lg

As well as in the case of heated and glycated β-Lg, the global adsorption of recombinant β-Lg on ELISA plates was checked using monoclonal antibodies mAb37 and mAb96. The results showed that coating of microtitration plate wells was identical

with either recombinant WT and Cys121Ser mutant β-Lg or with native β-Lg (Figure 3.29).

To evaluate the IgE binding of recombinant β-Lg comparing to native one, the IgE binding inhibition experiments using the pool of sera were done (Figure 3.30). The standard deviation was not reported on the graph because it was always < 10 %. The calculated IC_{50} values for native and recombinant proteins were almost the same and the difference was not significant, indicating that the recognition of β-Lg by IgE from CMA patients is not impaired by recombinant WT and Cys121Ser mutant β-Lgs.

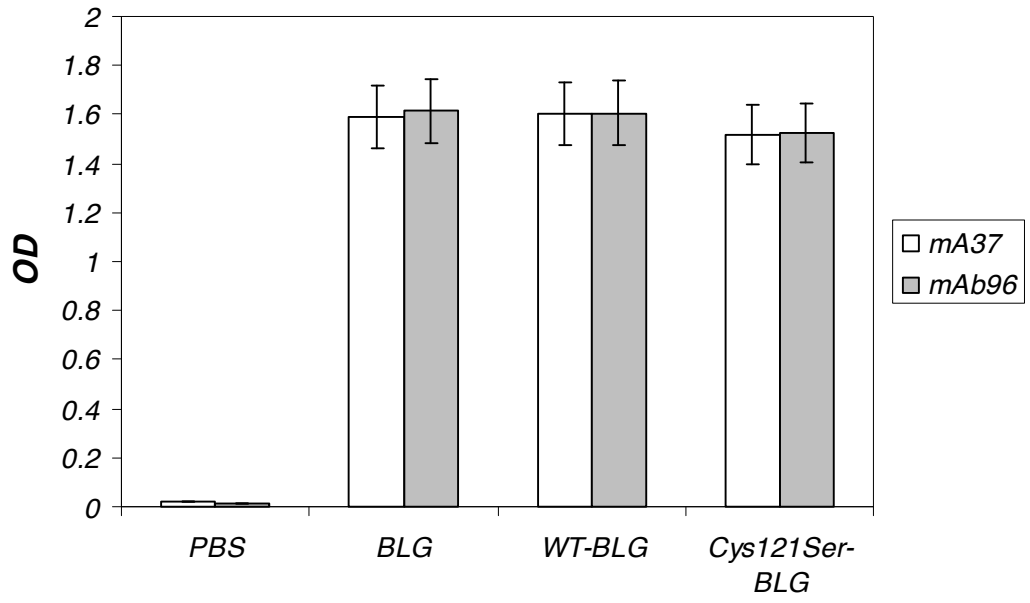


Figure 3.29. Colorimetric ELISA results of recombinant β-Lg, adsorption of recombinant β-Lg on ELISA plate. Signal from the binding (OD) of two anti-β-Lg monoclonal antibodies in wells coated with native β-Lg and recombinant WT and Cys121Ser mutant β-Lg.

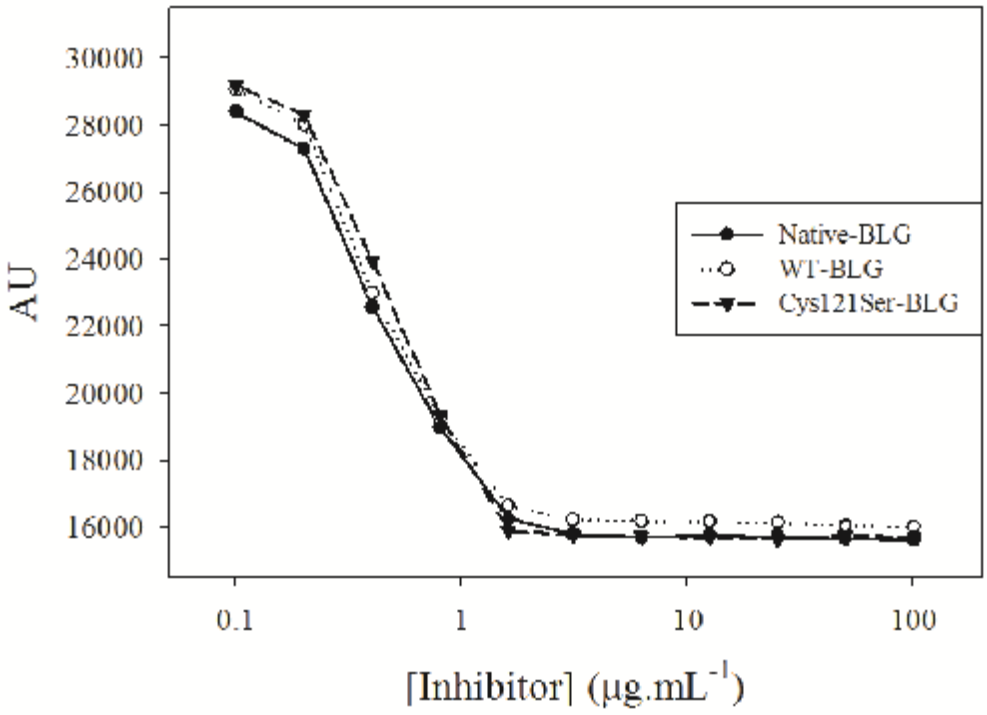


Figure 3.30. Inhibition of anti-β-Lg IgE in serum pool. Comparison between β-Lg native, WT recombinant β-Lg, and Cys121Ser mutant recombinant β-Lg. The signal (AU) resulting from the binding of IgE on native β-Lg coated plates is shown.

3.7. Interactions of β-Lg with serotonin (5-HT) and arachidonyl serotonin (AA-5HT)

3.7.1. Behavior of 5-HT and AA-5HT in Aqueous Solution and CMC Determination

Fluorescence is a useful method to study intermolecular interactions because of the photosensitivity of many fluorophores to changes in the polarity of their close environment. The observed λ_{max} shifts to a shorter wavelength, and fluorescence intensity increases during decreases in the polarity of the close neighborhood of a chromophore. As shown in Figure 3.31 A and B, λ_{max} of 10 μM 5-HT and AA-5HT is

around 338 and 349 nm, respectively, in 10 mM phosphate buffer, pH 7.4, and shifts to a shorter wavelength when the polarity of the solution decreases due to the increase in the ethanol concentration. λ_{max} for 5-HT and AA-5HT shifts to 335 and 337 nm, respectively, in 50 % ethanol with an obvious increase in fluorescence intensity. The same pattern was observed in 10 mM glycine buffer, pH 2.0 (data not shown). These results are similar with other previous results declaring that serotonin fluorescence in solvents of lower polarity is characterized by an enhancement in intensity and a blue shift in emission maximum. Also these previous results show that serotonin fluorescence, as measured by its intensity, emission maximum, and lifetime, is pH dependent that is in agreement with our obtained results [199].

The blue shift and increase in fluorescence intensity in increasing ethanol concentrations are much stronger for AA-5HT than for 5-HT. The regular blue shift of λ_{max} and increase in 5-HT and AA-5HT fluorescence emission intensity are observed as the ethanol percentage increases, when these ligands transfer from the hydrophilic environment of the aqueous solution to the more hydrophobic environment of hydroethanolic solutions.

The effect of increasing 5-HT and AA-5HT concentrations on their fluorescence emission spectra in 10 mM phosphate buffer, pH 7.4, is shown in Figure 3.32 A and B. The emission maxima (λ_{max}) of 5-HT and AA-5HT are at 338 and 350 nm, respectively.

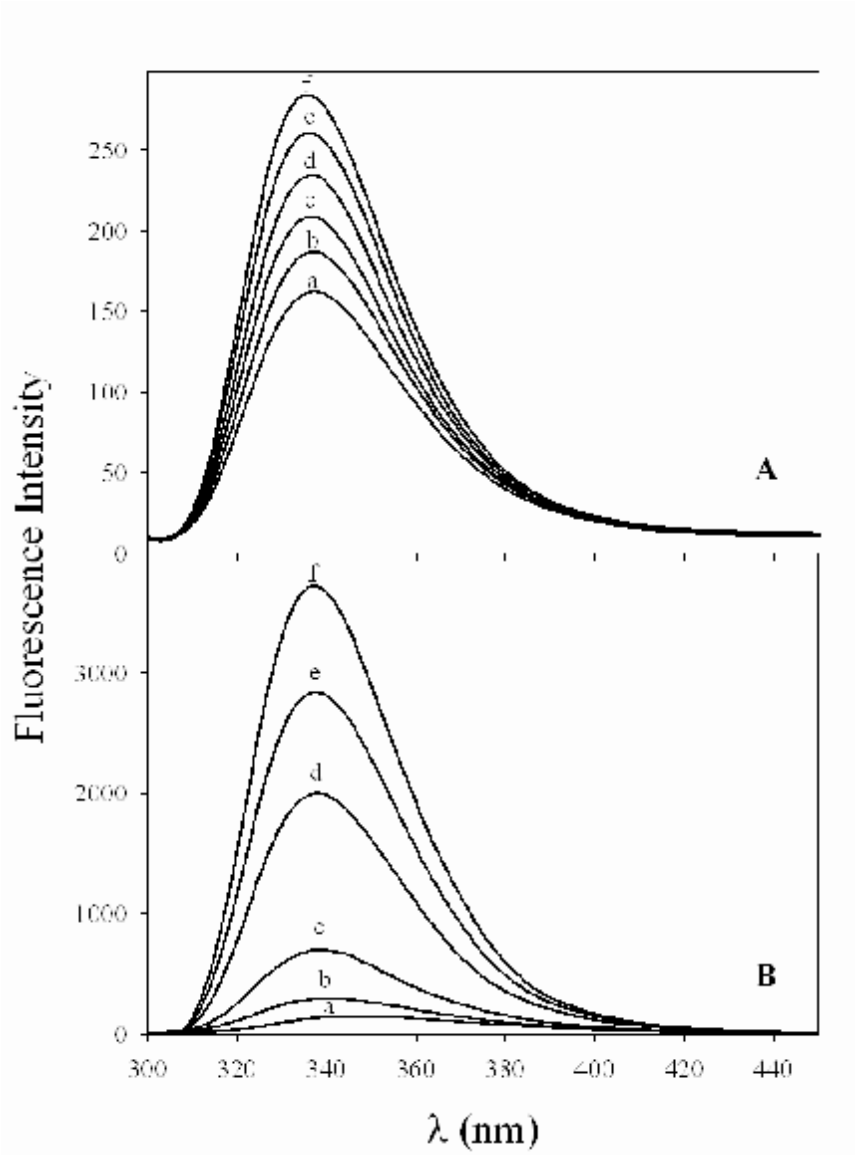


Figure 3.31. Fluorescence emission spectra of 10 μM (A) 5-HT and (B) AA-5HT at (a-f): 0, 10, 20, 30, 40 and 50 % ethanol in 10 mM phosphate buffer, pH 7.4. Similar spectra were observed in 10 mM glycine buffer, pH 2.0.

In the case of 5-HT, the fluorescence intensity increases as concentration increases till a value beyond which fluorescence intensity begins to decrease, indicating that self-quenching of 5-HT occurs at higher concentrations. 5-HT self-associates at a

concentration higher than 100 μM, which results in fluorescence self-quenching. These results are agreed with previous results [200].

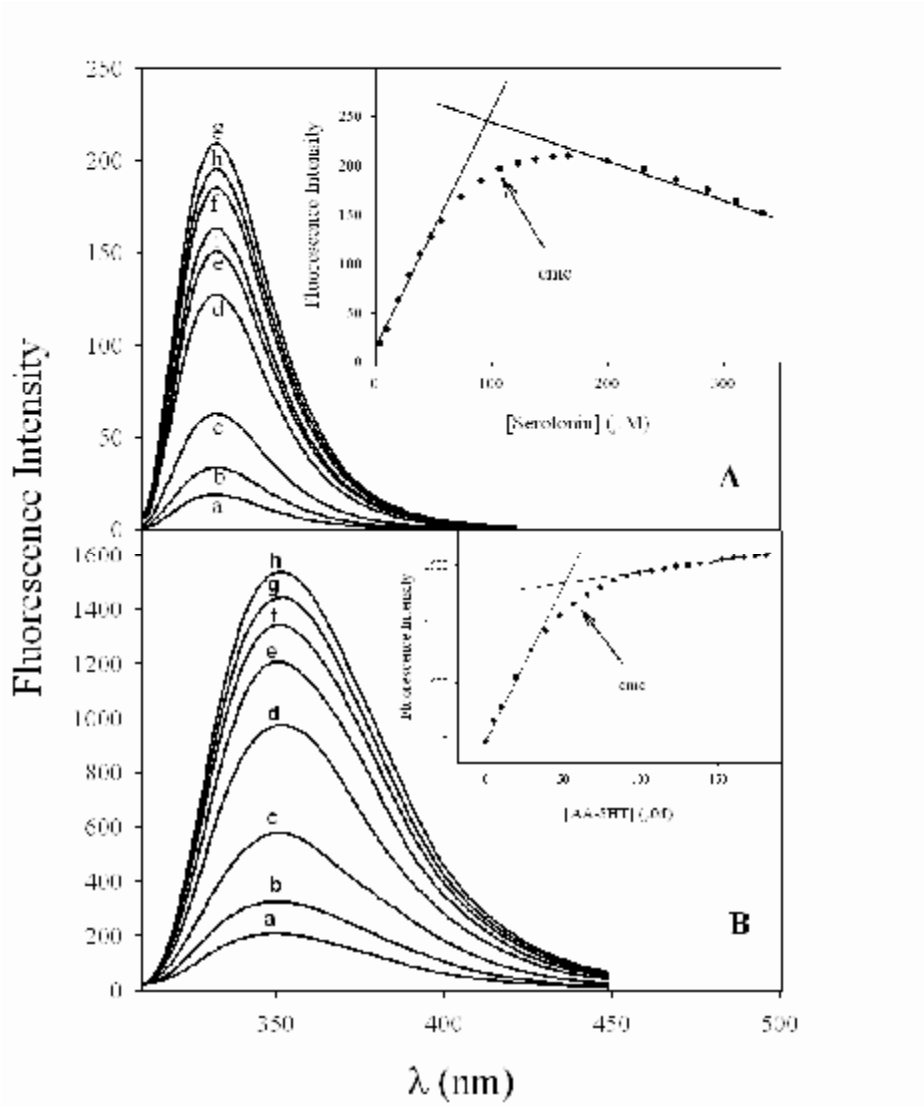


Figure 3.32. Fluorescence emission spectra of (A) 5-HT, at (a-i): 5, 10, 20, 50, 80, 100, 150, 250 and 300 μM, and (B) AA-5HT at (a-h): 5, 10, 20, 50, 60, 80, 100 and 150 μM concentration, in 10 mM phosphate buffer, pH 7.4. Similar effects were obtained for these ligands in 10 mM glycine buffer, pH 2.0.

Inset: Evolution of fluorescence intensity as a function of 5-HT and AA-5HT concentration. Sharp inclinations of spectra are observed at the CMC.

In the case of AA-5HT (Figure 3.32 B), the fluorescence intensity increases as AA-5HT concentration increases in the buffer solution, till a value beyond which fluorescence intensity begins to increase at a slower pace, indicating again that self-association of AA-5HT occurs at higher concentrations. The same pattern was observed for 5-HT and AA-5HT in 10 mM glycine buffer, pH 2.0 (data not shown). In both cases, the CMC (Critical Micelle Concentration) of 5-HT and AA-5HT were determined from the fluorescence intensity plots (Figure 3.32 A and B). The CMC of 5-HT and AA-5HT in 10 mM phosphate buffer, pH 7.4, were measured at about 91.9 and 52.1 μM. The same CMC values were obtained for both ligands in 10 mM glycine buffer, pH 2.0. The *ratio* of the slopes of the post-and pre-CMC straight lines was used to obtain the fraction of counter ions bound (*f*) to the micelles [201-204]. The standard Gibbs energy of micellization, ΔG, was obtained from the relation [204, 205]:

$$\Delta G_m = (1+f)RT\ln X_{CMC} \qquad (3)$$

where f is the fraction of counter ions bound to the micelle and X_{CMC} represents the CMC in mole fraction units. The CMC, f, and ΔG_m values are presented in Table 3.7. CMC increases as the hydrophobicity of the ligand decreases and the values of ΔG_m were found to become more negative due to the increased hydrophobicity of the ligand. These results are consistent with previous results showing that the CMC increases and ΔG_m becomes more negative within a series of surfactants as the apolar tail length (hydrophobicity) decreases [206].

Table 3.7. Critical Micelle Concentration (CMC), Fraction of Counter Ions Bound to the Micelle (F) and Gibbs Free Energy of Micellization (ΔG_m) for the Micellization of 5-HT and AA-5HT in 10 mM Phosphate Buffer, pH 7.4.

	CMC/μM	***F***	**ΔG_m/kJ mol^{-1}**
5-HT	91.9 (±2.3)	0.441 (±0.08)	-32.64 (±1.8)
AA-5HT	52.1 (±1.9)	0.139 (±0.03)	-27.38 (±1.2)

3.7.2. Effect of ligand/β-Lg interactions on the structure of β-Lg

CD spectroscopy is a valuable technique for studying structural transitions of proteins in solution because it can reveal small alterations in protein 2D and 3D structures [110]. Far-and near-UV CD spectra can be used to characterize the secondary structure and side-chain environments of proteins, respectively. Figures 3.33 and 3.34 show far-and near-UV CD spectra, respectively, of β-Lg in the absence and presence of 5-HT and AA-5HT at different concentrations in 10 mM phosphate buffer pH 7.4. The CD spectra of native β-Lg are consistent with those previously reported [209, 210].

The far-UV CD spectrum of β-Lg shows a dominance of typical β-sheet structure with a broad negative minimum around 216 nm (Figure 3.33 A and B). After protein complexing with 5-HT or AA-5HT, far-UV CD spectra remain roughly comparable to that of native β-Lg, suggesting that the interaction of 5-HT and AA-5HT with β-Lg has no significant effect on protein secondary structure at ligand concentrations lower than 80 μM. The similar results were obtained for the interaction of serotonin with human serum albumin (HSA) and calcium-binding protein calmodulin (CaM). In these cases, far-UV CD spectroscopic data indicate that the secondary structural features of HSA and CaM remain essentially intact after binding to serotonin [210, 211].

The near-UV CD spectrum of native β-Lg (Figure 3.34 A and B) shows the two negative minima at 285 and 292 nm, attributable to Trp residues at positions 19 and 61. Trp61 is partially exposed to the aqueous solvent and makes only a negligible contribution to the CD Trp signal, whereas Trp19 is in an apolar environment within the cavity of β-Lg, making it easily detectable in the spectrum [212]. The two negative bands at 285 and 292 nm regularly decrease with increasing 5-HT and AA-5HT concentrations (Figure 3.34 A and B), suggesting that the interaction of 5-HT and AA-5HT with β-Lg partially disrupts the anisotropic environment of Trp residues [213].

The same patterns were observed for far-and near-UV CD spectra of β-Lg in the absence and presence of 5-HT and AA-5HT at different concentrations in 10 mM glycine buffer, pH 2.0 (data not shown).

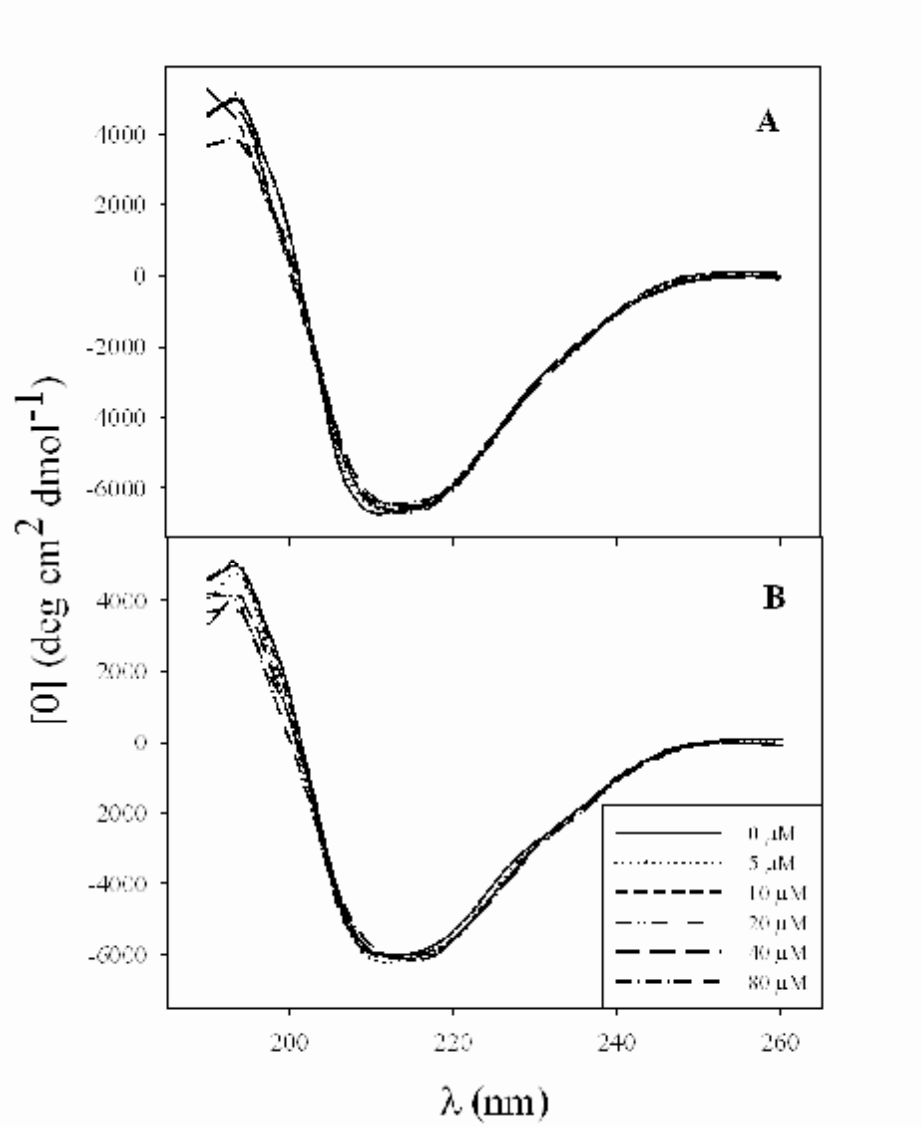


Figure 3.33. Far-UV CD spectra of 10 μM β-Lg in the presence of 0, 5, 10, 20, 40, and 80 μM of (A) 5-HT and (B) AA-5HT, in 10 mM phosphate buffer, pH 7.4. Similar spectra were observed for each ligand in 10 mM glycine buffer pH 2.0.

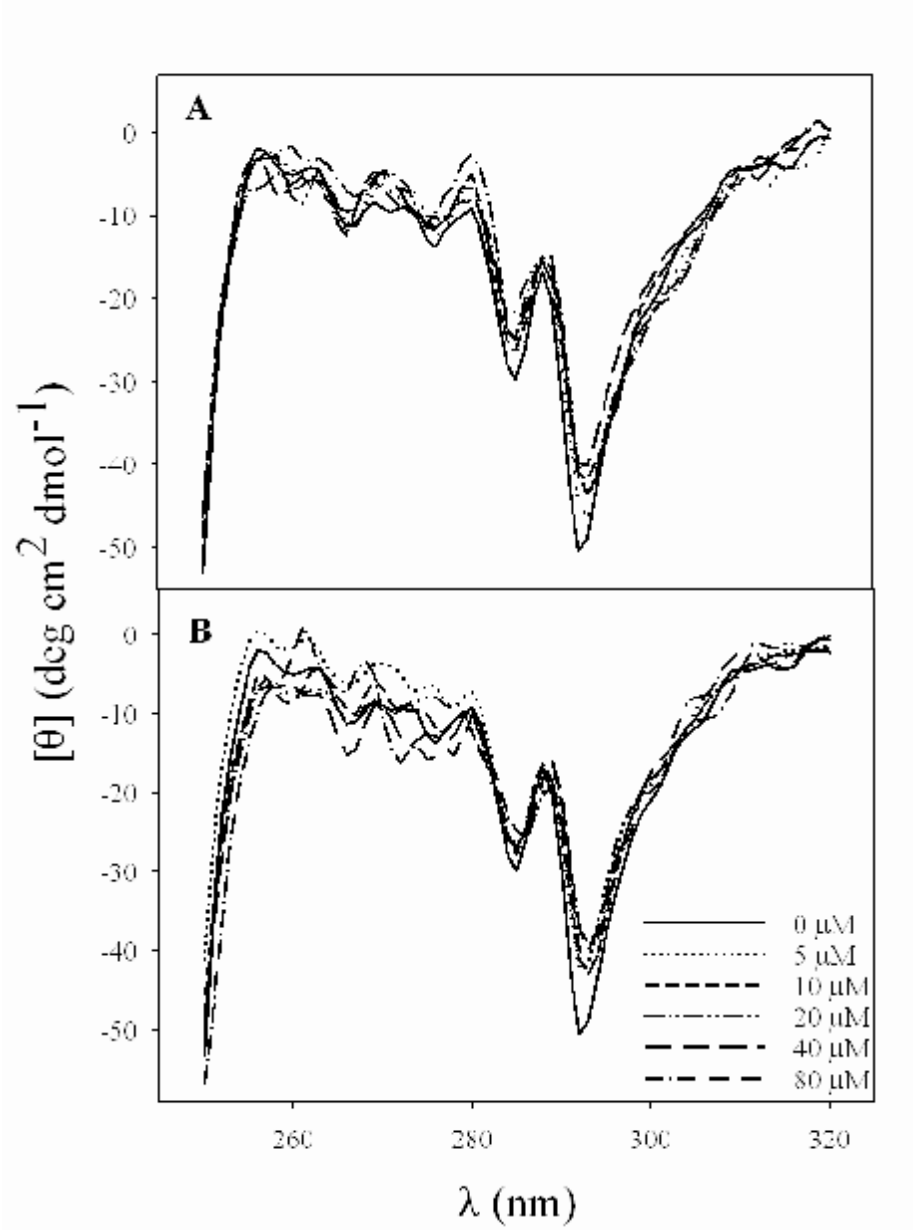


Figure 3.34. Near-UV CD spectra of 20 μM β-Lg in the presence of 0, 5, 10, 20, 40, and 80 μM of (A) 5-HT and (B) AA-5HT, in 10 mM phosphate buffer, pH 7.4. Similar effects were observed for each ligand in 10 mM glycine buffer, pH 2.0.

3.7.3. Binding Affinity Constant, Binding Number, and Site of 5-HT Binding to β-Lg

The intrinsic fluorescence of protein has been widely used to investigate the structural transition and binding properties of proteins in solution [214, 215].

Fluorescence emission spectra of 10 and 40 μM of AA-5HT in the presence of various β-Lg concentrations in 10 mM phosphate buffer, pH 7.4, is shown in Figure 3.35. The same profiles were observed for 10 and 40 μM of 5-HT in 10 mM phosphate buffer, pH 7.4, and also for 10 and 40 μM of 5-HT and AA-5HT in 10 mM glycine buffer, pH 2.0. Moreover, the same profiles were seen in 25 % ethanol media for both ligands (data not shown).

When 5-HT or AA-5HT interacts with β-Lg, the fluorescence emission spectra of 10 μM and 40 μM of 5-HT or AA-5HT show similar changes. The gradual blue shift of λ_{max} and an increase in ligand fluorescence intensity are observed as β-Lg concentration increases, suggesting that ligand transfers from the hydrophilic environment of the aqueous solution to a more hydrophobic environment. This behavior was more marked in the case of AA-5HT than in 5-HT, demonstrating the more hydrophobic environment of AA-5HT compared to 5-HT. However, even at the highest β-Lg concentration, the increase in ligand emission intensity is smaller than that observed in 50 % ethanol, suggesting that the environment of the bound ligand on the β-Lg molecule is not very hydrophobic. In other words, it is not the environment of the internal cavity in β-Lg because its hydrophobic influence on the spectral characteristics of the ligand chromophore would be seen. Therefore, this could imply that the binding site of 5-HT or AA-5HT is located on the surface of β-Lg. The similar situation has been previously observed for binding of resveratrol to β-Lg [216].

The double reciprocal linear plot of $1/\Delta F$ as a function of 1/[β-Lg] according to equation 2.1 is given in the inset in Figure 3.35. From the intercept and the slope of the straight line in Figure 3.35, ΔF_{max} and K_a can be calculated. At AA-5HT concentrations of 10 μM and 40 μM, K_a values are 3.1 (± 0.6) × 10^4 and 3.3 (± 0.7) × 10^4, respectively. The similarity of the binding constants suggests that the binding mode of ligand to β-Lg is the same at different ligand concentrations, with an average binding constant of about 3.2 × 10^4.

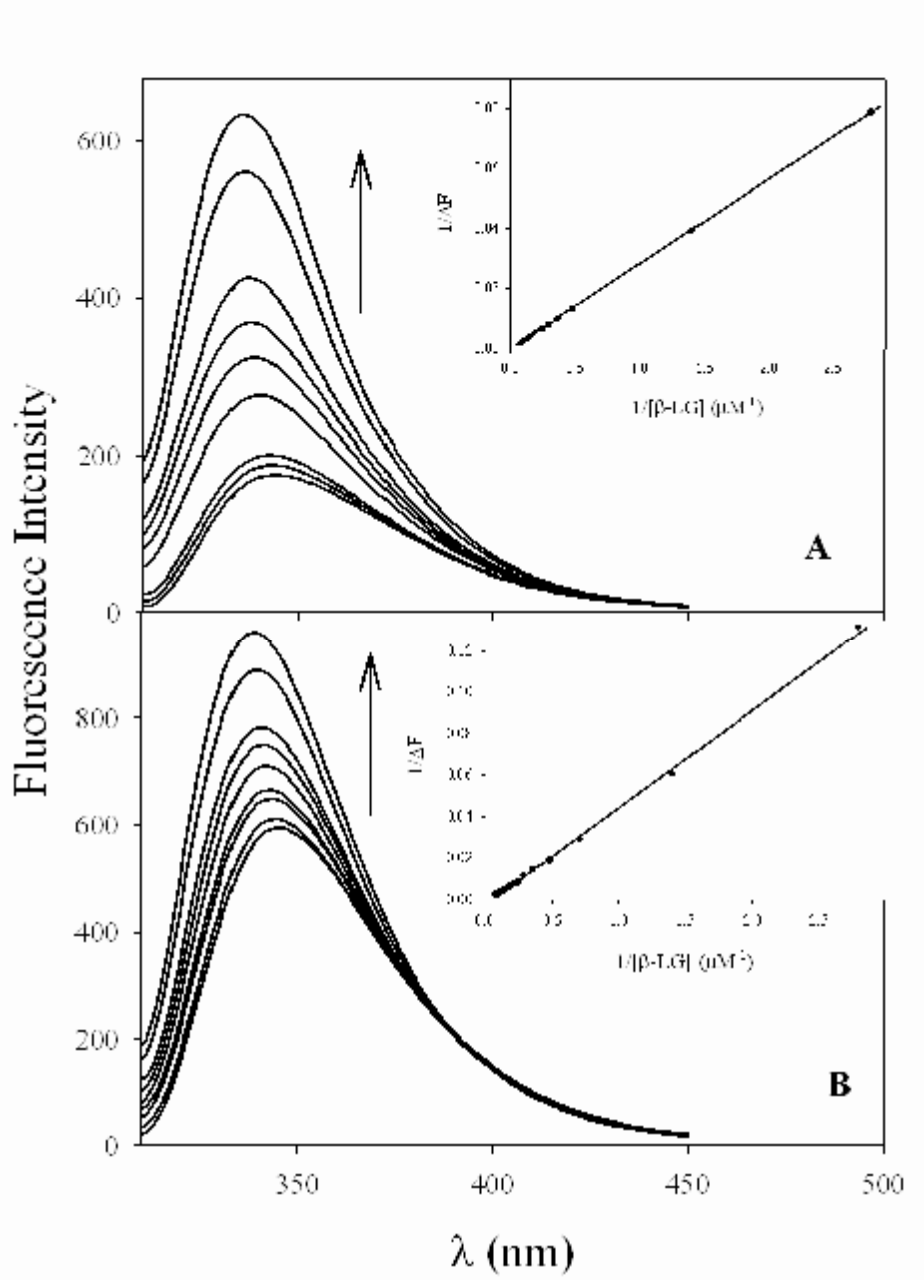


Figure 3.35. Fluorescence emission spectra of 10 μM (A) and 40 μM (B) AA-5HT in the presence of various β-Lg concentrations in 10 mM phosphate buffer, pH 7.4. Similar spectra were observed for 5-HT at this pH and for both ligands in 10 mM glycine buffer, pH 2.0. The β-Lg concentration in the solution is varied from 0-80 μM.
Inset: Double reciprocal linear plot of $1/\Delta F$ as a function of 1/[β-Lg] according to eq. 2.1.

The values of the average binding constant, K_a, for the interaction of 5-HT and AA-5HT with β-Lg at different pH and in different media are presented in Table 3.8. At ligand concentrations of 10 μM and 40 μM, ΔF_{max} values were found to be smaller than ΔF found when ligand was in buffer alone, in comparison with 50 % ethanol, indicating that β-Lg-bound ligand is not in a very hydrophobic environment and the binding site of ligand is possibly at the surface of β-Lg. For example, in the case of AA-5HT at 10 μM and 40 μM, ΔF_{max} values are 280 and 310, respectively, which are smaller than the changes observed for AA-5HT in 10 mM phosphate buffer, pH 7.4, as compared to 50 % ethanol.

Table 3.8. Binding Affinity Constants (K_a and K_s) and Binding Number (n) of 5-HT /β-Lg and AA-5HT/β-Lg Complexes at Various pH and 298 K (Results Expressed per Protein Monomer).

pH	Ligand	K_a (M)	K_s (M)	n
pH = 2.0	**5-HT**	$4.7(\pm0.18)\times 10^5$	$4.3\ (\pm0.17)\times 10^5$	1.03 (±0.06)
	AA-5HT	$6.4(\pm0.27)\times 10^4$	$6.1\ (\pm0.24)\times 10^4$	0.91 (±0.08)
	5-HT 25% ethanol	$8.6(\pm0.31)\times 10^5$	$9.1\ (\pm0.36)\times 10^5$	0.94 (±0.07)
	AA-5HT 25% ethanol	$8.1(\pm0.26)\times 10^4$	$8.3\ (\pm0.29)\times 10^4$	0.89 (±0.05)
pH = 7.4	**5-HT**	$3.1(\pm0.09)\times 10^5$	$4.0\ (\pm0.12)\times 10^5$	1.23 (±0.10)
	AA-5HT	$3.2(\pm0.12)\times 10^4$	$3.4\ (\pm0.15)\times 10^4$	0.92 (±0.07)
	5-HT 25% ethanol	$8.2(\pm0.24)\times 10^5$	$7.7\ (\pm0.28)\times 10^5$	1.16 (±0.09)
	AA-5HT 25% ethanol	$5.6(\pm0.21)\times 10^4$	$6.2\ (\pm0.19)\times 10^4$	0.98 (±0.06)

Figure 3.36 shows the intrinsic fluorescence emission spectra of β-Lg in the presence of different concentrations of 5-HT after subtracting the control (ligand in the buffer without protein) at an excitation wavelength of 280 nm. Similar results were observed in studies of the interaction of β-Lg with AA-5HT in 10 mM phosphate buffer, pH 7.4, and also of the interactions of β-Lg with 5-HT and AA-5HT in 10 mM glycine buffer, pH 2.0. Additionally, similar results were seen in 25 % ethanol media in the case

of both ligands (data not shown). β-Lg in its native state has a λ_{max} of 333 nm (Figure 3.36).

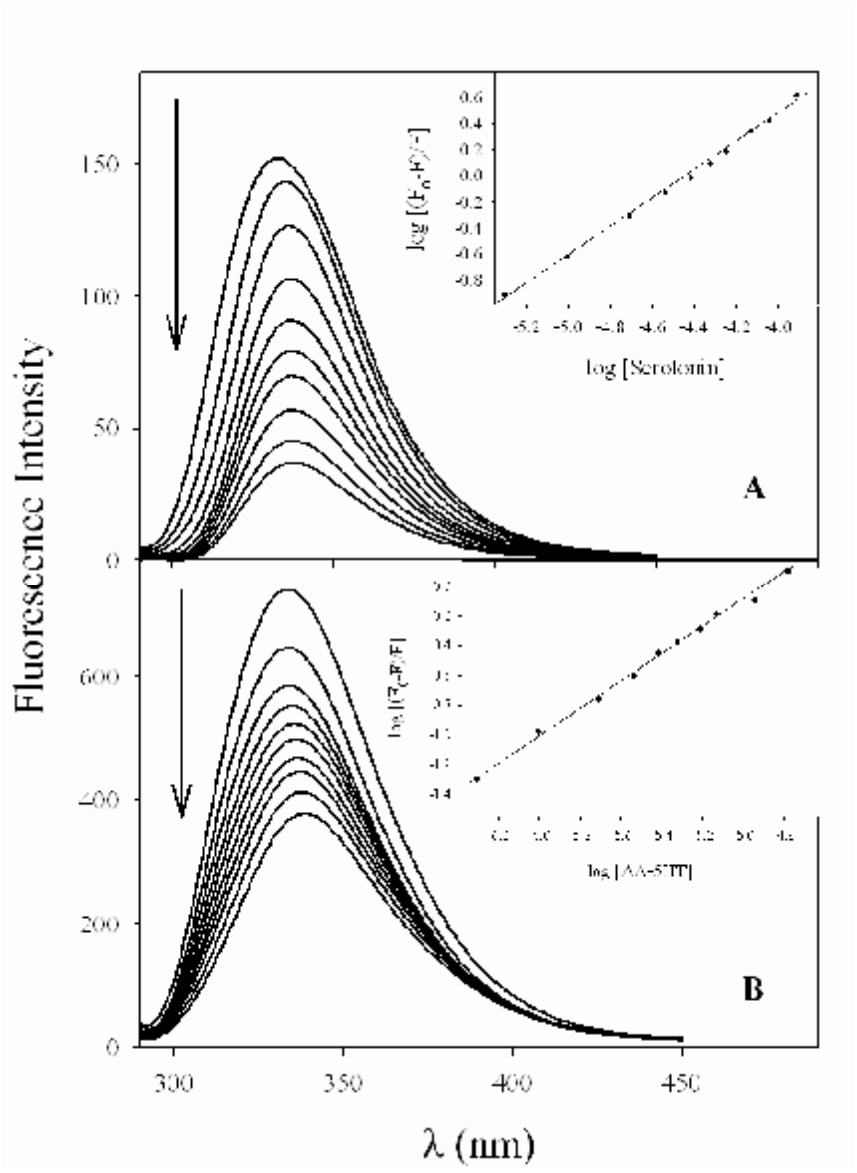


Figure 3.36. Fluorescence emission spectra of 5 μM β-Lg in the presence of (A) 5-HT and (B) AA-5HT, in 10 mM phosphate buffer at pH 7.4. Similar spectra were observed for each ligand in 10 mM glycine buffer, pH 2.0. The concentration ranges of 5-HT and AA-5HT in the solution is varied from 0-50 μM and 0-40 μM for 5-HT and AA-5HT, respectively. Spectral resolution was 2.5 nm and 5 nm in the presence of 5-HT and AA-5HT, respectively, for both excitation and emission.

Insets: Plot of $\log[(F_0\text{-}F)/F]$ *vs*. log[ligand] as per eq. 2.2.

As a result of the interaction of β-Lg with 5-HT or AA-5HT, the λ_{max} gradually shifts to a longer wavelength and Trp fluorescence intensity decreases when the concentration of the ligand increases. These results indicate that the interactions of 5-HT and AA-5HT with β-Lg position the Trp residues of β-Lg in a more hydrophilic environment, which is consistent with the results of near-UV CD titrations. The interactions of 5-HT and AA-5HT with β-Lg may thus partially modify the tertiary structure of this protein.

The results of the measurements of intrinsic fluorescence changes during titrations were analyzed after subtraction of the fluorescence intensity of ligand alone in the buffer solution according to equation 2.2. The linear plot of $\log[(F_0-F)/F]$ as a function of log [5-HT] is given in the inset in Figure 3.36. The values of K_s and n obtained from the intercept and the slope of this linear plot in the inset of Figure 3.36, respectively, are shown in Table 3.8. The n values indicate that β-Lg interacts with 5-HT forming equimolar complexes. K_s values are similar to K_a values and these two methods are consistent with each other.

The combination of the results of the studies of intrinsic fluorescence changes of β-Lg during titrations and the results of fluorescence emission of both ligands shows that the binding constants obtained by these methods are between 10^4 M^{-1} and 10^6 M^{-1}, what is lower than has been observed in the case of retinol binding by β-Lg in its internal cavity site [217]. At neutral pH, β-Lg exists as a mixture of monomers and dimers and the equilibrium between them shifts toward the monomeric form with decreasing β-Lg concentration. At acidic pH, β-Lg is monomeric [37, 218]. The equilibrium between monomers and associated ligands may exist in solution due to their self-association/micellization at high concentrations. This phenomenon has already been observed for retinol, which associates in water even at concentrations as low as 2 μM. During interactions with retinol-binding protein, equilibrium exists between monomeric and associated retinol, between monomeric retinol and retinol-protein complexes, and

possibly between associated retinol and retinol-protein complexes [219]. Similarly, the interactions of 5-HT and AA-5HT with β-Lg possibly compete with the self-association of ligands and of β-Lg. However, the probability for protein association is low, because the analysis of binding measurements on basis of proposed model is sufficiently satisfied. The binding between ligands and monomeric β-Lg is stronger than the association of ligands with dimeric β-Lg and consequently, the binding affinity constant of the interactions of ligands with β-Lg is higher at pH 2.0 when β-Lg is in monomeric form. The binding experiments were also performed in 25 % ethanol in which β-Lg has a folding similar to a molten globule [220]. In these conditions, β-Lg can make stronger complexes with both ligands since the binding affinity constants are greater greater with respect to the results obtained in fully aqueous media.

The fact that the binding site is external rather than internal has been tested by competitive titrations. Retinol binds tightly to the internal cavity and competitive studies involving both retinol and serotonin with β-Lg could make more convincing the conclusion about external position of serotonin binding site. The intrinsic fluorescence emission spectra of β-Lg/serotonin complexes (molar ratio 1) in the presence of different concentrations of retinol after subtracting the control (ligand in the solution without β-Lg) at an excitation wavelength of 280 nm and 10 mM phosphate buffer, pH 7.4 were done (Figures 3.37 and 3.38). These results analyzed according to equation 2.2 showed that the binding affinity constant (K_s) and the binding number of retinol in the presence of β-Lg/serotonin complexes are 3.2×10^7 M^{-1} and 1.12, respectively, what agrees with previous results without any other competing ligand. In another experiment, the intrinsic fluorescence emission spectra of β-Lg/retinol complexes (molar ratio 1) in the presence of different concentrations of serotonin after subtracting the control (ligand in the solution without β-Lg) at an excitation wavelength of 280 nm and 10 mM phosphate buffer, pH 7.4 were measured (data not shown). These results analyzed according to equation 2 showed that the binding affinity constant (K_s) and the binding

number of serotonin in the presence of β-Lg/retinol complexes are 3.8×10^5 M^{-1} and 1.17, respectively, agreeing with titration results without retinol. This means that the binding sites of retinol and serotonin are different from each other. It can be also concluded that the binding site of serotonin on β-Lg is external rather than internal.

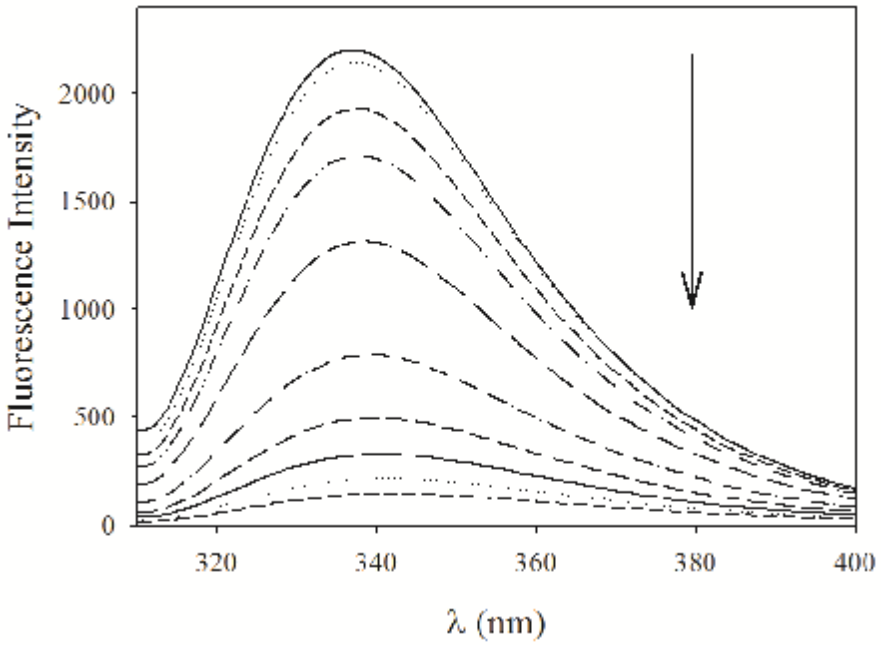


Figure 3.37: Fluorescence emission spectra of 5 μM β-Lg/serotonin (molar ratio 1) in the presence of various retinol concentrations in 10 mM phosphate buffer pH 7.4. The concentration range of retinol in the solution varied from 0 to 100 μM (molar ratio of about 20). Spectral resolution was 5 nm for both excitation and emission. Similar spectra were observed for fluorescence emission spectra of 5 μM β-Lg/retinol (molar ratio 1) in the presence of various serotonin concentrations in 10 mM phosphate buffer pH 7.4 after correcting blank intensity.

It is known that the interactions of β-Lg with ligands depend not only on the binding constants, but also on the solubility of the ligands [217]. For instance, the solubility of retinol and palmitic acid in water are 0.06 μM and 28 μM, respectively. Although the binding constant to β-Lg is about 2 orders of magnitude higher for retinol

than for palmitic acid, the higher solubility of palmitic acid leads to a 5-fold preferred binding as compared to retinol. Compared with retinol and palmitic acid, the higher solubility of both AA-5HT and especially 5-HT (about 50 and 90 μM, respectively) in aqueous solutions should mean a high affinity of 5-HT and AA-5HT for β-Lg, even though the binding constants for the interaction of 5-HT and AA-5HT with β-Lg are lower.

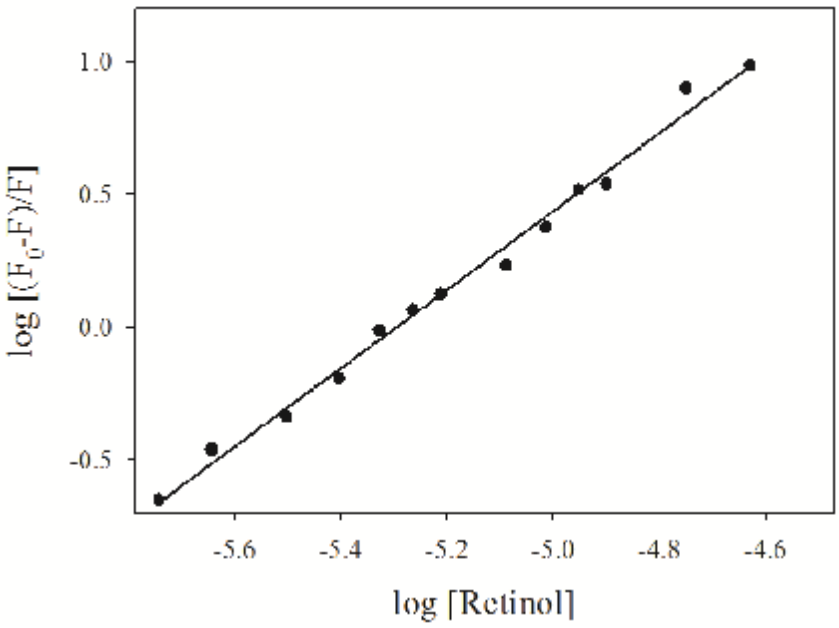


Figure 3.38: log[(F_0 - F)/F] *vs.* log[retinol] for titration of 5 μM β-Lg/serotonin (molar ratio 1) with various retinol concentrations in 10 mM phosphate buffer pH 7.4. Similar results were observed in case of titration of 5 μM β-Lg/retinol (molar ratio 1) with various serotonin concentrations in 10 mM phosphate buffer pH 7.4.

3.7.4. Ultrafiltration results

In ultrafiltration experiments, free serotonin can pass throughout the centricon (less than 10 kDa) and by comparing the absorbencies of both filtrate (non-retained) and concentrate (retained) solution the binding of serotonin to β-Lg can be assessed. Table

3.9 shows the absorbencies of each solution before and after ultrafiltration. As it shown in this Table, almost all β-Lg is retained after ultrafiltration and serotonin alone is completely non-retained as expected. As it shown in this Table, serotonin cannot bind to casein and all serotonin is recovered as non-retained after ultrafiltration.

In the case of β-Lg with different molar ratios of serotonin, the binding occurred and the amount of serotonin retained after ultrafiltration (bound to β-Lg) is proportional to the increasing amount and ratio of serotonin.

Consequently, this simple experiment is indicating that the binding of serotonin to β-Lg is specific and is not an artifact. After ultrafiltration of β-Lg/5-HT at molar ratio 2.0, the retained serotonin is present in the concentration of about 4 μM what is approximately corresponding to β-Lg/5-HT molar ratio of 1.

Table 3.9. Absorbencies of Different Solution before and after Ultrafiltration (Retained and Non-retained Solution) at 280 nm to Prove the Binding of Serotonin to β-Lg.

	before ultrafiltration	**after ultrafiltration (retained)**	**after ultrafiltration (non-retained)**
β-LG (5 μM)	0.087	0.084	0.005
5-HT (10 μM)	0.061	0.002	0.059
β-LG/5-HT (MR=0.5)	0.106	0.089	0.014
β-LG/5-HT (MR=1.0)	0.119	0.099	0.022
β-LG/5-HT (MR=2.0)	0.152	0.114	0.037
Casein (10 μM)	0.104	0.098	0.009
Casein/5-HT (MR=2)	0.158	0.101	0.062

Conclusions

The results of study of binding of IgE from CMA patients to heat-modified β-Lg are presented in this study. For nearly 70 % of CMA patients sensitized to β-Lg, it was observed that moderate heating of β-Lg at 75 °C caused a decrease in its recognition by IgE. The maximal effect was observed between 85 and 95 °C. This is consistent with the observation of Ehn et al. who found, using pools of sera but not individual sera, that heating of β-Lg at 74 °C caused a significant decrease in IgE binding, more evident after heating at 90 °C [163]. Because the denaturation (modification of secondary and tertiary structures) of β-Lg begins at 70-72 °C, it seems that its consecutive aggregation is responsible for the disappearance of conformational epitopes and, consequently, of reduced binding. Reorganization of protein structure and/or aggregation of β-Lg may have a masking effect on the recognition of sequential (or linear) epitopes. This should explain, at least partially, why some CMA patients are more tolerant to boiled milk than to raw milk. Our results are also consistent with the data obtained by Rytkonen et al. [221] who observed that rats sensitized with heat-denatured β-Lg produce less specific IgE than rats sensitized with native β-Lg, despite the fact that it induces a more intense inflammatory response. According to these results, it is clear that heat-induced denaturation of β-Lg and/or aggregates generated by heat treatments at moderately high temperatures induced a more or less pronounced decrease of β-Lg recognition by IgE from CMA patients. None of the studied sera from allergic patients showed a stronger IgE response against denatured β-Lg, indicating that this protein does not contain internal masked linear epitopes becoming exposed only after denaturation.

We found that all of the patients do not react identically to heated β-Lg. Mild reduction of IgE binding associated with β-Lg heating was observed for the majority (60 %) of the patients studied. Nevertheless, it was found that one serum among 17 (6 % of patients) recognizes only native β-Lg and β-Lg heated to 65 °C. This patient serum recognizes most likely conformational epitope(s), which are damaged by heating. It appears that in the case of a few sera, heating of β-Lg to temperatures higher than 75 °C

abrogate near totally its recognition by IgE. Surprisingly, the measured IgE concentration by the β-Lg-specific Phadia ImmunoCAP System (F77) was < 0.35 kIA L^{-1}, suggesting that patients sensitized only against native β-Lg are not well-detected by this assay. It should be underlined that β-Lg adsorbed on the surface of the ImmunoCAP System is probably not in its native state any more. Consequently, for a better diagnosis, it would be important to ensure the use of native β-Lg or execute them in solution and not adsorbed on any surface.

Glycation of proteins by the Maillard reaction occurred when they were incubated with the sugar at 60 °C for 72 h. This reaction is slow at room temperature, but its yield increases with temperature. The resulting products are responsible for the browning of cooked food. Because milk contains a high amount of lactose and because milk proteins are also present in multiple food preparations containing free reducing sugars, milk proteins, including β-Lg, are susceptible to glycations. The results presented in this study show that low or moderate glycation of β-Lg has no effect on its recognition by IgE, whereas the strongest substitution rates are associated with a decreased recognition of β-Lg by IgE. This result can be explained by a "masking" effect due to sugars. Modification of β-Lg occurs mainly on lysyl residues. All major and minor β-Lg epitopes [197] contain one or more lysyl residues, and some of them have been identified as critical for IgE binding (e.g., K75, K83, K135, K138, and K141) [222].

Despite of these *in vitro* allergenicity data, Bleumink and Berrens [197] observed in the 1960s that incubation of β-Lg at 50 °C in the presence of lactose increases 100 times its skin reactivity. In this work, β-Lg was heated from 48 to 216 h. The authors did not describe precisely what preparation was used for the intradermal reaction. They probably used β-Lg samples more intensively modified than those used in our study, quite probably containing AGE. Additionally, in vivo skin reactivity involves other complex mechanisms than IgE binding on the allergen. It is possible that

AGE present in the reaction medium affects these mechanisms, increasing the overall inflammation and allergic reactions without affecting IgE binding. Otani and Tokita [197] have reported contradictory results since they found that lactosylated β-Lg was able to strongly inhibit the passive coetaneous anaphylaxis test. This result suggests lower allergenicity of glycated β-Lg as compared to native β-Lg. The human *in vivo* allergenicity of different glycated β-Lg remains to be carefully evaluated.

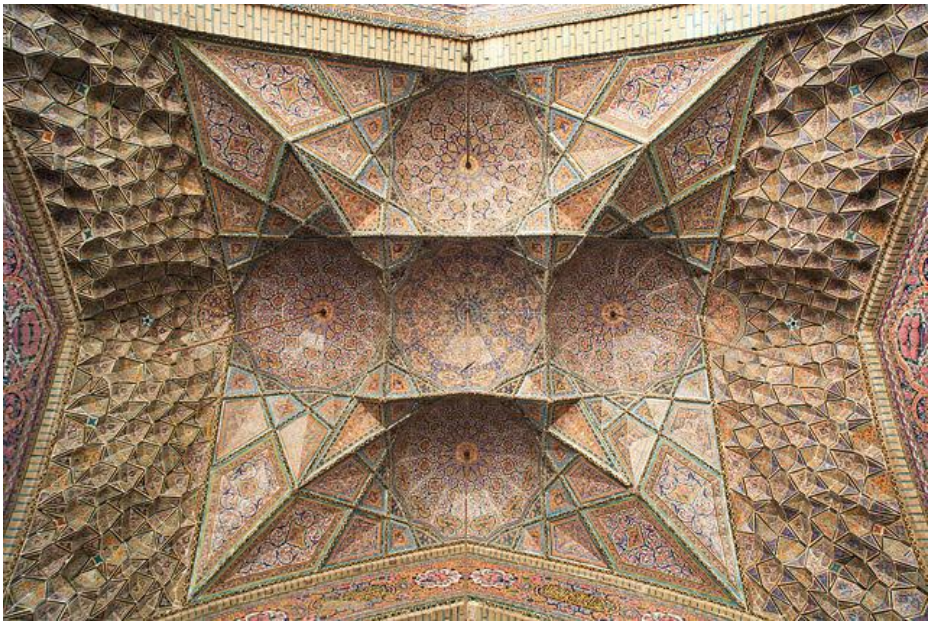
In conclusion, for most studied sera of the CMA patients, a moderate heat treatment and glycation of β-Lg during the moderate stages of the Maillard reaction do not have a drastic influence on the recognition of the protein by IgE. Nevertheless, in the case of rare individuals, heat treatment of β-Lg can neutralize near totally its recognition by IgE.

In the case of binding experiments, we showed that 5-HT and AA-5HT can interact with β-Lg forming equimolar complexes, without affecting the secondary structure of β-Lg but probably with a slight modification of its tertiary structure. The molecules of 5-HT and AA-5HT bind to the surface of β-Lg with a binding constant varying between 10^4 and 10^6 M^{-1}. The binding affinity constant for 5-HT/β-Lg complexes is higher than for AA-5HT/β-Lg complexes. The interactions of these ligands with β-Lg may compete with self-associations of both ligands and β-Lg. These ligands can bind better to β-Lg in 25 % ethanol and they show a good solubility in water (especially in the case of 5-HT) as compared with other more hydrophobic ligands such as retinol and palmitic acid. Thus, β-Lg can be considered as a good carrier of 5-HT-derived ligands, and 5-HT/β-Lg and AA-5HT/β-Lg complexes can serve as useful models of drug-β-Lg interactions.

Although many other data are still necessary to confirm this hypothesis, the observed for the first time binding by β-Lg of 5-HT and its derivatives such as AA-5HT could imply that the peripheral control of 5-HT quantity and 5-HT action on the gut enterochromaffin cells may also

be one of the potential, if not main, biological roles of β-Lg in mammals whose milk contains this protein.

References



Bibliographic references

[1] Thompson, A.; Boland, M.; Singh, H. (2009) *Milk Proteins: From Expression to food*. Elsevier Applied Science, Especially the chapter by Edwards et al., pp. 163-203.

[2] Fox, P. F., McSweeney, P. L. H. (1998) *Dairy Chemistry and Biochemistry*. London: Chapman & Hall., [reprinted by Kluwer Academic/Plenum Publishers, New York, 2000].

[3] Evans, D. E. (1959) Milk composition of mammals whose milk is not normally used for human consumption. *Dairy Sci. Abs.*, 21, 277–288.

[4] Jenness, R., Sloan, R. E. (1970) The composition of milk of various species: a review. *Dairy Sci. Abs.*, 32, 599–612.

[5] Laxminarayana, H., Dastur, N. N. (1968) Buffalo milk and milk products, Parts I & II. *Dairy Sci. Abs.*, 30, 177–186 and 231–241.

[6] Haenlein, G. F. W. (1980) Status of world literature on dairy goats: introductory remarks. *J. Dairy Sci.,* 63, 1591–99.

[7] Bencini, R., Pulina, G. (1997) The quality of sheep milk. *Australian J. Exp. Agric.,* 37, 485–504.

[8] Jandal, J. M. (1996) Comparative aspects of goat and sheep milk. *Small Rumi. Res.,* 22, 177–85.

[9] Park, Y. W., Juarez, M., Ramos, M., Haenlein, G. F. W. (2007) Physico-chemical characteristics of goat and sheep milk. *Small Rumin. Res.,* 68, 88–113.

[10] Rao, M. B., Gupta, R. C., Dastur, N. N. (1970) Camels' milk and milk products. *Indian J. Dairy Sci.,* 23, 71–78.

[11] Farah, Z. (1993) Composition and characteristics of camel milk. *J. Dairy Res.,* 60, 603–26.

[12] Solaroli, G., Pagliarini, E., Peri, C. (1993) Composition and quality of mare's milk. *Italian J. Food Sci.,* V, 3–10.

[13] Park, Y. W., Zhang, H., Zhang, B., Zhang, L. (2006) Mare milk. In: *Handbook of Milk of Non-bovine Mammals* (Y. W. Park and G. F. W. Haenlein, eds) pp. 275–96. Oxford : Blackwell Publishing.

[14] Atkinson, S. A. and Lonnerdal, B. (1989) *Protein and Non-protein Nitrogen in Human Milk*. Boca Raton, Florida : CRC Press.

[15] Jensen, R. G. (1989) *The Lipids of Human Milk*. Boca Raton, Florida : CRC Press.

[16] Jensen, R. G. (1995) *Handbook of Milk Composition*. San Diego, California: Academic Press.

[17] Verstegen, M. W. A., Moughan, P. J. and Schrama, J. W. (1998) *The Lactating Sow*. Wageningen : Wageningen Pers.

[18] Barker, M. E. (2003) Contribution of dairy foods to nutrient intake. In *E ncyclopedia of Dairy Sciences* (H. Roginski, J. W. Fuquay and P. F. Fox, eds) pp. 2133–37. London : Academic Press.

[19] Hammarsten, O. (1883) Zur Frage, ob das Casein ein einheitleicher Stoff sei. *Z. Physiol Chemie,* 7, 227–273.

[20] McMeekin, T. L. (1970) Milk proteins in retrospect. In *Milk Proteins: Chemistry and Molecular Biology,* Volume I (H. A. McKenzie, ed.) pp. 3–15. New York : Academic Press.

[21] Fox, P. F. (1992) *Advanced Dairy Chemistry,* Volume 1, *Proteins,* 2nd edn. London : Elsevier Applied Science.

[22] Fox, P. F. (2003) Milk proteins: general and historical aspects. In *Advanced Dairy Chemistry,* Volume 1, *Proteins,* 3rd edn (P. F. Fox and P. L. H. McSweeney, eds) pp. 1–48. New York : Kluwer Academic/Plenum Publishers.

[23] Fox, P. F. and McSweeney, P. L. H. (2003) *Advanced Dairy Chemistry,* Volume 1, *Proteins,* 3rd edn. New York : Kluver Academic-Plenum Publishers, Especially the chapter by Sawyer, pp. 319-386.

[24] Holt, C., Sawyer, L. (1993) Caseins as rheomorphic proteins: interpretation of primary and secondary structures of α_{s1}-, β-and κ-caseins. *J. Chem. Soc. Faraday Trans*. 89, 2683–92.

[25] Horne, D. S. (2002) Casein structure, self-association and gelation. *Curr. Opin. Coll. Inter. Sci.,* 7, 456–461.

[26] Farrell, H. M. Jr., Qi, P. X., Uversky, V. N. (2006) New views on protein structure: application to the caseins: protein structure and functionality. In *Advances in Biopolymers: Molecules, Clusters, Networks, and Interactions* (M. L. Fishman, P. X. Qi and L. Whisker, eds), ACS Symposium Series 935 pp. 1–18. Washington, DC : American Chemical Society.

[27] Imafidon, G. F., Farkye, N. Y., Spanier, A. M. (1997) Isolation, purification and alteration of some functional groups of major milk proteins: a review. *CRC Crit. Rev. Food Sci. Nutr.,* 37, 663–689.

[28] Mulvihill, D. M., Ennis, M. P. (2003) Functional milk proteins: production and utilization. In *Advanced Dairy Chemistry,* Volume 1, *Proteins,* 3rd edn (P. F. Fox and P. L. H. McSweeney, eds) pp. 1175–228. New York : Kluwer Academic/Plenum Publishers.

[29] Varnum, A. H. and J. P. Sutherland. 1994. Milk and Milk Products. Chapman & Hall., New York, NY. P159-189.

[30] Fennema, O. R. (1996) Water and ice. In *F ood Chemistry,* 3rd edn (O. R. Fennema, ed.) pp. 17–94. New York : Marcel Dekker.

[31] Hambling, S. G., McAlpine, A. S., Sawyer, L. (1992) β-Lactoglobulin. In *Advanced Dairy Chemistry,* Volume 1, *Proteins,* 2nd edn (P. F. Fox, ed.) pp. 141–90. Barking, Essex : Elsevier Applied Science.

[32] Banaszak, L., Winter, N., Xu, Z., Bernlohr, D. A., Cowan, S., Jones, T. A. (1994) Lipid-binding proteins: a family of fatty acid and retinoid transport proteins. *Adv. Prot. Chem.,* 45, 89–151.

[33] Flower, D. R. (1996) The lipocalin protein family: structure and function. *Biochem. J.,* 318, 1–14.

[34] Papiz, M. Z., Sawyer, L., Eliopoulos, E. E., North, A. C. T., Findlay, J. B. C., Sivaprasadarao, R., Jones, T. A., Newcomer, M. E., Kraulis, P. J. (1986) The structure of β-lactoglobulin and its similarity to plasma retinol-binding protein. *Nature,* 324, 383–385.

[35] Farrell, H. M. Jr., Jimenez-Flores, R., Bleck, G. T., Brown, E. M., Butler, J. E., Creamer, L. K., Hicks, C. L., Hollar, C. M., Ng-Kwai-Hang, K. F., Swaisgood, H. E. (2004) Nomenclature of the proteins of cows' milk–sixth revision. *J. Dairy Sci.,* 87, 1641–1674.

[36] Kumosinski, T. F., Timasheff, S. N. (1966) Molecular interactions in β-lactoglobulin. X. The stoichiometry of the β-lactoglobulin mixed tetramerization. *J. Am. Chem. Soc.,* 88, 5635–5642.

[37] McKenzie, H. A., Sawyer, W. H. (1967) Effect of pH on β-lactoglobulins. *Nature,* 214, 1101–1104.

[38] Gottschalk, M.; Nilsson, H.; Roos, H.; Halle, B. (2003) Protein self-association in solution: the bovine β-lactoglobulin dimer and octamer. *Prot. Sci.,* 12, 2404–2411.

[39] Sakurai, K.; Oobatake, M.; Goto, Y. (2001) Salt-dependent monomer–dimer equilibrium of bovine β-lactoglobulin at pH 3. *Prot. Sci.,* 10, 2325–2335.

[40] Sakurai, K.; Goto, Y. (2002) Manipulating monomer–dimer equilibrium of bovine β-lactoglobulin by amino acid substitution. *J. Biol. Chem.,* 277, 25735–25740.

[41] Larson, B. L. (1972) Methionine stimulation of milk protein synthesis in bovine mammary cell cultures. *J. Dairy Sci.*, 55, 629-631.

[42] Yoshikawa, M.; Mizukami, T.; Sasaki, R.; Chiba, H. (1978) Pre-β-lactoglobulin synthesis by mRNA from bovine mammary gland. *Agric. Biol. Chem.*, 42, 2185-2186.

[43] Sawyer, L.; Kontopidis, G. (2000) The core lipocalin, bovine beta-lactoglobulin. *Biochim. Biophys. Acta*, 1482, 136-148.

[44] www.pdb.org, code 1BEB.

[45] Green, D. W.; Aschaffenburg, R.; Camerman, A.; Coppola, J. C.; Dunnill, P.; Simmons, R. M.; Komorowski, E. S.; Sawyer, L.; Turner, E. M. C.; Woods, K. F. (1979) Structure of bovine β-lactoglobulin at 6 Å resolution. *J. Mol. Biol.,* 131, 375–397.

[46] Creamer, L. K.; Parry, D. A. D.; Malcolm, G. N. (1983) Secondary structure of bovine β-lactoglobulin B. *Arch. Biochem. Biophys.,* 227, 98–105.

[47] Hamada, D.; Kuroda, Y.; Tanaka, T.; Goto, Y. (1995) High helical propensity of the peptide fragments derived from β-lactoglobulin, a predominantly β-sheet protein. *J. Mol. Biol.,* 254, 737–746.

[48] Kuroda, Y.; Hamada, D.; Tanaka, T.; Goto, Y. (1996) High helicity of peptide fragments corresponding to β-strand regions of β-lactoglobulin observed by 2D-NMR spectroscopy. *Fold. Des.,* 1, 255–263.

[49] Chamani, J.; Moosavi-Movahedi, A. A.; Rajabi, O.; Gharanfoli, M.; Momen-Heravi, M.; Hakimelahi, G. H.; Neamati-Baghsiah, A.; Varasteh, A. R. (2006) Cooperative α-helix formation of β-lactoglobulin induced by sodium n-alkyl sulfates. *J. Coll. Inter. Sci.,* 293, 52–60.

[50] Bewley, M. C.; Qin, B. Y.; Jameson, G. B.; Sawyer, L.; Baker, E. N. (1997) Bovine β-lactoglobulin and its variants: a three-dimensional structural perspective. In: *Milk Protein Polymorphism,* IDF Special Issue 9702, pp. 100–9. Brussels: International Dairy Federation.

[51] Brownlow, S.; Morais Cabral, J. H.; Cooper, R.; Flower, D. R.; Yewdall, S. J.; Polikarpov, I.; North, A. C. T.; Sawyer, L. (1997) Bovine β-lactoglobulin at 1.8 Å resolutions still an enigmatic lipocalin. *Structure*, 5, 481-495.

[52] Qin, B. Y.; Bewley, M. C.; Creamer, L. K.; Baker, E. N.; Jameson, G. B. (1999) Functional implications of structural differences between variants A and B of bovine β-lactoglobulin. *Prot. Sci.,* 8, 75–83.

[53] Tanford, C.; Nozaki, Y. (1959) Physico-chemical comparison of β-lactoglobulins A and B. *J. Biol. Chem.,* 234, 2874–2877.

[54] Tanford, C.; Bunville, L. G.; Nozaki, Y. (1959) Reversible transformation of β-lactoglobulin at pH 7.5. *J. Am. Chem. Soc.,* 81, 4032–4036.

[55] Pedersen, K. O. (1936) Ultracentrifugal and electrophoretic studies on the milk proteins: the lactoglobulin of Palmer. *Biochem. J.,* 30, 961–970.

[56] Groves, M. L.; Hipp, N. J.; McMeekin, T. L. (1951) Effect of pH on the denaturation of β-lactoglobulin and its dodecyl sulfate derivative. *J. Am. Chem. Soc.,* 73, 2790–2793.

[57] Qin, B. Y.; Bewley, M. C.; Creamer, L. K.; Baker, H. M.; Baker, E. N.; Jameson, G. B. (1998) Structural basis of the Tanford transition of bovine β-lactoglobulin. *Biochemistry,* 37, 14014–14023.

[58] Monaco, H. L.; Zanotti, G.; Spadon, P.; Bolognesi, M.; Sawyer, L.; Eliopoulos, E. E.; (1987) Crystal structure of the trigonal form of bovine β-lactoglobulin and of its complex with retinol at 2.5 Å resolution. *J. Mol. Biol.,* 197, 695–706.

[59] Dufour, E.; Genot, C.; Haertlé, T. (1994) β-Lactoglobulin binding properties during its folding changes studied by fluorescence spectroscopy. *Biochim. Biophys. Acta,* 1205, 105–112.

[60] Lange, D. C.; Kothari, R.; Patel, R. C.; Patel, S. C. (1998) Retinol and retinoic acid bind to a surface cleft in bovine β-lactoglobulin: a method of binding site determination using fluorescence resonance energy transfer. *Biophys. Chem.,* 74, 45–51.

[61] Narayan, M.; Berliner, L. J. (1998) Mapping fatty acid binding to β-lactoglobulin: ligand binding is restricted by modification of Cys 121. *Prot. Sci.,* 7, 150–157.

[62] Kontopidis, G.; Holt, C.; Sawyer, L. (2004) β-Lactoglobulin: binding properties, structure, and function. *J. Dairy Sci.,* 87, 785–796.

[63] Qin, B. Y.; Creamer, L. K.; Baker, E. N.; Jameson, G. B. (1998) 12-Bromododecanoic acid binds inside the calyx of bovine β-lactoglobulin. *FEBS Lett.,* 438, 272–278.

[64] Ragona, L.; Pusterla, F.; Zetta, L.; Monaco, H. L.; Molinari, H. (1997) Identification of a conserved hydrophobic cluster in partially folded bovine β-lactoglobulin at pH 2. *Fold. Des.,* 2, 281–290.

[65] Fogolari, F.; Ragona, L.; Zetta, L.; Romagnoli, S.; de Kruif, K. G.; Molinari, H. (1998) Monomeric bovine β-lactoglobulin adopts a β-barrel fold at pH 2. *FEBS Lett.,* 436, 149–154.

[66] Kuwata, K.; Hoshino, M.; Era, S.; Batt, C. A.; Goto, Y. (1998) α→β transition of β-lactoglobulin as evidenced by heteronuclear NMR. *J. Mol. Biol.,* 283, 731–739.

[67] Uhrinova, S.; Uhrin, D.; Denton, H.; Smith, M.; Sawyer, L.; Barlow, P. N. (1998) Complete assignment of ^{1}H, ^{13}C and ^{15}N chemical shifts for bovine β-lactoglobulin: secondary structure and topology of the native state is retained in a partially unfolded form. *J. Biomol. NMR,* 12, 89–107.

[68] Cavanagh, J.; Fairbrother, W.; Palmer, A. G.; Skelton, N. (1995) *Protein NMR Spectroscopy: Principles and Practice*. San Diego, California : Academic Press.

[69] Kuwata, K.; Hoshino, M.; Forge, V.; Era, S.; Batt, C. A.; Goto, Y. (1999) Solution structure and dynamics of bovine β-lactoglobulin A. *Prot. Sci.,* 8, 2541–2545.

[70] Uhrinova, S.; Smith, M. H.; Jameson, G. B.; Uhrin, D.; Sawyer, L.; B arlow, P. N. (2000) Structural changes accompanying pH-induced dissociation of the β-lactoglobulin dimer. *Biochemistry,* 39, 3565–3574.

[71] Kim, T-R.; Goto, Y.; Hirota, N.; Kuwata, K.; Denton, H.; Wu, S-Y.; Sawyer, L.; Batt, C. A. (1997) High-level expression of bovine β-lactoglobulin in Pichia pastoris and characterization of its physical properties. *Prot. Eng.,* 10, 1339–1345.

[72] Denton, H.; Smith, M.; Husi, H.; Uhrin, D.; Barlow, P. N.; Batt, C. A.; Sawyer, L. (1998) Isotopically labeled bovine β-lactoglobulin for NMR studies expressed in Pichia pastoris. *Prot. Expr. Pur.,* 14, 97–103.

[73] Jameson, G. B.; Adams, J. J.; Creamer, L. K. (2002) Flexibility, functionality and hydrophobicity of bovine β-lactoglobulin. *Inter. Dairy J.,* 12, 319–329.

[74] Palmer, A. H. (1934) The preparation of a crystalline globulin from the alabumin fraction of cow's milk. *J. Biol. Chem.*, 104, 359-372.

[75] Kinekawa, Y.; Kitabatake, N. (1996) Purification of beta-lactoglobulin from whey protein concentrate by pepsin treatment. *J. Dairy Sci.*, 79, 350-356.

[76] Outinen, M.; Tossavainen, O.; Tupasela, T.; Koskela, P.; Koskinen, H.; Rantamaki, P.; Syvaoja, E-L.; Antila, P.; Kankare, V. (1996) Fractionation of proteins from whey with different pilot scale processes. *Food Sci. Technol.*, 29, 411-417.

[77] de Jongh, H. H.; Groneveld, T.; de Groot, J. (2001) Mild isolation procedure discloses new protein structural properties of beta-lactoglobulin. *J. Dairy Sci.*, 84, 562-571.

[78] Sakai, K.; Sakurai, K.; Sakai, M.; Hoshino, M.; Goto, Y. (2000) Conformation and stability of thiol-modified bovine beta-lactoglobulin. *Prot. Sci.*, 9, 1719-1729.

[79] Astwood, J. D.; Leach, J. N.; Fuchs, R. L. (1996) Stability of food allergens to digestion in vitro. *Nat. Biotechnol.*, 14, 1269-1273.

[80] Takagi, K.; Teshima, R.; Okunuki, H.; Sawada, J. (2003) Comparative study of in vitro digestibility of food proteins and effect of preheating on the digestion. *Biol. Pharm. Bulletin*, 26, 969-973.

[81] Fu, T. J.; Abbott, U. R.; Hatzos, C. (2002) Digestibility of food allergens and nonallergenic proteins in simulated gastric fluid and simulated intestinal fluid-a comparative study. *J. Agric. Food Chem.*, 50, 7154-7160.

[82] Kitabatake, N.; Kinekawa, Y. (1998) Digestibility of bovine milk whey protein and β-lactoglobulin in vitro and in vivo. *J. Agric. Food Chem.*, 46, 4917-4923.

[83] Sorva, R.; Makinen-Kiljunen, S.; Juntunen-Backman, K. (1994) Beta-lactoglobulin secretion in human milk varies widely after cow's milk ingestion in mothers of infants with cow's milk allergy. *J. Allergy Clin. Immunol.*, 93, 787-792.

[84] Fukushima, Y.; Kawata, Y.; Onda, T.; Kitagawa, M. (1997) Consumption of cow milk and egg by lactating women and the presence of beta-lactoglobulin and ovalbumin in breast milk. *Am. J. Clin. Nutr.*, 65, 30-35.

[85] Conti, A.; Giuffrida, M. G.; Napolitano, L.; Quaranta, S.; Bertino, E.; Coscia, A.; Costa, S.; Fabris, C. (2000) Identification of the human beta-casein C-terminal fragments that specifically bind to purified antibodies to bovine beta-lactoglobulin. *J. Nutr. Biochem.*, 11, 332-337.

[86] Reddy, I.; Kella, N.; Kinsella, J. (1988) Structural and conformational basis of the resistance of beta-lactoglobulin to peptic and chymotryptic digestion. *J. Agric. Food Chem.*, 36, 737-741.

[87] Mahe, S.; Messing, B.; Thuillier, F.; Tome, D. (1991) Digestion of bovine milk proteins in patients with a high jejunostomy. *Am. J. Clin. Nutr.*, 54, 534-538.

[88] de Wit, J. N.; Swinkels, G. A. M. (1980) A differential scanning calorimetric study of the thermal denaturation of bovine β-lactoglobulin. Thermal behavior at temperatures up to 100 °C. *Biochim. Biophys. Acta, Prot. Stru.,* 624, 40–50.

[89] Qi, X. L.; Brownlow, S.; Holt, C.; Sellers, P. (1995) Thermal denaturation of β-lactoglobulin: effect of protein concentration at pH 6.75 and 8.05. *Biochim. Biophys. Acta,* 1248, 43–49.

[90] Iametti, S.; De Gregori, B.; Vecchio, G.; Bonomi, F. (1996) Modifi cations occur at different structural levels during the heat denaturation of β-lactoglobulin. *Eur. J. Biochem.,* 237, 106–112.

[91] Creamer, L. K.; Bienvenue, A.; Nilsson, H.; Paulsson, M.; Van Wanroij, M.; Lowe, E. K.; Anema, S. G.; Boland, M. J.; Jimenez-Flores, R. (2004) Heat-induced redistribution of disulfide bonds in milk proteins. 1. Bovine β-lactoglobulin. *J. Agric. Food Chem.,* 52, 7660–7668.

[92] Cho, Y.; Gu, W.; Watkins, S.; Lee, S. P.; Kim, T. R.; Brady, J. W.; Batt, C. A. (1994) Thermostable variants of bovine β-lactoglobulin. *Prot. Eng.,* 7, 263–270.

[93] Havea, P.; Singh, H.; Creamer, L. K. (2001) Characterization of heat-induced aggregates of β-lactoglobulin, α-lactalbumin and bovine serum albumin in a whey protein concentrate environment. *J. Dairy Res.,* 68, 483–497.

[94] Edwards, P. J. B.; Jameson, G. B.; Palmano, K. P.; Creamer, L. K. (2002) Heat-resistant structural features of bovine β-lactoglobulin A revealed by NMR H/D exchange observations. *Inter. Dairy J.,* 12, 331–344.

[95] Schokker, E. P.; Singh, H.; Pinder, D. N.; Creamer, L. K. (2000) Heat-induced aggregation of β-lactoglobulin AB at pH 2.5 as influenced by ionic strength and protein concentration. *J. Agric. Food Chem.,* 10, 233–240.

[96] Relkin, P. (1996) Thermal unfolding of β-lactoglobulin, α-lactalbumin, and bovine serum albumin. A thermodynamic approach. *Crit. Rev. Food Sci. Nut.,* 36, 565–601.

[97] Considine, T.; Patel, H. A.; Singh, H.; Creamer, L. K. (2005) Influence of binding of sodium dodecyl sulfate, all-trans-retinol, palmitate, and 8-anilino-1-naphthalenesulfonate on the heat-induced unfolding and aggregation of β-lactoglobulin B. *J. Agric. Food Chem.,* 53, 3197–3205.

[98] Busti, P.; Gatti, C. A.; Delorenzi, N. J. (2006) Binding of alkylsulfonate ligands to bovine β-lactoglobulin: effects on protein thermal unfolding. *Food Res. Inter.,* 39, 503–509.

[99] Manderson, G. A.; Hardman, M. J.; Creamer, L. K. (1997) Spectroscopic examination of the heat-induced changes in β-lactoglobulin A, B and C. In *Milk Protein Polymorphism,* IDF Special Issue 9702, pp. 204–11. Brussels: International Dairy Federation.

[100] Kaminogawa, S.; Shimizu, M.; Ametani, A.; Hattori, M.; Ando, O.; Hachimura, S.; Nakamura, Y.; Totsuka, M.; Yamauchi, K. (1989) Monoclonal antibodies as probes for monitoring the denaturation process of bovine β-lactoglobulin. *Biochim. Biophys. Acta,* 998, 50–56.

[101] Casal, H. L.; Kohler, U.; Mantsch, H. H. (1988) Structural and conformational changes of β-lactoglobulin B: an infrared spectroscopic study of the effect of pH and temperature. *Biochim. Biophys. Acta,* 957, 11–20.

[102] Qi, X. L.; Holt, C.; McNulty, D.; Clarke, D. T.; Brownlow, S.; Jones, G. R. (1997) Effect of temperature on the secondary structure of β-lactoglobulin at pH 6.7, as determined by CD and IR spectroscopy: a test of the molten globule hypothesis. *Biochem. J.,* 324, 341–346.

[103] Belloque, J.; Smith, G. M. (1998) Thermal denaturation of β-lactoglobulin. A ^{1}H NMR study. *J. Agric. Food Chem.,* 46, 1805–1813.

[104] Bhattacharjee, C.; Saha, S.; Biswas, A.; Kundu, M.; Ghosh, L.; Das, K. P. (2005) Structural changes of β-lactoglobulin during thermal unfolding and refolding-an FT-IR and circular dichroism study. *Prot. J.,* 24, 27–35.

[105] Patel, H. A.; Singh, H.; Havea, P.; Considine, T.; Creamer, L. K. (2005) Pressure induced unfolding and aggregation of the proteins in whey protein concentrate solutions. *J. Agric. Food Chem.,* 53, 9590–9601.

[106] Vant, S. C.; Glen, N. F.; Kontopidis, G.; Sawyer, L.; Schaschke, C. J. (2002) Volumetric changes to the molecular structure of β-lactoglobulin processed at high pressure. *High Temp. High Pressu.,* 34, 705–712.

[107] Knudsen, J. C.; Otte, J.; Olsen, K.; Skibsted, L. H. (2002) Effect of high hydrostatic pressure on the conformation of β-lactoglobulin A as assessed by proteolytic peptide profiling. *Inter. Dairy J.,* 12, 791–803.

[108] Belloque, J.; Lopez-Fandino, R.; Smith, G. M. (2000) A ^{1}H-NMR study on the effect of high pressures on β-lactoglobulin. *J. Agric. Food Chem.,* 48, 3906–3912.

[109] Skibsted, L. H.; Orlien, V.; Stapelfeldt, H. (2007) Temperature effects on pressure denaturation of β-lactoglobulin. *Milchwissenschaft,* 62, 13–15.

[110] Aouzelleg, A.; Bull, L-A.; Price, N. C.; Kelly, S. M. (2004) Molecular studies of pressure/temperature-induced structural changes in bovine β-lactoglobulin. *J. Sci. Food Agric.,* 84, 398–404.

[111] Dufour, E.; Hervé, G.; Haertlé, T. (1995) Hydrolysis of β-lactoglobulin by thermolysin and pepsin under high hydrostatic pressure. *Biopolymers,* 35, 475–483.

[112] Kuwata, K.; Li, H.; Yamada, H.; Batt, C. A.; Goto, Y.; Akasaka, K. (2001) High pressure NMR reveals a variety of fluctuating conformers in β-lactoglobulin. *J. Mol. Biol.,* 305, 1073–1083.

[113] Considine, T.; Singh, H.; Patel, H. A.; Creamer, L. K. (2005) Influence of binding of sodium dodecyl sulfate, all-trans-retinol, and 8-anilino-1-naphthalenesulfonate on the high-pressure-induced unfolding and aggregation of β-lactoglobulin B. *J. Agric. Food Chem.,* 53, 8010–8018.

[114] Tanford, C.; De, P. K.; Taggart, V. G. (1960) The role of the α-helix in the structure of proteins. Optical rotatory dispersion of β-lactoglobulin. *J. Am. Chem. Soc.,* 82, 6028–6034.

[115] Gosal, W. S.; Clark, A. H.; Pudney, P. D. A.; Ross-Murphy, S. B. (2002) Novel amyloid fibrillar networks derived from a globular protein: β-lactoglobulin. *Langmuir,* 18, 7174–7181.

[116] Dar, T. A.; Singh, L. R.; Islam, A.; Anjum, F.; Moosavi-Movahedi, A. A.; Ahmad, F. (2007) Guanidinium chloride and urea denaturations of β-lactoglobulin A at pH 2.0 and 25 °C: the equilibrium intermediate contains non-native structures (helix, tryptophan and hydrophobic patches). *Biophys. Chem.,* 127, 140–148.

[117] Hagihara, Y.; Aimoto, S.; Fink, A. L.; Goto, Y. (1993) Guanidine hydrochloride induced folding of proteins. *J. Mol. Biol.,* 231, 180–184.

[118] D'Alfonso, L.; Collini, M.; Ragona, L.; Ugolini, R.; Baldini, G.; Molinari, H. (2004) Porcine β-lactoglobulin chemical unfolding: identification of a non-native α-helical intermediate. *Prot. Stru. Func. Bioinf.,* 58, 70–79.

[119] Patel, H. A.; Singh, H.; Anema, S. G.; C reamer, L. K. (2006) Effects of heat and high hydrostatic pressure treatments on disulfide bonding interchanges among the proteins in skim milk. *J. Agric. Food Chem.,* 54, 3409–3420.

[120] Manderson, G. A.; Hardman, M. J.; Creamer, L. K. (1998) Effect of heat treatment on the conformation and aggregation of β-lactoglobulin A, B, and C. *J. Agric. Food Chem.,* 46, 5052–5061.

[121] Sawyer, L.; Brownlow, S.; Polikarpov, I.; Wu, S-Y. (1998) β-Lactoglobulin: structural studies, biological clues. *Inter. Dairy J.,* 8, 65–72.

[122] Zsila, F.; Bikadi, Z.; Simonyi, M. (2002) Retinoic acid binding properties of the lipocalin member β-lactoglobulin studied by circular dichroism, electronic absorption spectroscopy and molecular modeling methods. *Biochem. Pharm.,* 64, 1651–1660.

[123] Zsila, F.; Hazai, E.; Sawyer, L. (2005) Binding of the pepper alkaloid piperine to bovine β-lactoglobulin: circular dichroism spectroscopy and molecular modeling study. *J. Agric. Food Chem.,* 53, 10179–10185.

[124] Ragona, L.; Fogolari, F.; Catalano, M.; Ugolini, R.; Zetta, L.; Molinari, H. (2003) EF loop conformational change triggers ligand binding in β-lactoglobulins. *J. Biol. Chem.,* 278, 38840–38846.

[125] Konuma, T.; Sakurai, K.; Goto, Y. (2007) Promiscuous binding of ligands by β-lactoglobulin involves hydrophobic interactions and plasticity. *J. Mol. Biol.,* 368, 209–218.

[126] Wu, S. Y.; Perez, M. D.; Puyol, P.; Sawyer, L. (1999) β-Lactoglobulin binds palmitate within its central cavity. *J. Biol. Chem.,* 274, 170–174.

[127] Adams, J. J.; Anderson, B. F.; Norris, G. E.; Creamer, L. K.; Jameson, G. B. (2006) Structure of bovine β-lactoglobulin (variant A) at very low ionic strength. *J. Struc. Biol.,* 154, 246–254.

[128] Tromelin, A.; Guichard, E. (2006) Interaction between flavor compounds and β-lactoglobulin: approach by NMR and 2D/3D-QSAR studies of ligands. *Flav. Frag. J.,* 21, 13–24.

[129] Lefevre, T.; Subirade, M. (2001) Conformational rearrangement of β-lactoglobulin upon interaction with anionic membrane. *Biochim. Biophys. Acta*, 15, 37-50.

[130] Collini, M.; D'Alfonso, L.; Baldini, G. (2000) New insight on beta-lactoglobulin binding sites by 1-anilinonaphthalene-8-sulfonate fluorescence decay. *Protein. Sci.*, 9, 1968-1974.

[131] Taheri-Kafrani, A.; Bordbar, A. K.; Mousavi, H.; Haertlé, T. (2008) β-lactoglobulin structure and retinol binding changes in presence of anionic and neutral detergents. *J. Agric. Food Chem.*, 56, 7528-7534.

[132] Taheri-Kafrani, A.; Asgari-Mobarakeh, E.; Bordbar, A. K.; Haertlé, T. (2010) Structure-function relationship of β-lactoglobulin in the presence of dodecyltrimethyl ammonium bromide. *Coll. Surf. B: Biointerfaces*, 75, 268-274.

[133] Berger, M.; Gray, J. A.; Roth, B. L. (2009) The expanded biology of serotonin. *Annu. Rev. Med.*, 60, 355-366.

[134] Kang, K.; Park, S.; Kim, Y. S.; Lee, S.; Back, K. (2009) Biosynthesis and biotechnological production of serotonin derivatives. *Appl. Microbiol. Biotechnol.*, 83, 27-34.

[135] Pai, V. P.; Horseman, N. D. (2008) Biphasic regulation of mammary epithelial resistance by serotonin through activation of multiple pathways. *J. Biol. Chem.*, 283, 30901-30910.

[136] Gershon, M. D.; Tack, J. (2007) The serotonin signaling system: from basic understanding to drug development for functional GI disorders. *Gastroenterology*, 132, 397-414.

[137] Novellis, V.; Palazzo, E.; Rossi, F.; Petrocellis, L.; Petrosino, S.; Guida, F.; Luongo, L.; Migliozzi, A.; Cristino, L.; Marabese, I.; Starowicz, K.; Marzo, V.; Maione, S. (2008) The analgesic effect of *N*-arachidonoyl-serotonin, a FAAH inhibitor and TRPV1 receptor antagonist, associated with changes in rostral ventromedial medulla and locus coeruleus cell activity in rats. *Neuropharmacology*, 55, 1105-1113.

[138] Maione, S.; Petrocellis, L.; Novellis, V.; Moriello, A. S.; Petrosino, S.; Palazzo, E.; Rossi, F. S.; Woodward, D. F.; Marzo, V. (2007) Analgesic actions of N-arach-idonoyl-serotonin, a fatty acid amide hydrolase inhibitor with antagonistic activity at vanilloid TRPV1 receptors. *Br. J. Pharmacol.*, 150, 766-781.

[139] Hoffmann, M. A. M.; van Mil, P. J J. M. (1997) Heat-induced aggregation of beta-lactoglobulin : role of the free thiol group and disulfide bonds. *J. Agric. Food Chem.*, 45, 2942-2948.

[140] Jayat, D.; Gaudin, J. C.; Chobert, J. M.; Burova, T. V.; Holt, C.; McNae, I.; Sawyer, L.; Haertlé, T. (2004) A recombinant C121S mutant of bovine β-lactoglobulin is more susceptible to peptic digestion and to denaturation by reducing agents and heating. *Biochemistry*, 43, 6312-6321.

[141] Qi, X. L.; Brownlow, S.; Holt, C.; Sellers, P. (1995) Multi-state thermal unfolding and aggregation of β-lactoglobulin A. *Biochem. Soc. Trans.*, 23, 74S.

[142] Manderson, G. A.; Hardman, M. J.; Creamer, L. K. (1999) Effect of heat treatment on bovine β-lactoglobulin A, B, and C explored using thiol availability and fluorescence. *J. Agric. Food Chem.*, 47, 3617-3627.

[143] Burova, T. V.; Choiset, Y.; Tran, V.; Haertlé, T. (1998) Role of free Cys121 in stabilization of bovine β-lactoglobulin B. *Prot. Eng.*, 11, 1065-1073.

[144] McKenzie, H. A.; Ralston, G. B.; Shaw, D. C. (1972) Location of sulfhydryl and disulfide groups in bovine β-lactoglobulins and effects of urea. *Biochemistry*, 11, 4539-4547.

[145] Phelan, P.; Malthouse, P. G. (1994) ^{13}C-NMR of the cyanylated β-lactoglobulins: evidence that Cys-121 provides the thiol group of BLG A and B. *Biochem. J.*, 302, 511-516.

[146] Yen, T.-Y.; Rajesh, K. J.; Yan, H.; Seto, N. O. L.; Palcic, M. M.; Macher, B. A. (2000) Characterization of cysteine residues and disulfide bonds in proteins by liquid chromatography/electrospray ionization tandem mass spectrometry, *J. Mass Spectr.*, 35, 990-1002.

[147] Gell P. G. H.; Coombs, R. R. A. (1963) The classification of allergic reactions underlying disease. In: R. R. A. Coombs (ed) Clinical Aspects of Immunology. Oxford, Blackwell, p.

[148] Descotes, J.; Choquet-Kastylevsky, G. (2001) Gell and coombs's classification: is it still valid? *Toxicology*, 158, 43-49.

[149] Geha, R. S.; Jabara, H. H.; Brodeur, S. R. (2003) The regulation of immunoglobulin E class-switch recombination. *Nat. Rev. Immunol.*, 3, 721-732.

[150] Shakib, F.; Schulz, O.; Sewell, H. (1998) A mite subversive: cleavage of CD23 and CD25 by Der p 1 enhances allergenicity. *Immunol. Today*, 19, 313-316.

[151] Johansson, S. G.; Bieber, T.; Dahl, R.; Friedmann, P. S.; Lanier, B. Q.; Lockey, R. F.; Motala, C.; Ortega Martell, J. A.; Platts-Mills, T. A.; Ring, J.; Thien, F.; Van Cauwenberge, P.; Williams, H. C. (2004) Revised nomenclature for allergy for global use: report of the nomenclature review committee of the world allergy organization. *J. Allergy Clin. Immunol.*, 113, 832-836.

[152] Sampson, H. A. (2004) Update on food allergy. *J. Allergy Clin. Immunol.*, 113, 805-819.

[153] Dubuisson, C.; La Vielle, S.; Martin, A. *Allergies alimentaires: état des lieux et propositions*; Agence Française de Sécurité Sanitaire des Aliments Report, France, 2002.

[154] Docena, G. H.; Fernandez, R.; Chirdo, F. G.; Fossati, C. A. (1996) Identification of casein as the major allergenic and antigenic protein of cow's milk. *Allergy*, 51, 412-416.

[155] Natale, M.; Bisson, C.; Monti, G.; Peltran, A.; Garoffo, L. P.; Valentini, S.; Fabris, C.; Bertino, E.; Coscia, A.; Conti, A. (2004) Cow's milk allergens identification by two-dimensional immunoblotting and mass spectrometry. *Mol. Nutr. Food Res.*, 48, 363-369.

[156] Gaudin, J.-C.; Rabesona, H.; Choiset, Y.; Yeretssian, G.; Chobert, J.-M.; Sakanyan, V.; Drouet, M.; Haertlé, T. (2008) Assessment of the immunoglobulin E-mediated immune response to milk-specific proteins in allergic patients using microarrays. *Clin. Exp. Allergy*, 38, 686-693.

[157] Otani, H; Uchio, T.; Tokita, F. (1985) Antigenic reactivities of chemically modified β-lactoglobulins with antiserum to bovine β-lactoglobulins. *Agric. Biol. Chem.*, 49, 2531-2536.

[158] Sakaguchi, H.; Inoue, R.; Kaneko, H.; Watanabe, M.; Suzuki, K.; Kato, Z.; Matsushita, S.; Kondo, N. (2002) Interaction among human leucocyte antigen-peptide-T cell receptor complexes in cow's milk allergy: the significance of human leucocyte antigen and T cell receptor-complementarity determining region 3 loops. *Clin. Exp. Allergy*, 32, 762-770.

[159] Fritsche, R.; Adel-Patient, K.; Bernard, H.; Martin-Paschoud, C.; Schwarz, C.; Ah-Leung, S.; Wal, J. M. (2005) IgE-mediated rat mast cell triggering with tryptic and synthetic peptides of bovine beta-lactoglobulin. *Int. Arch. Allergy Immunol.*, 138, 291-297.

[160] Totsuka, M.; Ametani, A.; Kaminogawa, S. (1997) Fine mapping of T-cell determinants of bovine beta-lactoglobulin. *Cytotechnology*, 25, 101-113.

[161] Davis, P. J.; Smales, C. M.; James, D. C. (2001) How can thermal processing modify the antigenicity of proteins? *Allergy*, 56, 56-60.

[162] Song, C. Y.; Chen, W. L.; Yang, M. C.; Huang, J. P.; Mao, S. J. (2005) Epitope mapping of a monoclonal antibody specific to bovine dry milk: involvement of residues 66-76 of strand D in thermal denatured beta-lactoglobulin. *J. Biol. Chem.*, 280, 3574-3582.

[163] Ehn, B. M.; Ekstrand, B.; Bengtsson, U.; Ahlstedt, S. (2004) Modification of IgE binding during heat processing of the cow's milk allergen beta-lactoglobulin. *J. Agric. Food Chem.*, 52, 1398-1403.

[164] Maillard, L. C. (1912) Action des amines sur les sucres: formation des mélanoïdines par voie méthodique. *C. R. Acad. Sci.*, 154, 66-68.

[165] Chung, S. Y.; Champagne, E. T. (2001) Association of end-product adducts with increased IgE-binding of roasted peanuts. *J. Agric. Food Chem.*, 49, 3911–3916.

[166] Gruber, P.; Vieths, S; Wangorsch, A.; Nerkamp, J.; Hofmann, T. (2004) Maillard reaction and enzymatic browning affect the allergenicity of Pru av 1, the major allergen from cherry (Prunus avium). *J. Agric. Food Chem.*, 52, 4002–4007.

[167] Mondoulet, L.; Paty, E.; Drumare, M. F.; Ah-Leung, S.; Scheinmann, P.; Willemot, R. M.; Wal, J. M.; Bernard, H. (2005) Influence of thermal processing on the allergenicity of peanut proteins. *J. Agric. Food Chem.*, 53, 4547–4553.

[168] Nakamura, A.; Watanabe, K.; Ojima, T.; Ahn, D. H.; Saeki, H. (2005) Effect of Maillard reaction on allergenicity of scallop Tropomyosin. *J. Agric. Food Chem.*, 53, 7559–7564.

[169] Nakamura, A.; Sazaki, F.; Watanabe, K.; Ojima, T.; Ahn, D. H.; Saeki, H. (2006) Changes in allergenicity and digestibility of squid tropomyosin during Maillard reaction with ribose. *J. Agric. Food Chem.*, 54, 9529–9534.

[170] Nakamura, S.; Suzuki, Y.; Ishikawa, E.; Yakushi, T.; Jing, H.; Miyamoto, T.; Hashizume, K. (2008) Reduction of in vitro allergenicity of buckwheat Fag e 1 through the Maillard-type glycosylation with polysaccharides. *Food Chem.*, 109, 538–545.

[171] Maillard, P.; Ribadeau-Dumas, B. (1988) Preparation of [beta]-lactoglobulin and [beta]-lactoglobulin-free proteins from whey retentate by NaCI salting out at low pH. *J. Food Sci.*, 53, 743-745.

[172] Frister, H.; Meisel, H.; Schlimme, E. (1988) OPA method modified by use of N,N-dimethyl-2-mercaptoethylammonium chloride as thiol component. *Fresenius Z. Anal. Chem.*, 330, 631-633.

[173] Wilson, C.; Quarrie, L.; Allan, G. J.; Flint, D. J.; Sawyer, L.; Holt, C. (1999) Expression of recombinant wild-type and mutant β-lactoglobulins in the yeast Pichia pastoris. *Int. J. Food. Sci. Tech.*, 34, 445-450.

[174] Cogan, U.; Kopelman, M.; Mokady, S.; Shinitzky, M. (1976) Binding affinities of retinol and related compounds to retinol binding proteins. *Eur. J. Biochem.*, 65, 71-78.

[175] Guharay, J.; Sengupta, B.; Sengupta, P. K. (2001) Protein-flavonol interaction: fluorescence spectroscopic study. *Prot. Struc. Func. Bioinf.*, 43, 75-81.

[176] Bhattacharya, J.; Bhattacharya, M.; Chakrabarty, A. S.; Chaudhury, U.; Poddar, R. K. (1994) Interaction of chlorpromazine with myoglobin and hemoglobin, a comparative study. *Biochem. Pharmacology*, 47, 2049-2052.

[177] Wei, X. F.; Liu, H. Z. (2000) The interaction between triton x-100 and bovine serum albumin. *Chin. J. Anal. Chem.*, 28, 699-701.

[178] Mishra, B.; Barik, A.; Priyadarsini, K. I.; Mohan, H. (2005) Fluorescence spectroscopic studies on binding of a flavonoid antioxidant quercetin to serum albumins. *J. Chem. Sci.*, 117, 641-647.

[179] Nacka, F.; Chobert, J. M.; Burova, T.; Léonil, J.; Haertlé, T. (1998) Induction of new physicochemical and functional properties by the glycosylation of whey proteins. *J. Prot. Chem.*, 17, 495–503.

[180] Prabhakaram, M.; Ortwerth, B. J. (1994) Determination of glycation crosslinking by the sugar-dependent incorporation of [^{14}C]lysine into protein. *Anal. Biochem.*, 216, 305–312.

[181] Chevalier, F.; Chobert, J. M.; Molle, D.; Haertle, T. (2001) Maillard glycation of β-lactoglobulin with several sugars: comparative study of the properties of the obtained polymers and of the substituted sites. *Lait*, 81, 651–666.

[182] Chevalier, F.; Chobert, J. M.; Dalgalarrondo, M.; Choiset, Y.; Haertle, T. (2002) Maillard glycation of beta-lactoglobulin induces conformation changes. *Nahrung*, 46, 58–63.

[183] Kelly, S. M.; Jess, T. J.; Price, N. C. (2005) How to study proteins by circular dichroism. *Biochem. Biophys. Acta*, 1751, 119-139.

[184] Moore, C.; Fasman, G. D. (1993) The random coil conformation of proteins. Chemtracts-Biochem. *Mol. Biol.*, 4, 67-74.

[185] Woody, R. W. (1995) Circular dichroism. *Meth. Enzymol.*, 246, 34-71.

[186] Griffin, W. G.; Griffin, M. C. A.; Martin, S. R.; Price, J. (1993) The molecular basis of thermal aggregation of bovine β-lactoglobulin A. *J. Chem. Soc. Faraday Trans.*, 89, 3395-3406.

[187] Prabakaran, S.; Damodaran, S. (1997) Thermal unfolding of β-lactoglobulin: characterization of initial unfolding events responsible for heat-induced aggregation. *J. Agric. Food Chem.*, 45, 4303-4308.

[188] McSwiney, M.; Singh, H.; Campanella, O.; Creamer, L. K. (1994) Thermal gelation and denaturation of bovine β-lactoglobulins A and B. *J. Dairy Res.*, 61, 221 232.

[189] Creamer, L. K. (1995) Effect of sodium dodecyl sulfate and palmitic acid on the equilibrium unfolding of bovine β-lactoglobulin. *Biochemistry*, 34, 7170-7176.

[190] Strickland, E. H. (1974) Aromatic contribution to circular dichroism spectra of proteins. *CRC Crit. Rev. Biochem.*, 2, 113-175.

[191] Kahn, P. C. (1979) The interpretation of near-ultraviolet circular dichroism. *Meth. Enzymol.*, 61, 339-378.

[192] Matsuura, J. E.; Manning, M. C. (1994) Heat-induced gel formation of β-lactoglobulin: a study on the secondary and tertiary structure as followed by circular dichroism spectroscopy. *J. Agric. Food Chem.*, 42, 1650–1656.

[193] Kuwajima, K.; Yamaya, H.; Sugai, S. (1996) The burst-phase intermediate in the refolding of β-lactoglobulin studied by stopped-flow circular dichroism and absorption spectroscopy. *J. Mol. Biol.*, 264, 806-822.

[194] Provencher, S. W.; Glockner, J. (1981) Estimation of globular protein secondary structure from circular dichroism. *Biochemistry*, 20, 33-37.

[195] Venien, A.; Levieux, D.; Astier, C.; Briand, L.; Chobert, J.-M.; Haertle, T. (1997) Production and epitopic characterization of monoclonal antibodies against bovine β-lactoglobulin. *J. Dairy Sci.*, 80, 1977–1987.

[196] Otani, H.; Tokita, F. (1980) Natural and secondary antigenicity of betalactoglobulin heated in the presence of lactose. *Jpn. J. Zootechnical Sci.*, 51, 711–716.

[197] Otani, H.; Tokita, F. (1982) Contribution of the sugar moiety in the browning product between beta-lactoglobulin and lactose as an antigenic determinant. *Jpn. J. Zootechnical Sci.*, 53, 344–350.

[198] Jarvinen, K.-M.; Chatchatee, P.; Bardina, L.; Beyer, K.; Sampson, H. A. (2001) IgE and IgG binding epitopes on alpha-lactalbumin and betalactoglobulin in cow's milk allergy. *Int. Arch. Allergy Immunol.*, 126, 111–118.

[199] Chattopadhyay, A.; Rukmini, R.; Mukherjee, S. (1996) Photophysics of a neurotransmitter: ionization and spectroscopic properties of serotonin. *Biophys. J.*, 71, 1952-1960.

[200] Nag, S.; Balaji, J.; Madhu, P. K.; Maiti, S. (2008) Intermolecular association provides specific optical and NMR signatures for serotonin at intravesicular concentrations. *Biophys. J.*, 94, 4145-4153.

[201] Evans, H. C. (1956) Alkyl sulphates: Part I, Critical micelle concentrations of the sodium salts. *J. Chem. Soc.*, 80, 579-586.

[202] Jana, P. K.; Moulik, S. P. (1991) Interaction of bile salts with hexadecyltrimethylammonium bromide and sodium dodecyl sulfate. *J. Phys. Chem.*, 95, 9525-9532.

[203] Ghosh, S.; Moulik, S. P. (1998) Interfacial and micellization behaviors of binary and ternary mixtures of amphiphiles (Tween-20, Brij-35, and Sodium Dodecyl Sulfate) in aqueous medium. *J. Coll. Interface Sci.*, 208, 357-366.

[204] Chatterjee, A.; Moulik, S. P.; Sanyal, S. K.; Mishra, B. K.; Puri, P. M. (2001) Thermodynamics of micelle formation of ionic surfactants: A critical assessment for sodium dodecyl sulfate, cetyl pyridinium chloride and dioctyl sulfosuccinate (Na Salt) by microcalorimetric, conductometric, and tensiometric measurements. *J. Phys. Chem. B*, 105, 12823-12831.

[205] Moulik, S. P. (1996) Micelles: self-organized surfactant assemblies. *Curr. Sci.*, 71, 368-376.

[206] Stodghill, S. P.; Smith, A. E.; O'Haver, J. H. (2004) Thermodynamics of micellization and adsorption of three alkyltrimethylammonium bromides using isothermal titration calorimetry. *Langmuir*, 20, 11387-11392.

[207] Wallace, B. A.; Janes, R. W. (2003) Circular dichroism and synchrotron radiation dichroism circular spectroscopy: tools for drug discovery. *Biochem. Soc. Trans.*, 31, 631-633.

[208] Yagi, M.; Sakurai, K.; Kalidas, C.; Batt, C. A.; Goto, Y. (2003) Reversible unfolding of bovine β-lactoglobulin mutants without a free thiol group. *J. Biol. Chem.*, 278, 47009-47015.

[209] Taheri-Kafrani, A.; Gaudin, J. C.; Rabesona, H.; Nioi, C.; Agarwal, D.; Drouet, M.; Chobert, J. M.; Bordbar, A. K.; Haertlé, T. (2009) Effects of heating and glycation of β-lactoglobulin on its recognition by IgE of sera from cow milk allergy patients. *J. Agric. Food Chem.*, 57, 4974-4982.

[210] Sengupta, B.; Chaudhuri, S.; Banerjee, A.; Sengupta, P. K. (2004) Characterization of serotonin in protein and membrane mimetic environments: A spectroscopic study. *Chem. Biodiver.*, 1, 868-877.

[211] Ouyang, H.; Vogel, H. J. (1998) Melatonin and serotonin interactions with calmodulin: NMR, spectroscopic and biochemical studies. *Biochim. Biophys. Acta*, 1387, 37-47.

[212] Fessas, D.; Iametti, S.; Schiraldi, A.; Bonomi, F. (2001) Thermal unfolding of monomeric and dimeric β-lactoglobulins. *Eur. J. Biochem.*, 268, 5439-5448.

[213] Fujiwara, K.; Arai, M.; Shimizu, A.; Ikeguchi, M.; Kuwajima, K.; Sugai, S. (1999) Folding-unfolding equilibrium and kinetics of equine β-lactoglobulin: equivalence between the equilibrium molten globule state and a burst-phase folding intermediate. *Biochemistry*, 38, 4455-4463.

[214] N'soukpoe-Kossi, C. N.; St-Louis, C.; Beauregard, M.; Subirade, M.; Carpentier, R.; Hotchandani, S.; Tajmir-Riahi, H. A. (2006) Resveratrol binding to human serum albumin. *J. Biomol. Struct. Dyn.*, 24, 277-283.

[215] Liang, L.; Yao, P.; Jiang, M. (2005) Structural transformation of apocytochrome c induced by alternating copolymers of maleic acid and alkene. *Biomacromolecules*, 6, 2748-2755.

[216] Liang, L.; Tajmir-Riahi, H. A.; Subirade, M. (2008) Interaction of beta-lactoglobulin with resveratrol and its biological implication. *Biomacromolecules*, 9, 50–56.

[217] Kontopidis, G.; Holt, C.; Sawyer, L. (2002) The ligand binding site of bovine β-lactoglobulin: evidence for a function. *J. Mol. Biol.*, 318, 1043-1055.

[218] Wang, Q. W.; Allen, J. C.; Swaisgood, H. E. (1998) Protein concentration dependence of palmitate binding to β-lactoglobulin. *J. Dairy. Sci.*, 81, 76-81.

[219] Muresan, S.; van der Bent, A.; de Wolf, A. (2001) Interaction of β-lactoglobulin with small hydrophobic ligands as Monitored by fluorometry and equilibrium dialysis: Nonlinear quenching effects related to protein-protein association. *J. Agric. Food Chem.*, 49, 2609-2618.

[220] Mousavi, H.; Chobert, J. M.; Bordbar, A. K.; Haertlé, T. (2008) Ethanol effect on the structure of β-lactoglobulin B and its ligand binding. *J. Agric. Food Chem.*, 56, 8680-8684.

[221] Rytkonen, J.; Karttunen, T. J.; Karttunen, R.; Valkonen, K. H.; Jenmalm,M. C.; Alatossava, T.; Bjorksten, B.; Kokkonen, J. (2002) Effect of heat denaturation on beta-lactoglobulin-induced gastrointestinal sensitization in rats: Denatured beta-Lg induces a more intensive local immunologic response than native β-Lg. *Pediatr. Allergy Immunol.*, 13, 269–277.

[222] Cocco, R. R.; Jarvinen, K.-M.; Han, N.; Beyer, K.; Sampson, H. A. (2007) Mutational analysis of immunoglobulin E-binding epitopes of betacasein and beta-lactoglobulin showed a heterogeneous pattern of critical amino acids between individual patients and pooled sera. *Clin. Exp. Allergy*, 37, 831–838.

Published Papers

Interactions of β-Lactoglobulin with Serotonin and Arachidonyl Serotonin

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ABSTRACT:

β-Lactoglobulin (β-LG) is a lipocalin, which is the major whey protein of cow's milk and the milk of other mammals. However, it is absent from human milk. The biological function of β-LG is not clear, but its potential role in carrying fatty acids through the digestive tract has been suggested. β-LG has been found in complexes with lipids such as butyric and oleic acids and has a high affinity for a wide variety of compounds. Serotonin (5-hydroxytryptamine, 5-HT), an important compound found in animals and plants, has various functions, including the regulation of mood, appetite, sleep, muscle contraction, and some cognitive functions such as memory and learning. In this study, the interaction of serotonin and one of its derivatives, arachidonyl serotonin (AA-5HT), with β-LG was investigated using circular dichroism (CD) and fluorescence intensity measurements. These two ligands interact with β-LG forming equimolar complexes. The binding constant for the serotonin/β-LG interaction is between 10^5 and 10^6 M^{-1}, whereas for the AA-5HT/β-LG complex it is between 10^4 and 10^5 M^{-1} as determined by measurements of either protein or ligand fluorescence. The observed binding affinities were higher in hydroethanolic media (25% EtOH). The interactions between serotonin/β-LG and AA-5HT/β-LG may compete with self-association (micellization) of both the ligand and the protein. According to far- and near-UV CD results, these ligands have no apparent influence on β-LG secondary structure, however they partially destabilize its tertiary structure. Their binding by β-LG may be one of the peripheral mechanisms of the regulation of the content of serotonin and its derivatives in the bowel of milk-fed animals. © *2011 Wiley Periodicals, Inc. Biopolymers 95: 871–880, 2011.*

Keywords: *β-lactoglobulin; serotonin; arachidonyl serotonin; micellization; binding constant; fluorimetry; circular dichroism*

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INTRODUCTION

Serotonin (5-hydroxytryptamine, 5-HT, Figure 1A) is a monoamine neurotransmitter. 5-HT and its derivatives are found extensively in the gastrointestinal tract of animals, and about 80% to 90% of the total 5-HT in the human body is located in the

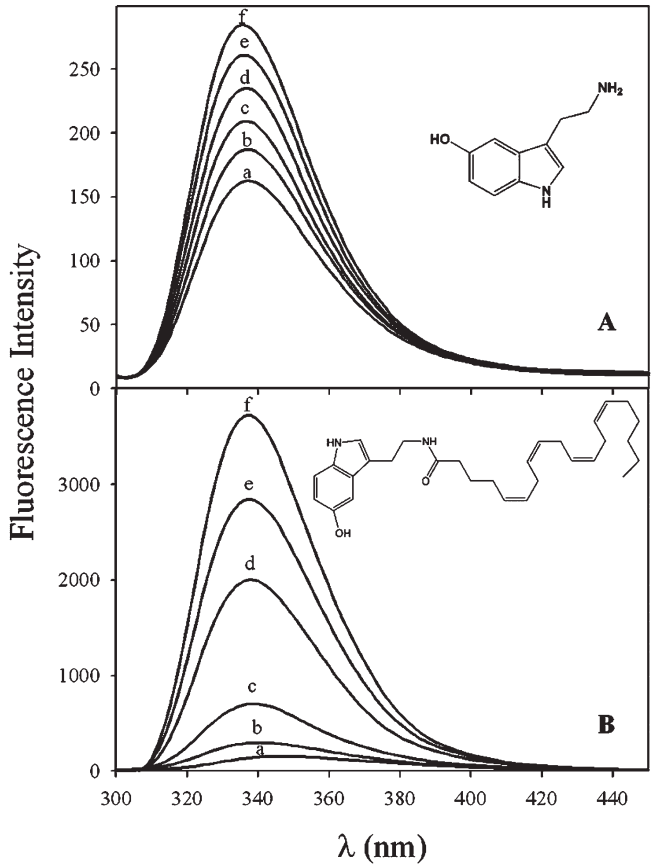

FIGURE 1 Fluorescence emission spectra of 10 μM (A) 5-HT and (B) AA-5HT at (a–f): 0, 10, 20, 30, 40, and 50% ethanol in 10 mM phosphate buffer, pH 7.4. Similar spectra were observed in 10 mM glycine buffer, pH 2.0. The structure of 5-HT and AA-5HT is presented in insets.

enterochromaffin cells (Kulchitsky cells) in the gut, where it is used to regulate intestinal movements.[1,2] The remainder is synthesized in serotoninergic neurons in the central nervous system (CNS) where it has various functions, including the regulation of mood, appetite, sleep, muscle contraction, and some cognitive functions such as memory and learning. 5-HT is found not only in animals, but also in fungi and plants including fruits and vegetables.[3]

Although 5-HT was discovered 60 years ago,[4] the studies of 5-HT and its receptors continue to yield new proof of its physiological importance in almost all major organs and systems, including the cardiovascular, pulmonary, gastrointestinal, and genitourinary systems as well as the CNS.[1] 5-HT is best known as a neurotransmitter modulating the activities of a wide range of neuropsychological processes. Drugs targeting 5-HT receptors are widely used in psychiatry and neurology. However, most 5-HT is found outside the CNS, and essentially all of the known fifteen 5-HT receptors are expressed outside as well as inside the brain.[5] 5-HT modulates many human behavioral processes. Mammary gland homeostasis and the lactation-to-involution switch are regulated by 5-HT. Mammary epithelial tight junctions are targets of 5-HT, and their disruption marks an early stage of mammary gland involution.[6] 5-HT is also an important gastrointestinal signaling molecule. It is a paracrine messenger utilized by enterochromaffin (EC) cells, which function as sensory transducers. 5-HT activates intrinsic and extrinsic primary afferent neurons to initiate peristaltic and secretory reflexes, respectively, and to transmit information to the central nervous system.[7] Abnormalities in 5-HT signaling are putatively implicated as causes of bowel diseases.

Arachidonyl serotonin (AA-5HT, Figure 1B) is an inhibitor of fatty acid amide hydrolase (FAAH), the enzyme responsible for inactivation of anandamide and other endogenous cannabinoids.[8] AA-5HT inhibits the FAAH activity isolated from mouse neuroblastoma cells with an IC_{50} value of 12 μM. Both the K_m (Michaelis–Menten constant is defined as the concentration of substrate that leads to half-maximal velocity) and the V_{max} (the maximum catalytic rate that can be achieved by a particular enzyme) of the enzyme are affected, indicating that AA-5HT is a tightly bound, uncompetitive inhibitor of FAAH. AA-5HT does not inhibit $cPLA_2$ (cytosolic phospholipase A_2) and is essentially devoid of cannabimimetic activity. This compound has been chosen as a lipophilic analog of 5-HT with promising applications in biochemistry and drug design. It modulates the activity of the enteric nervous system, influencing gastric and intestinal clearance. It affects the areas of central activities, including pain reduction, motor regulation, learning/memory, and reward. Finally, the role of the endocannabinoid system in appetite stimulation in the adult organism and, perhaps more importantly, its critical involvement in milk ingestion and the survival of the newborn, may not only advance our understanding of the physiology of food intake and growth, but may also lead to therapeutic applications in wasting diseases and infant's "failure to thrive."[9–12]

β-Lactoglobulin (β-LG) is a major whey protein of bovine milk with known primary, secondary, and three-dimensional structures but with still unknown biological function(s).[13] Its polypeptide chain is composed of 162 amino acid residues including two disulfide bonds (Cys66-Cys160 and Cys109-Cys119) and one free cysteine (Cys121).[13] β-LG exhibits affinity for a variety of hydrophobic and amphiphilic compounds, including retinol,[14] fatty acids,[15] phospholipids,[16] and aromatic compounds.[17] Binding to β-LG provides protection to retinol and β-carotene from degradation due to heat, oxidation, and irradiation.[18] It has therefore been anticipated that

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β-LG could be used as a versatile transporter of hydrophobic molecules in applications of controlled delivery.[19]

The binding constants of β-LG with different compounds vary extensively, from as small as 1.5×10^2 M^{-1} for 2-heptanone to as high as 6.8×10^5 M^{-1} for palmitate and 5×10^7 M^{-1} for retinol.[20] Three possible binding sites have been reported for ligand binding to β-LG: the interior cavity of the β-barrel, the surface hydrophobic pocket in a groove between the α-helix and the β-barrel, and the external surface near Trp19-Arg124.[17,21–24]

Various interactions, such as hydrophobic interactions, hydrogen bonding, and electrostatic interactions, may occur between β-LG and its ligands and they are also the driving forces for stabilization and for the structural transitions of proteins.[14,17,25] Consequently, hydrophobic and amphiphilic ligands could have an effect on the structure of β-LG. Cationic and anionic surfactants, such as dodecyltrimethylammonium chloride, dodecyldimethylammonium bromide and sodium dodecyl sulfate, and anionic phospholipids such as dimyristoylphosphatidylglycerol have been reported to destabilize the tertiary structure of β-LG, inducing its refolding from a dominant β-sheet to a prevailing α-helical secondary structure.[26–29]

In the present study, the interactions of 5-HT and AA-5HT with β-LG were investigated using circular dichroism (CD) and fluorescence spectroscopy to describe the potential of β-LG to bind such important regulators of lactation and intestinal transit as 5-HT and AA-5HT. The binding constants and binding sites of 5-HT and AA-5HT on β-LG were determined, and the effects of the ligand-protein interactions on β-LG structure are discussed.

EXPERIMENTAL

Materials

5-HT (purity >98%) was purchased from Sigma–Aldrich Chemical Co. AA-5HT was synthesized according to the procedure described previously[12] and its purity (HPLC check) was not less than 98%. β-LG variant A was isolated from the milk of a homozygous cow according to Mailliart and Ribadeau Dumas.[30] The homogeneity of the protein preparation was assessed by high performance gel permeation chromatography and SDS-polyacrylamide gel electrophoresis. The preparations of β-LG obtained were over 98% pure. Protein stock solution was made by dissolving β-LG in 10 m*M* phosphate buffer, pH 7.4, or 10 m*M* glycine buffer, pH 2.0, to obtain concentrations of 100 μ*M* measured by a spectrophotometer using a molar extinction coefficient of 17,600 M^{-1} cm^{-1} at 280 nm.[31]

5-HT and AA-5HT stock solutions were prepared by dissolving them at a concentration of 15 m*M* in 75% ethanol and then diluting to a final concentration of 1 m*M* with 10 m*M* phosphate buffer, pH 7.4, or 10 m*M* glycine buffer, pH 2.0. Samples were prepared by mixing β-LG and 5-HT or AA-5HT stock solutions in varying proportions. The highest resulting ethanol concentration in solutions was less than 4%, which had no major effect on protein structure.[23] The samples were incubated for 2 h at room temperature prior to analysis.

Steady-State Fluorescence Measurements

Fluorescence measurements were performed using an RF-5000 Shimadzu spectrofluorimeter with a cell compartment thermostated at 293 K. Protein intrinsic fluorescence experiments were performed at constant β-LG concentration (5 μ*M*) titrated with different 5-HT or AA-5HT concentrations, in order to have at least a molar ratio of ligand/protein equal to 20. Emission spectra were recorded from 300 to 450 nm with an excitation wavelength of 280 nm and at a scan rate of 60 nm/min. The results of all measurements of fluorescence were corrected by the results of the same titrations of medium without protein. Spectral resolution was 5 nm for both excitation and emission in these experiments.

The fluorescence of 5-HT or AA-5HT was measured at each of the above concentrations and at 10 μ*M* or 40 μ*M* in the presence of various concentrations of β-LG used for reverse titrations. These spectra were recorded from 310 to 450 nm with an excitation wavelength of 300 nm. All fluorescence measurements were corrected by the same titration of medium without ligand. Spectral resolution for 5-HT and AA-5HT was 2.5 nm and 5 nm, respectively, for both excitation and emission. The observed fluorescence intensities were corrected for dilution in all fluorescence experiments.

Competitive experiments were performed to highlight the binding site of serotonin and its derivative. In these experiments, retinol, which binds tightly to the internal cavity of the protein was used. In the first set of competitive displacement titration experiments, protein intrinsic fluorescence experiments were performed at constant β-LG/serotonin concentration (5 μ*M* to produce a molar ratio of 1) titrated with different retinol concentrations, in order to attain at least a molar β-LG/retinol ratio of 20. The reverse titration experiments were performed at a constant molar ratio of β-LG/retinol equal to 1, titrated with different serotonin concentrations, in order to have at least a molar β-LG/serotonin ratio of 20. Emission spectra were recorded from 300 to 450 nm with an excitation wavelength of 280 nm and scan rate of 60 nm/min. The results of all measurements of fluorescence were corrected by the results of the same titrations of medium without β-LG. Spectral resolution was 5 nm for both excitation and emission in these experiments.

Circular Dichroism Spectroscopy

CD spectra were recorded on a CD6 dichrograph (Jobin Yvon, Longjumeau, France), using a scan time of 1 nm/s, 0.2 nm resolution, 10 accumulations, and 1.0 nm bandwidth. All measurements were carried out at 293 K with thermostatically controlled cell holders. The instruments were calibrated with ammonium d-10-camphorsulfonic acid. The data were expressed as molar ellipticity (θ) (with units of degrees/cm^2/decimole), which is defined as $(\theta) = 100\ \theta_{obs}/cl$, where θ_{obs} is the observed ellipticity in degrees, c is the molar concentration (mol/l), and l is the pathlength of the light in cm.

Far-UV (190–260 nm) and near-UV (250–320 nm) CD spectra were obtained at a constant protein concentration of 10 μ*M* and 20 μ*M*, respectively, titrated with 5-HT or AA-5HT reaching concen-

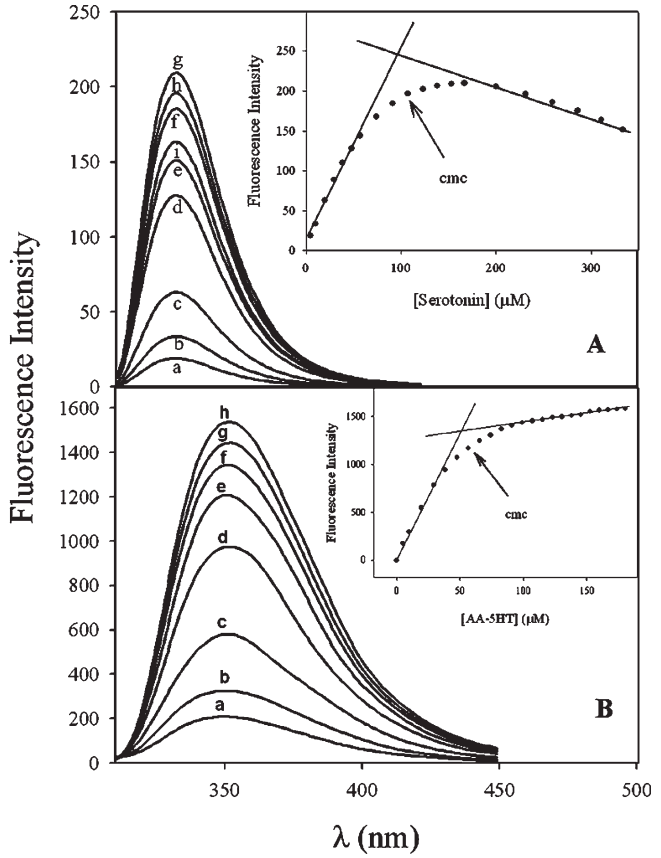

FIGURE 2 Fluorescence emission spectra of (A) 5-HT, at (a–i): 5, 10, 20, 50, 80, 100, 150, 250, and 300 μM, and (B) AA-5HT at (a–h): 5, 10, 20, 50, 60, 80, 100, and 150 μM concentration, in 10 mM phosphate buffer, pH 7.4. Similar effects were obtained for these ligands in 10 mM glycine buffer, pH 2.0. Inset: Evolution of fluorescence intensity as a function of 5-HT and AA-5HT concentration. Sharp inclinations of spectra are observed at the CMC.

trations of 0, 5, 10, 20, 40, and 80 μM in 10 mM phosphate buffer, pH 7.4, or 10 mM glycine buffer pH 2.0. Pathlengths were 0.05 cm and 1 cm for the far-UV and near-UV region, respectively. The blank background was subtracted from the raw spectra for each spectrum.

Determination of the Binding Affinity Constants

In general, the complexes involving non-covalent bonds (and many others) are reversible. For example, the binding of retinol to retinol-binding protein is a function of the dynamic equilibrium between retinol and retinol-protein complexes.[32] Likewise, there may be equilibrium between free and β-LG-bound 5-HT and AA-5HT. In the case of equimolar (1:1) complexes, the 5-HT and AA-5HT fluorescence titration data can be analyzed by the following equation[33,34]:

$$1/\Delta F = 1/\Delta F_{max} + 1/(K_a \times \Delta F_{max} \times [\beta - \mathrm{LG}]) \qquad (1)$$

where ΔF is the change in 5-HT or AA-5HT fluorescence intensity in the presence and absence of β-LG; ΔF_{max} is the maximal change in fluorescence intensity; K_a is the binding affinity constant; and (β-LG) is the concentration of β-LG. According to this equation, the plot of $1/\Delta F$ as a function of $1/(\beta - \mathrm{LG})$ will be linear. The intercept of this plot corresponds to $1/\Delta F_{max}$ while the slope gives the estimate of the binding affinity constant, K_a. Otherwise, the binding constant and the binding number can be calculated according to the following equation[35,36]:

$$\log[(F_0 - F)/F] = \log K_s + n\log[\mathrm{Ligand}] \qquad (2)$$

where F_0 and F are the fluorescence emission intensities of protein without and with ligand, respectively (after subtracting the fluorescence intensity of ligand alone in buffer solution); (Ligand) is ligand concentration; K_s is the binding constant; and n is the binding number. The plot of log $[(F_0 - F)/F]$ as a function of log (Ligand) will be a linear plot with an intercept of log K_s and a slope of n.

K_a and K_s are similar in meaning (binding affinity constants) but they were determined using different models and the similarity of their values obtained by two different methods can confirm the results.

RESULTS AND DISCUSSION

Behavior of 5-HT and AA-5HT in Aqueous Solution and CMC Determination

Fluorescence is a useful method to study intermolecular interactions because of the photosensitivity of many fluorophors to changes in the polarity of their close environment. The observed λ_{max} shifts to a shorter wavelength, and fluorescence intensity increases during decreases in the polarity of the close neighborhood of a chromophore. As shown in Figures 1A and 1B, λ_{max} of 10 μM 5-HT and AA-5HT is around 338 and 349 nm, respectively, in 10 mM phosphate buffer, pH 7.4, and shifts to a shorter wavelength when the polarity of the solution decreases due to the increase in the ethanol concentration. λ_{max} for 5-HT and AA-5HT shifts to 335 and 337 nm, respectively, in 50% ethanol with an obvious increase in fluorescence intensity. The same pattern was observed in 10 mM glycine buffer, pH 2.0 (data not shown). These results are similar to other previous results describing that serotonin fluorescence in solvents of lower polarity is characterized by an enhancement in intensity and a blue shift in emission maximum. Also, these previous results show that serotonin fluorescence, as measured by its intensity, emission maximum, and lifetime, is pH-dependent in agreement with our previous results.[37]

The blue shift and increase in fluorescence intensity in increasing ethanol concentrations are much stronger for AA-5HT than for 5-HT. The regular blue shift of λ_{max} and increase in 5-HT and AA-5HT fluorescence emission intensity are observed as the ethanol percentage increases, when these ligands transfer from the hydrophilic environment of the aqueous solution to the more hydrophobic environment of hydroethanolic solutions.

Table I Critical Micelle Concentration (CMC), Fraction of Counter Ions Bound to the Micelle (*F*), and Gibbs Free Energy of Micellization (ΔG_m) for the Micellization of 5-HT and AA-5HT in 10 m*M* Phosphate Buffer, pH 7.4

	CMC (μM)	*F*	ΔG_m (kJ/mol)
5-HT	52.1 ($\pm$1.9)	0.139 ($\pm$0.03)	$-$27.38 ($\pm$1.2)
AA-5HT	91.9 ($\pm$2.3)	0.441 ($\pm$0.08)	$-$32.64 ($\pm$1.8)

The effect of increasing 5-HT and AA-5HT concentrations on their fluorescence emission spectra in 10 m*M* phosphate buffer, pH 7.4, is shown in Figures 2A and 2B. The emission maxima (λ_{max}) of 5-HT and AA-5HT are at 338 and 350 nm, respectively. In the case of 5-HT, the fluorescence intensity increases as concentration increases till a value beyond which fluorescence intensity begins to decrease, indicating that self-quenching of 5-HT occurs at higher concentrations. 5-HT self-associates at a concentration higher than 100 μM, which results in fluorescence self-quenching. These results are in agreement with previous results.[38] In the case of AA-5HT (Figure 2B), the fluorescence intensity increases as AA-5HT concentration increases in the buffer solution, till a value beyond which fluorescence intensity begins to increase at a slower pace, indicating again that self-association of AA-5HT occurs at higher concentrations. The same pattern was observed for 5-HT and AA-5HT in 10 m*M* glycine buffer, pH 2.0 (data not shown). In both cases, the CMC (Critical Micelle Concentration) of 5-HT and AA-5HT were determined from the fluorescence intensity plots (Figures 2A and 2B). The CMC of 5-HT and AA-5HT in 10 m*M* phosphate buffer, pH 7.4, were measured at about 91.9 and 52.1 μM. The same CMC values were obtained for both ligands in 10 m*M* glycine buffer, pH 2.0. The ratio of the slopes of the post- and pre-CMC straight lines was used to obtain the fraction of counter ions bound (f) to the micelles.[39–42] The standard Gibbs energy of micellization, ΔG, was obtained from the relation[42,43]:

$$\Delta G_m = (1 + f)RT\ln X_{CMC} \quad (3)$$

where f is the fraction of counter ions bound to the micelle and X_{CMC} represents the CMC in mole fraction units. The CMC, f, and ΔG_m values are presented in Table I. CMC increases as the hydrophobicity of the ligand decreases and the values of ΔG_m were found to become more negative due to the increased hydrophobicity of the ligand. These results are consistent with previous results showing that the CMC increases and ΔG_m becomes more negative within a series of surfactants as the apolar tail length (hydrophobicity) decreases.[44]

Effect of Ligand/β-LG Interactions on the Structure of β-LG

CD spectroscopy is a valuable technique for studying structural transitions of proteins in solution because it can reveal small alterations in protein two-dimensional (2D) and 3D structures.[45] Far- and near-UV CD spectra can be used to characterize the secondary structure and side-chain environments of proteins, respectively. Figures 3 and 4 show far- and near-UV CD spectra, respectively, of β-LG in the absence and presence of 5-HT and AA-5HT at different concentrations in 10 m*M* phosphate buffer pH 7.4. The CD spectra of native β-LG are consistent with those previously reported.[46,47] The far-UV CD spectrum of β-LG shows a dominance of typical β-sheet structure with a broad negative minimum around 216 nm (Figures 3A and 3B). After protein complexing with 5-HT or AA-5HT, far-UV CD spectra remain roughly comparable to that of native β-LG, suggesting that the interaction of 5-HT and AA-5HT with β-LG has no significant effect on protein secondary structure at ligand concentrations lower than 80 μM. Similar results were obtained for the interaction of serotonin with human serum albumin (HSA) and

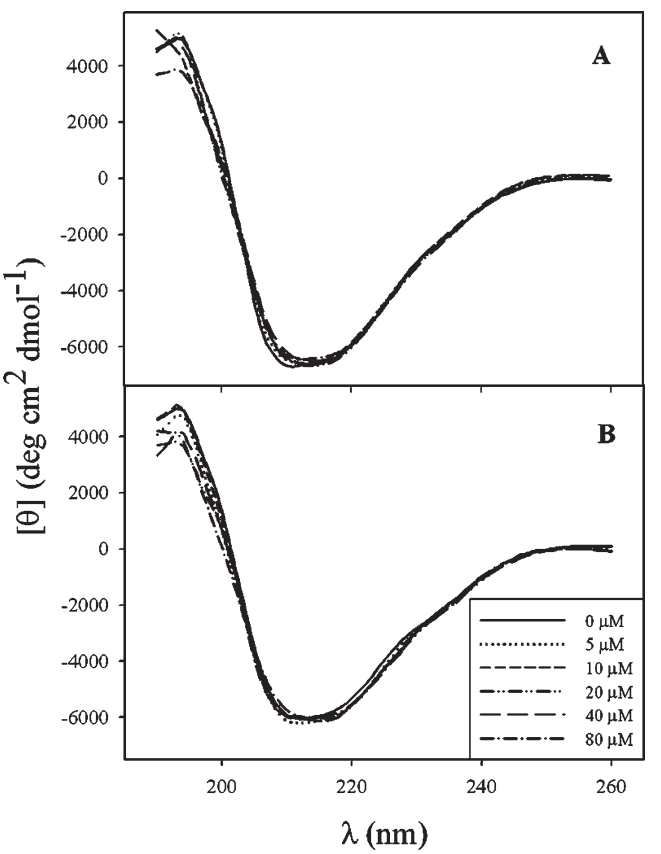

FIGURE 3 Far-UV CD spectra of 10 μM β-LG in the presence of 0, 5, 10, 20, 40, and 80 μM of (A) 5-HT and (B) AA-5HT, in 10 m*M* phosphate buffer, pH 7.4. Similar spectra were observed for each ligand in 10 m*M* glycine buffer pH 2.0.

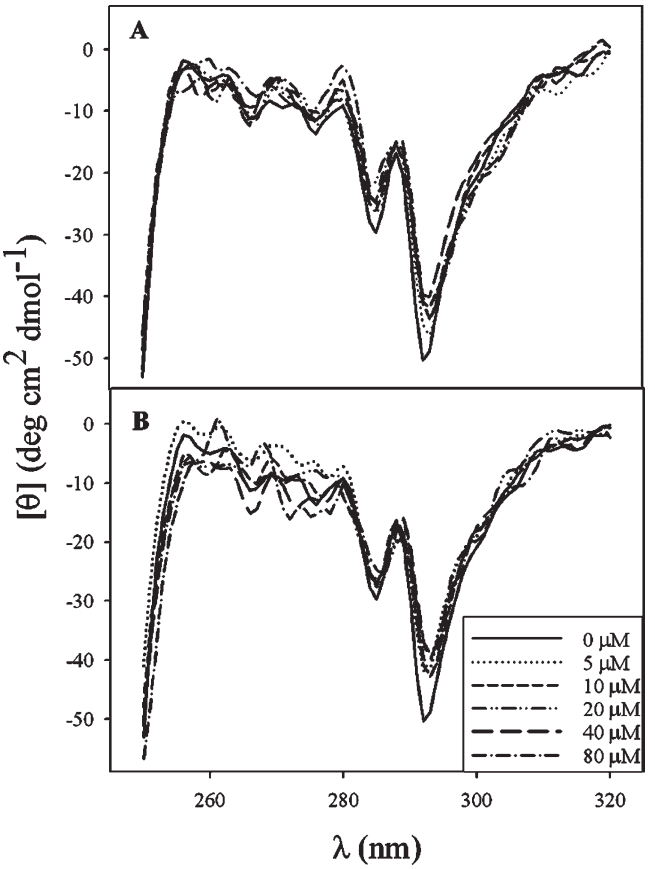

FIGURE 4 Near-UV CD spectra of 20 μM β-LG in the presence of 0, 5, 10, 20, 40, and 80 μM of (A) 5-HT and (B) AA-5HT, in 10 mM phosphate buffer, pH 7.4. Similar effects were observed for each ligand in 10 mM glycine buffer, pH 2.0.

calcium-binding protein calmodulin (CaM). In these cases, far-UV CD spectroscopic data indicate that the secondary structural features of HSA and CaM remain essentially intact after binding to serotonin.[48,49]

The near-UV CD spectrum of native β-LG (Figures 4A and 4B) shows the two negative minima at 285 and 292 nm, attributable to Trp residues at positions 19 and 61. Trp61 is partially exposed to the aqueous solvent and makes only a negligible contribution to the CD Trp signal, whereas Trp19 is in an apolar environment within the cavity of β-LG, making it easily detectable in the spectrum.[50] The two negative bands at 285 and 292 nm regularly decrease with increasing 5-HT and AA-5HT concentrations (Figures 4A and 4B), suggesting that the interaction of 5-HT and AA-5HT with β-LG partially disrupts the anisotropic environment of Trp residues.[51]

The same patterns were observed for far- and near-UV CD spectra of β-LG in the absence and presence of 5-HT and AA-5HT at different concentrations in 10 mM glycine buffer, pH 2.0 (data not shown).

Binding Affinity Constant, Binding Number, and Site of 5-HT Binding to β-LG

The intrinsic fluorescence of protein has been widely used to investigate the structural transition and binding properties of proteins in solution.[52,53] Fluorescence emission spectra of 10 and 40 μM of AA-5HT in the presence of various β-LG concentrations in 10 mM phosphate buffer, pH 7.4, are shown in Figure 5. The same profiles were observed for 10 and 40 μM of 5-HT in 10 mM phosphate buffer, pH 7.4, and also for 10 and 40 μM of 5-HT and AA-5HT in 10 mM glycine buffer, pH 2.0. Moreover, the same profiles were seen in 25% ethanol media for both ligands (data not shown). When 5-HT or AA-5HT interacts with β-LG, the fluorescence emission spectra of 10 μM and 40 μM of 5-HT or AA-5HT show similar changes. The gradual blue shift of λ_{max} and an increase in ligand fluorescence intensity are observed as β-LG concentration increases, suggesting that ligand transfers from the

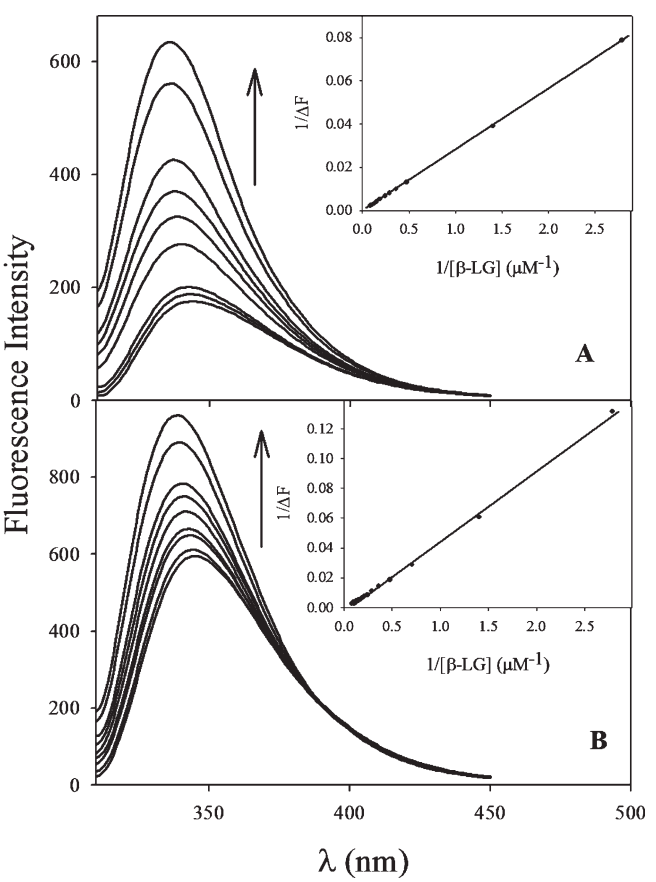

FIGURE 5 Fluorescence emission spectra of 10 μM (A) and 40 μM (B) AA-5HT in the presence of various β-LG concentrations in 10 mM phosphate buffer, pH 7.4. Similar spectra were observed for 5-HT at this pH and for both ligands in 10 mM glycine buffer, pH 2.0. The concentration range of β-LG in the solution varied from 0 to 80 μM. Inset: Double reciprocal linear plot of $1/\Delta F$ as a function of $1/(\beta$-LG) according to Eq. (1).

Table II Binding Affinity Constants (K_a and K_s) and Binding Number (n) of 5-HT/β-LG and AA-5HT/β-LG Complexes at Various pH and 298 K (Results Expressed per Protein Monomer)

pH	Ligand	K_a (M)	K_s (M)	n
pH = 2.0	5-HT	4.7 (±0.18)× 10^5	4.3 (±0.17) × 10^5	1.03 (±0.06)
	AA-5HT	6.4 (±0.27) × 10^4	6.1 (±0.24) × 10^4	0.91 (±0.08)
	5-HT 25% ethanol	8.6 (±0.31) × 10^5	9.1 (±0.36) × 10^5	0.94 (±0.07)
	AA-5HT 25% ethanol	8.1 (±0.26) × 10^4	8.3 (±0.29) × 10^4	0.89 (±0.05)
pH = 7.4	5-HT	3.1 (±0.09) × 10^5	4.0 (±0.12) × 10^5	1.23 (±0.10)
	AA-5HT	3.2 (±0.12) × 10^4	3.4 (±0.15) × 10^4	0.92 (±0.07)
	5-HT 25% ethanol	8.2 (±0.24) × 10^5	7.7 (±0.28) × 10^5	1.16 (±0.09)
	AA-5HT 25% ethanol	5.6 (±0.21) × 10^4	6.2 (±0.19)× 10^4	0.98 (±0.06)

hydrophilic environment of the aqueous solution to a more hydrophobic environment. This behavior was more marked in the case of AA-5HT than in 5-HT, demonstrating the more hydrophobic environment of AA-5HT compared to 5-HT. However, even at the highest β-LG concentrations, the increase in ligand emission intensity is smaller than that observed in 50% ethanol, suggesting that the environment of the bound ligand on the β-LG molecule is not very hydrophobic. In other words, it is not the environment of the internal cavity in β-LG since its hydrophobic influence on the spectral characteristics of the ligand chromophore would be seen. Therefore, this could imply that the binding site of 5-HT or AA-5HT is located on the surface of β-LG. A similar situation has been previously observed for binding of resveratrol to β-LG.[54]

The double reciprocal plot of $1/\Delta F$ as a function of $1/(\beta - \text{LG})$ according to Eq. (1) is given in the inset in Figure 5. From the intercept and the slope of the straight line in Figure 5, ΔF_{max} and K_a can be calculated. At AA-5HT concentrations of 10 μM and 40 μM, K_a values are 3.1 (± 0.6) × 10^4 and 3.3 (± 0.7) × 10^4, respectively. The similarity of the binding constants suggests that the binding mode of ligand to β-LG is the same at different ligand concentrations, with an average binding constant of about 3.2 × 10^4. The values of the average binding constant, K_a, for the interaction of 5-HT and AA-5HT with β-LG at different pH and in different media are presented in Table II. At ligand concentrations of 10 μM and 40 μM, ΔF_{max} values were found to be smaller than ΔF observed in the case of ligand in buffer as compared with 50% ethanol, indicating that β-LG-bound ligand is not in a very hydrophobic environment and the binding site of ligand is possibly at the surface of β-LG. For example, in the case of AA-5HT at 10 μM and 40 μM, ΔF_{max} values are 280 and 310, respectively, which are smaller than the changes observed for AA-5HT in 10 mM phosphate buffer, pH 7.4, as compared to 50% ethanol.

Figure 6 shows the intrinsic fluorescence emission spectra of β-LG in the presence of different concentrations of 5-HT after subtracting the control (ligand in the buffer without protein) at an excitation wavelength of 280 nm. Similar

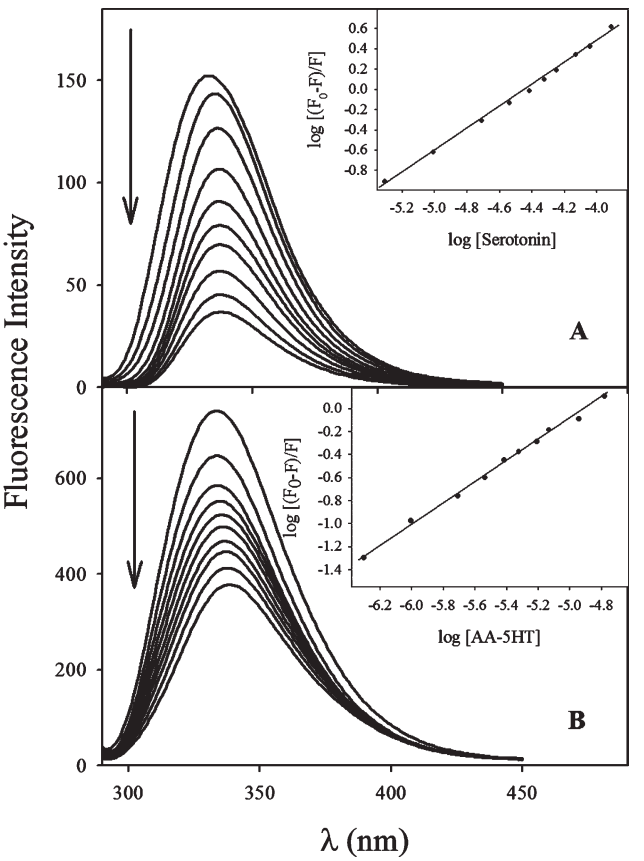

FIGURE 6 Fluorescence emission spectra of 5 μM β-LG in the presence of (A) 5-HT and (B) AA-5HT, in 10 mM phosphate buffer at pH 7.4. Similar spectra were observed for each ligand in 10 mM glycine buffer, pH 2.0. The concentration range of 5-HT and AA-5HT in the solutions varied from 0 to 50 μM and from 0 to 40 μM, respectively. Spectral resolution was 2.5 nm and 5 nm in the presence of 5-HT and AA-5HT, respectively, for both excitation and emission. Inset: log $[(F_0 - F)/F]$ vs. log (ligand) as per eq. 2.

results were observed in studies of the interaction of β-LG with AA-5HT in 10 m*M* phosphate buffer, pH 7.4, and also of the interactions of β-LG with 5-HT and AA-5HT in 10 m*M* glycine buffer, pH 2.0. Additionally, similar results were seen in 25% ethanol media in the case of both ligands (data not shown). β-LG in its native state has a λ_{max} of 333 nm (see Figure 6). As a result of the interaction of β-LG with 5-HT or AA-5HT, the λ_{max} gradually shifts to a longer wavelength and Trp fluorescence intensity decreases when the concentration of the ligand increases. These results indicate that the interactions of 5-HT and AA-5HT with β-LG position the Trp residues of β-LG in a more hydrophilic environment, which is consistent with the results of near-UV CD titrations. The interactions of 5-HT and AA-5HT with β-LG may thus partially modify the tertiary structure of this protein.

The results of the measurements of intrinsic fluorescence changes during titrations were analyzed after subtraction of the fluorescence intensity of ligand alone in the buffer solution according to Eq. (2). The linear plot of $\log[(F_0 - F)/F]$ as a function of log (5-HT) is given in the inset in Figure 6. The values of K_s and n obtained from the intercept and the slope of this linear plot in the inset of Figure 6, respectively, are shown in Table II. The n values indicate that β-LG interacts with 5-HT forming equimolar complexes. K_s values are similar to K_a values and these two methods are consistent with each other.

The combination of the results of the studies of intrinsic fluorescence changes of β-LG during titrations and the results of fluorescence emission of both ligands shows that the binding constants obtained by these methods are between 10^4 M^{-1} and 10^6 M^{-1}, which is lower than has been observed in the case of retinol binding by β-LG in its internal cavity site.[14] At neutral pH, β-LG exists as a mixture of monomers and dimers and the equilibrium between them shifts toward the monomeric form with decreasing β-LG concentration. At acidic pH, β-LG is monomeric.[15,55] The equilibrium between monomers and associated ligands may exist in solution due to their self-association/micellization at high concentrations. This phenomenon has already been observed for retinol, which associates in water even at concentrations as low as 2 μ*M*. During interactions with retinol-binding protein, equilibrium exists between monomeric and associated retinol, between monomeric retinol and retinol-protein complexes, and possibly between associated retinol and retinol-protein complexes.[56] Similarly, the interactions of 5-HT and AA-5HT with β-LG possibly compete with the self-association of ligands and of β-LG. However, the probability for protein association is low, because the analysis of binding measurements on basis of proposed model is sufficiently satisfied. The binding between ligands and monomeric β-LG is stronger than that between ligands and dimeric β-LG. Consequently, the binding affinity constant of the interactions of ligands with β-LG is higher at pH 2.0 (when β-LG is in its monomeric form) than at pH 7.4 (when β-LG exists as a mixture of monomers and dimers). The binding experiments were also performed in 25% ethanol in which β-LG has a folding similar to a molten globule.[57] In these conditions, β-LG can make stronger complexes with both ligands since the binding affinity constants are greater with respect to the results obtained in fully aqueous media.

The fact that the binding site is external rather than internal has been tested by competitive titrations. Retinol binds tightly to the internal cavity and competitive studies involving both retinol and serotonin with β-LG could make more convincing the conclusion about external position of serotonin binding site. The intrinsic fluorescence emission spectra of β-LG/serotonin complexes (molar ratio 1) in the presence of different concentrations of retinol after subtracting the control (ligand in the solution without β-LG) at an excitation wavelength of 280 nm and 10 m*M* phosphate buffer, pH 7.4 were done (data not shown). These results analyzed according to Eq. (2) showed that the binding affinity constant (K_s) and the binding number of retinol in the presence of β-LG/serotonin complexes are 3.2×10^7 M^{-1} and 1.12, respectively, what agrees with previous results without any other competing ligand.[20,23,28,29,31] In another experiment, the intrinsic fluorescence emission spectra of β-LG/retinol complexes (molar ratio 1) in the presence of different concentrations of serotonin after subtracting the control (ligand in the solution without β-LG) at an excitation wavelength of 280 nm and 10 m*M* phosphate buffer, pH 7.4 were measured (data not shown). These results analyzed according to Eq. (2) showed that the binding affinity constant (K_s) and the binding number of serotonin in the presence of β-LG/retinol complexes are 3.8×10^5 M^{-1} and 1.17, respectively, agreeing with results of titration without retinol. This means that the binding sites of retinol and serotonin are different from each other. It can be also concluded that the binding site of serotonin on β-LG is external rather than internal.

It is known that the interactions of β-LG with ligands depend not only on the binding constants, but also on the solubility of the ligands.[14] For instance, the solubilities of retinol and palmitic acid in water are 0.06 μ*M* and 28 μ*M*, respectively. Although the binding constant to β-LG is about two orders of magnitude higher for retinol than for palmitic acid, the higher solubility of palmitic acid leads to a 5-fold preferred binding as compared to retinol. Compared with retinol and palmitic acid, the higher solubility of both AA-5HT and especially 5-HT (about 50 and 90 μ*M*, respectively) in aqueous solutions should improve the affinity of 5-HT

and AA-5HT for β-LG, even though the binding constants for the interaction of 5-HT and AA-5HT with β-LG are lower. In other words, there is a higher concentration of 5-HT or AA-5HT-bound β-LG than of retinol-bound β-LG in the same conditions, demonstrating that the binding affinity constant of β-LG for retinol is higher than binding affinity constants for 5-HT and AA-5HT.

CONCLUSIONS

5-HT and AA-5HT can interact with β-LG forming equimolar complexes, without affecting the secondary structure of β-LG but probably with a slight modification of its tertiary structure. The molecules of 5-HT and AA-5HT bind to the surface of β-LG with a binding constant varying between 10^4 and 10^6 M^{-1}. The binding affinity constant for 5-HT/β-LG complexes is higher than for AA-5HT/β-LG complexes. The interactions of these ligands with β-LG may compete with self-associations of both ligands and β-LG. These ligands can bind better to β-LG in 25% ethanol and they show a good solubility in water (especially in the case of 5-HT) as compared with other more hydrophobic ligands such as retinol and palmitic acid. Thus, β-LG can be considered as a good carrier of 5-HT-derived ligands, and 5-HT/β-LG and AA-5HT/β-LG complexes can serve as useful models of drug-β-LG interactions.

Although other data are still necessary to confirm this hypothesis, the binding by β-LG of 5-HT and its derivatives such as AA-5HT, observed here for the first time, could imply that the peripheral control of 5-HT quantity and 5-HT action on the gut enterochromaffin cells may also be one of the potential, if not main, biological roles of β-LG in mammals whose milk contains this protein.

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REFERENCES

1. Berger, M.; Gray, J. A.; Roth, B. L. Annu Rev Med 2009, 60, 355–366.
2. Roth, B. L. The Serotonin Receptors: From Molecular Pharmacology to Human Therapeutics; Humana Press: 2006.
3. Kang, K.; Park, S.; Kim, Y. S.; Lee, S.; Back, K. Appl Microbiol Biotechnol 2009, 83, 27–34.
4. Rapport, M. M.; Green, A. A.; Page, I. H. J Biol Chem 1974, 176, 1243–1251.
5. Kroeze, W. K.; Kristiansen, K.; Roth, B. L. Curr Top Med Chem 2002, 2, 507–528.
6. Pai, V. P.; Horseman, N. D. J Biol Chem 2008, 283, 30901–30910.
7. Gershon, M. D.; Tack, J. Gastroenterology 2007, 132, 397–414.
8. Bisogno, T.; Melck, D.; De Petrocellis, L.; Bobrov, M. Yu.; Gretskaya, N. M.; Bezuglov, V. V.; Sitachitta, N.; Gerwick, W. H.; Di Marzo, V. Biochem Biophys Res Commun 1998, 248, 515–522.
9. Fride, E. E. Prostaglandins Leukot Essent Fatty Acids 2002, 66, 221–233.
10. Novellis, V.; Palazzo, E.; Rossi, F.; Petrocellis, L.; Petrosino, S.; Guida, F.; Luongo, L.; Migliozzi, A.; Cristino, L.; Marabese, I.; Starowicz, K.; Marzo, V.; Maione, S. Neuropharmacology 2008, 55, 1105–1113.
11. Maione, S.; Petrocellis, L.; Novellis, V.; Moriello, A. S.; Petrosino, S.; Palazzo, E.; Rossi, F. S.; Woodward, D. F.; Marzo, V. Br J Pharmacol 2007, 150, 766–781.
12. Bezuglov, V. V.; Manevich, Y.; Archakov, A. V.; Bobrov, M. Yu.; Kuklev, D. V.; Petrukhina, G. N.; Makarov, V. A.; Buznikov, G. A. Russ J Bioorg Chem 1997, 23, 190–198.
13. Hambling, S. G.; McAlpine, A. S.; Sawyer, L. Advanced Dairy Chemistry 1: Proteins; Elsevier Applied Science: London, 1992; pp 141–190.
14. Kontopidis, G.; Holt, C.; Sawyer, L. J Mol Biol 2002, 318, 1043–1055.
15. Wang, Q. W.; Allen, J. C.; Swaisgood, H. E. J Dairy Sci 1998, 81, 76–81.
16. Lefevre, T.; Subirade, M. Biochim Biophys Acta 2001, 15, 37–50.
17. Collini, M.; D'Alfonso, L.; Baldini, G. Protein Sci 2000, 9, 1968–1974.
18. Hattori, M.; Watabe, A.; Takahashi, K. Biosci Biotechnol Biochem 1995, 59, 2295–2297.
19. McAlpine, A. S.; Sawyer, L. Biochem Soc Trans 1990, 18, 879.
20. O'Neill, T. E.; Kinsella, J. E. J Agric Food Chem 1987, 35, 770–774.
21. Roufik, S.; Gauthier, S. F.; Leng, X. J.; Turgeon, S. L. Biomacromolecules 2006, 7, 419–426.
22. Sawyer, L.; Brownlow, S.; Polikarpov, I.; Wu, S. Y. Int Dairy J 1998, 8, 65–72.
23. Dufour, E.; Bertrand-Harb, C.; Haertlé, T. Biopolymers 1993, 33, 589–598.
24. Yang, M. C.; Guan, H. H.; Liu, M. Y.; Lin, Y. H.; Yang, J. M.; Chen, W. L.; Chen, C. J.; Mao, S. J. T. Proteins 2008, 71, 1197–1210.
25. Dill, K. A. Biochemistry 1990, 29, 7133–7155.
26. Viseu, M. I.; Carvalho, T. I.; Costa, S. M. B. Biophys J 2004, 86, 2392–2402.
27. Liu, X. H.; Shang, L.; Jiang, X.; Dong, S. J.; Wang, E. K. Biophys Chem 2006, 121, 218–223.
28. Taheri-Kafrani, A.; Bordbar, A. K.; Mousavi, H.; Haertlé, T. J Agric Food Chem 2008, 56, 7528–7534.
29. Taheri-Kafrani, A.; Asgari-Mobarakeh, E.; Bordbar, A. K.; Haertlé, T. Coll Surf B: Biointerfaces 2010, 75, 268–274.
30. Mailliart, P.; Ribadeau Dumas, B. J Food Sci 1988, 33, 743–752.
31. Dufour, E.; Genot, C.; Haertlé, T. Biochim Biophys Acta 1994, 1205, 105–112.
32. Cogan, U.; Kopelman, M.; Mokady, S.; Shinitzky, M. Eur J Biochem 1976, 65, 71–78.
33. Guharay, J.; Sengupta, B.; Sengupta, P. K. Prot Struc Func Bioinf 2001, 43, 75–81.
34. Bhattacharya, J.; Bhattacharya, M.; Chakrabarty, A. S.; Chaudhury, U.; Poddar, R. K. Biochem Pharmacology 1994, 47, 2049–2052.
35. Wei, X. F.; Liu, H. Z. Chin J Anal Chem 2000, 28, 699–701.
36. Mishra, B.; Barik, A.; Priyadarsini, K. I.; Mohan, H. J Chem Sci

2005, 117, 641–647.
37. Chattopadhyay, A.; Rukmini, R.; Mukherjee, S. Biophys J 1996, 71, 1952–1960.
38. Nag, S.; Balaji, J.; Madhu, P. K.; Maiti, S. Biophys J 2008, 94, 4145–4153.
39. Evans, H. C. J Chem Soc 1956, 80, 579–586.
40. Jana, P. K.; Moulik, S. P. J Phys Chem 1991, 95, 9525–9532.
41. Ghosh, S.; Moulik, S. P. J Coll Interface Sci 1998, 208, 357–366.
42. Chatterjee, A.; Moulik, S. P.; Sanyal, S. K.; Mishra, B. K.; Puri, P. M. J Phys Chem B 2001, 105, 12823–12831.
43. Moulik, S. P. Curr Sci 1996, 71, 368–376.
44. Stodghill, S. P.; Smith, A. E.; O'Haver, J. H. Langmuir 2004, 20, 11387–11392.
45. Wallace, B. A.; Janes, R. W. Biochem Soc Trans 2003, 31, 631–633.
46. Yagi, M.; Sakurai, K.; Kalidas, C.; Batt, C. A.; Goto, Y. J Biol Chem 2003, 278, 47009–47015.
47. Taheri-Kafrani, A.; Gaudin, J. C.; Rabesona, H.; Nioi, C.; Agarwal, D.; Drouet, M.; Chobert, J. M.; Bordbar, A. K.; Haertlé, T. J Agric Food Chem 2009, 57, 4974–4982.
48. Sengupta, B.; Chaudhuri, S.; Banerjee, A.; Sengupta, P. K. Chem Biodiver 2004, 1, 868–877.
49. Ouyang, H.; Vogel, H. J Biochim Biophys Acta 1998, 1387, 37–47.
50. Fessas, D.; Iametti, S.; Schiraldi, A.; Bonomi, F. Eur J Biochem 2001, 268, 5439–5448.
51. Fujiwara, K.; Arai, M.; Shimizu, A.; Ikeguchi, M.; Kuwajima, K.; Sugai, S. Biochemistry 1999, 38, 4455–4463.
52. N'soukpoe-Kossi, C. N.; St-Louis, C.; Beauregard, M.; Subirade, M.; Carpentier, R.; Hotchandani, S.; Tajmir-Riahi, H. A. J Biomol Struct Dyn 2006, 24, 277–283.
53. Liang, L.; Yao, P.; Jiang, M. Biomacromolecules 2005, 6, 2748–2755.
54. Liang, L.; Tajmir-Riahi, H. A.; Subirade, M. Biomacromolecules 2008, 9, 50–56.
55. McKenzie, H. A.; Sawyer, W. H. Nature 1967, 214, 1101–1104.
56. Muresan, S.; van der Bent, A.; de Wolf, A. J Agric Food Chem 2001, 49, 2609–2618.
57. Mousavi, H.; Chobert, J. M.; Bordbar, A. K.; Haertlé, T. J Agric Food Chem 2008, 56, 8680–8684.

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Structure–function relationship of β-lactoglobulin in the presence of dodecyltrimethyl ammonium bromide

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ABSTRACT

Bovine β-lactoglobulin (β-LG) present in milks has been found "in vivo" in complexes with lipids such as butyric and oleic acids. To elucidate the still unknown structure–function relationship in this protein, the structural changes of β-lactoglobulin variant A (β-LG A) in the presence of cationic surfactant such as dodecyltrimethyl ammonium bromide (DTAB) have been investigated using various experimental techniques such as UV–vis spectrophotometry, fluorimetry, isothermal titration calorimetry (ITC) and circular dichroism (CD). Subsequently, the retinol binding by β-LG has been investigated in the presence of various amounts of this surfactant as its extrinsic functional binding fluorophore. Comparison of the results allowed to determine the binding of retinol by β-LG in the presence of DTAB. The results of UV–vis and fluorescence studies showed a red shift in wavelength and an increase in absorbance and enhancement in the intensity of the quantum yield of protein during its interaction with DTAB. The results of UV–vis also showed two distinct conformational changes corresponding first to precipitation and second to solubilization of the precipitated β-LG at pH 6.7 and 8.0. The results indicate the cooperative character of binding at pH 2.0. The results of fluorescence studies showed that the binding strength of β-LG/DTAB complex increases with the increase of the pH. CD results showed the shifts in positions of the major minima and change in magnitude of ellipticity and subsequently signified two significant changes in structure of β-LG between 10–30 and 50–100 molar ratio of [DTAB]/[β-LG]. ITC measurements indicated the endothermic nature of β-LG/DTAB interactions at pH 6.7 and the exothermic nature of β-LG/DTAB interactions at pH 8.0. The analysis of the binding data demonstrates the absence of significant changes in retinol-binding properties of β-LG in the presence of various amounts of this surfactant. This implies that surfactant binding does not change the conformation of β-LG in the regions defining retinol-binding site nor interferes with retinol binding by a competition for the same binding site(s).

1. Introduction

Interactions of proteins with surfactants have been studied extensively for decades because of their importance in many biological, pharmaceutical, food and industrial systems [1–10]. The interactions between biopolymers and surfactants depend strongly on the type of biopolymer and surfactant as well as on medium and its physico-chemical properties such as pH, ionic strength and temperature [11–17].

Protein–surfactant interactions often change the stabilities of many proteins. An understanding of the mechanisms involved in protein–surfactant interactions provide a basis for the evaluation of protein stability and for rational strategies to optimize the applications of surfactants.

The globular protein β-lactoglobulin (β-LG), which is the major protein in the whey of ruminant milk [18] and may play important functions in the binding and transport of hydrophobic ligands such as retinoids, alkenes and fatty acids is attracting more and more attention [10,19–25] and its interactions with amphiphilic and hydrophobic ligands such as retinoids, long chain fatty acids and surfactants are investigated by different methods.

β-LG is a small globular protein with known primary, secondary and three-dimensional structures but its biological role still remains uncertain [26]. Its 3D structure consists of a β-barrel composed of eight anti-parallel β-strands, what makes it similar to other lipocalin proteins [27,28]. The core of β-LG molecule includes a structural motif similar to that found in retinol-binding protein

Abbreviations: β-LG, β-lactoglobulin; DTAB, dodecyltrimethyl ammonium bromide; ITC, isothermal titration calorimetry; CD, circular dichroism; NATA, *N*-acetyl-L-tryptophanamide; Trp, tryptophan; Cys, cysteine; Arg, arginine; Lys, lysine; Glu, glutamic acid; Asp, aspartic acid.

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(RBP) [24]. Like RBP, β-LG is able to bind a wide variety of hydrophobic molecules. Analysis of the retinol binding by β-lactoglobulin show some evidence of protrusion of retinol hydroxyl group out of binding site what can be deduced from retinol susceptibility to an attack by dehydrogenase [26]. This could indicate also the external placement of retinol-binding site on β-LG molecule [23]. β-LG is known to bind tightly, in vitro, one retinol molecule per monomer [26]. Developments in structural studies of β-LG [27] RBP [28] and bilin-binding protein [29] show that these transporters of hydrophobic molecules share a three-dimensional structural pattern termed β-barrel. Unambiguous crystallographic data analysis [28] indicates that the ligands of RBP and bilin-binding protein are bound inside the calyx formed by the β-barrel. The exact placement of the binding sites in other proteins from this super family is less clear.

As implied by the substantial increase in the unfolding temperature the β-LG structure is strengthened in the presence of equimolar concentrations of sodium dodecyl sulfate (SDS). The increase of the SDS concentration over equimolar causes unfolding of the protein. In contrast to these effects of the anionic surfactant on β-LG stability, a slight decrease in the unfolding temperature was observed in the presence of cationic surfactant dodecyl trimethyl ammonium chloride in similar conditions [30].

A study of the interactions of the mixtures of cationic–anionic surfactants with β-LG was reported recently by Lu et al. [31] They concluded also that the strength of interactions is dependent on the ratio of surfactants in mixture.

The interactions of anionic and neutral surfactants with β-LG [10] were recently investigated. The obtained results demonstrated the contribution of both electrostatic and specific hydrophobic forces in the measured interactions. Additionally, no significant changes in retinol-binding properties of β-LG in the presence of various amounts of these surfactants were observed. This implies that surfactant binding does not change the conformation of β-LG in the regions forming the retinol-binding site, nor expels retinol from its binding site.

The present study was undertaken taking in account previous observations in attempt to unravel further the nature of the binding of amphiphilic molecules to the hydrophobic pocket/binding site(s) on β-LG. For this purpose the structural changes of β-LG have been investigated initially in the presence of cationic surfactant, DTAB, using various experimental techniques such as UV–vis, fluorescence, ITC and CD. Subsequently, the retinol-binding properties of β-LG as its functional extrinsic fluorescence probe was investigated in the presence of the various amounts of this surfactant using fluorescence titration. Comparison of the obtained results allows determining some aspects of the structure–function relationship of β-LG in the presence of DTAB.

2. Experimental

2.1. Materials

β-Lactoglobulin variant A (β-LG A) was isolated from the milk of a homozygous cow according to Maillart and Ribadeau-Dumas method [32]. Homogeneity of the protein preparation was assessed by high performance gel permeation chromatography and SDS gel electrophoresis. The obtained preparations of β-lactoglobulin were over 98% pure. Dodecyltrimethyl ammonium bromide (DTAB), *N*-acetyl-L-tryptophanamide (NATA) and trans-retinol palmitate were purchased from Sigma Chemical Co. Glycine, ethanol, Na_2HPO_4 and NaH_2PO_4 were obtained from Merck Chemical Co. All the used reagents were of highest degree of purity. All of the solutions were prepared using double distilled water. The 50 mM glycine buffer pH 2.0, the 50 mM phosphate buffer pH 6.7, and 8.0 were used as buffers. All of solutions were used freshly after preparation. The β-LG and retinol concentrations were determined from the optical density of appropriate solutions using the extinction coefficient at 280 nm of 17,600 M^{-1} cm^{-1} and 330 nm of 48,000 M^{-1} cm^{-1}, respectively [21].

2.2. Apparatus

The measurements of absorbance were carried out using Carry-500 Scan UV–vis–NIR double beam spectrophotometer, which was equipped with a cell compartment thermostated at 298 K. Fluorescence measurements were performed using RF-5000 Shimadzu spectrofluorimeter with a cell compartment thermostated at 298 K. Isothermal titration calorimetry (ITC) was performed at temperature 298 K on a thermal activity monitor calorimeter (Thermometrics AB, Järfälla, Sweden) equipped with a high performance titration unit and a nano-watt amplifier. Circular dichroism (CD) spectra were recorded on a CD6 dichrograph (Jobin Yvon, Longjumeau, France), using cells of a 0.5 and 0.01 cm length for near and far-UV, respectively, and a scan time of 1 nm/s at 298 K with thermostatically controlled cell holders. The instrument was calibrated with ammonium d-10-camphorsulfonic acid [33].

2.3. UV–vis spectroscopy measurements

During UV–vis spectroscopy measurements, the samples were put in quartz cuvettes of 1 cm optical path. In typical experiments 800 μL of β-LG solution were placed in the cuvette. The absorbance spectra were recorded between 250 and 350 nm after each addition of DTAB stock solutions (10 mM) at 298 K (25 °C). The observed absorbance was corrected for dilution. The β-LG solutions were freshly prepared just before the measurements.

2.4. Fluorescence spectroscopy measurements

Fluorescence spectroscopy is used to study the conformational changes of proteins containing tryptophan residues during the binding of ligands, because the intrinsic fluorescence of indol chromophores in tryptophan (Trp) residues is particularly sensitive to their microenvironments [34]. During fluorescence measurements, the samples were put in quartz cuvettes of 1 cm optical path. In typical experiments 3.0 mL of β-LG solution were placed into the cuvette. Emission spectra were recorded after each addition of DTAB stock solutions at 298 K. The excitation was performed at 280 nm and the emitted fluorescence was recorded between 300 and 400 nm. The observed fluorescence intensities were corrected for dilution. The band slits for excitation and emission were set at 3 and 5 nm, respectively.

The binding of retinol was measured by following the quenching of protein tryptophan fluorescence at 330 nm. The following procedure was used during titration of β-LG solutions or various [DTAB]/[β-LG] solutions, with retinol: 3 mL of β-LG solutions or various [DTAB]/[β-LG] solutions were placed in a cuvette and small increments (1–5 μL) of the DTAB solution was injected in the cuvette with a Hamilton syringe. The experiments were performed in 50 mM glycine buffer at pH 2.0 and 50 mM phosphate buffer at pH 6.7 and 8.0. In order to eliminate the dilution of β-LG solution by the added DTAB solution and tryptophan fluorescence changes induced by alcohol, a blank containing *N*-acetyl-L-tryptophan amid, NATA, solution titrated with ligand was monitored as described above. The fluorescence intensity changes of the blank were subtracted from fluorescence intensity measurements of the ligand/protein complexes for every titration point. The β-LG solutions were freshly prepared just before the measurements and their absorbencies at 280 nm did not exceeded 0.1, β-LG concentration was around 3 μM, to avoid inner filter effect.

2.5. Circular dichroism measurements

Circular dichroism (CD) spectra were measured using a CD6 dichrograph (Jobin Yvon, Longjumeau, France) at 298 K, with an integration time of 1 s and a 2 nm bandwidth. The data were expressed as molar residue ellipticity [θ], which is defined as $[\theta] = 100\theta_{obs}/cl$, where θ_{obs} is the observed ellipticity in degrees, c is the concentration in residue mol cm^{-3}, and l is the length of the light path in cm. CD spectra in the far-UV region were measured at the sample concentration of 1 mg/mL in 50 mM phosphate buffer pH 6.7. The spectra were recorded between 190 and 250 nm using quartz cuvette with 0.1 mm path length. Each spectrum was result of the accumulation of five successive measurements and baseline was corrected by subtracting buffer spectrum.

2.6. Isothermal titration calorimetric experiments

The nano-watt isothermal titration was performed using micro calorimeter Thermometric 2277, Thermal Activity Monitor (Thermometrics AB, Järfälla, Sweden), controlled by Digitam 4.1 software. The instrument has an electrical calibration with a precision better than ±1%. The used sample and reference cells of the calorimeter are made from stainless steel. This isothermal titration calorimeter was used to measure enthalpies of mixing at 298 K for the measurements of interactions of DTAB with β-LG at pH 6.7 and 8.0. 250 μL aliquots of DTAB stock solution (10 mM) were sequentially injected (10 μL in each injection) by 250 μL Hamilton syringe controlled by a Thermometric 612 Lund Pump into a 2600 μL reaction cell containing initially either buffer solution or β-LG solution. The concentrations of β-LG in the cell were around 3 μM. Each injection lasted 5 min and there was an interval of 10 min between every successive injection. The solution in the reaction cell was stirred at a speed of 60 rpm with a gold propeller throughout the experiments. All the solutions were degassed before the measurements. All the experiments were carried out at least twice using freshly prepared samples and the reported results are the averages. Typically, the reproducibility of the enthalpy changes measured on a particular sample by ITC was acceptable with less than 10% of error. Data were analyzed using the proprietary software Digitam 4.1 supplied by Thermometric AB (Järfälla, Sweden).

2.7. Determination of the apparent dissociation constants

The following procedure was used for the titration of β-LG solutions or various [DTAB]/[β-LG] solutions with retinol: 3 mL of protein solution or various [DTAB]/[β-LG] solution was placed in a cuvette and small increments of retinol solution were injected with a micropipette. The final amount of ethanol added with retinol during titration never exceeded 3% (v/v). Differences in fluorescence intensity at 330 nm (excitation at 280 nm) were monitored in order to measure apparent dissociation constants of β-LG and various [DTAB]/[β-LG] molar ratios with retinol. It was assumed that the change in the fluorescence depends on the amount of protein/ligand complex, and the apparent dissociation constants were determined according to Cogan et al. using the following equation [35]:

$$P_0\alpha = \left(\frac{L_0}{n}\right)\left(\frac{\alpha}{(1-\alpha)}\right) - \frac{K_d}{n} \quad (1)$$

where α is the fraction of free binding sites, L_0 is the total ligand concentration, P_0 is the total protein concentration.

By plotting $P_0\alpha$ *vs* $\alpha/(1-\alpha)$, a straight line is obtained with an intercept of K_d/n and a slope of L_0/n; where K_d is the apparent dissociation constant, n is the apparent molar ratio of β-LG/retinol at saturation. α is defined as the fraction of unoccupied binding sites on the protein molecules. The value of α was calculated for every desired point on the titration curve of fluorescence quenching intensity using this relationship:

$$\alpha = \frac{(F - F_{min})}{(F_0 - F_{min})} \quad (2)$$

where F represents the fluorescence intensity (corrected for blank) at a certain L_0, F_{min} represents the fluorescence intensity upon saturation of β-LG molecules, and F_0 is the initial fluorescence intensity without retinol.

3. Results and discussion

3.1. Influence of various cationic surfactant concentration on the structure of β-LG

When ionic surfactants are added to the β-LG solutions, the ionic surfactant monomers first bind electrostatically to the oppositely charged residues on the β-LG surface (site-specific binding), and this binding induces the expansion of the β-LG structure. This expansion, allows more hydrophobic interactions of the surfactant hydrophobic tails with the exposed β-LG non-polar interior (nonspecific, cooperative binding) sites.

β-LG molecule contains 14 Glu and 10 Asp residues. At the pH of measurements they are mostly negatively charged and can constitute the binding sites for cationic surfactant [18]. At pH 6.7 and 8.0, β-LG is negatively charged (the isoelectric point of β-LG is 5.2) [18], so it can form precipitates with oppositely charged surfactants because of creation of neutral complexes between β-LG and cationic surfactant. During further increase of concentrations of cationic surfactants, more surfactants bind to the electrostatically neutral complexes, so the secondary complexes of β-LG/cationic surfactant become gradually positively charged, and the precipitate is dissolved. At pH 2.0, below the pK_a of aspartates and glutamates β-LG is positively charged and when cationic surfactant binds to β-LG, the β-LG/cationic surfactant complex is always positively charged and the precipitation is not observed.

3.2. UV–vis absorption spectra

UV–vis spectroscopy was used to analyze the binding of DTAB to β-LG. The absorption maximum of β-LG depends on the microenvironment in which the probe is located albeit this dependence is significantly smaller that in case of fluorescence measurements. The study of the absorbance of β-LG during its interactions with surfactants allows the determination of the micro polarity of the local environment surrounding the probe.

As mentioned above at pH 6.7 and 8.0 β-LG is negatively charged, so it can form precipitates with oppositely charged surfactant DTAB because of the formation of neutral complex β-LG/DTAB. With the further increase of DTAB concentration, more cationic surfactant binds to already electrostatically neutral complexes, so the complex of β-LG/DTAB becomes positively charged, and the precipitate is dissolved. Therefore, upon the increase of DTAB concentration, the mixtures of β-LG/DTAB undergo a transition from homogeneous solution to precipitates, and then to another homogeneous solution again. No precipitate was observed at pH 2.0. These results are similar to what was observed in case of other oppositely charged protein–surfactant systems [36,37].

Fig. 1 shows the plot of the maximum of absorbance of β-LG *vs* molar ratio of [DTAB]/[β-LG] at pH 2.0, 6.7 and 8.0 at 298 K. As shown in this figure, the same evolution of absorbance was observed at pH 6.7 and 8.0 but it was different at pH 2.0. Two distinct conformational changes can be distinguished at pH 6.7 and 8.0. First, precipitation is observed at 105 and 120 molar ratios of [DTAB]/[β-LG] at pH 6.7 and 8.0, respectively. Actually, the observed sharp increasing of absorbance is due to the formation of

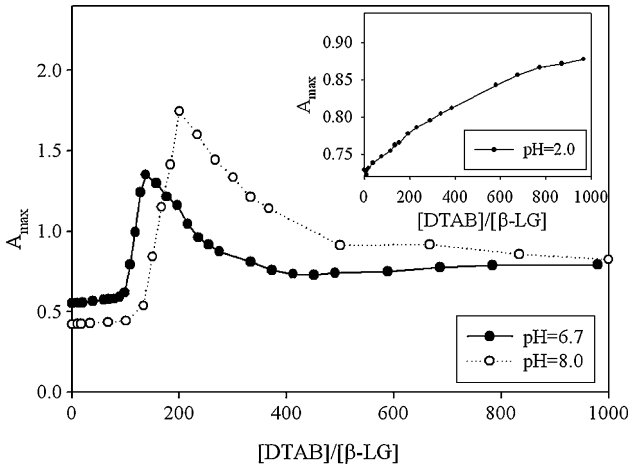

Fig. 1. Variation of the maximum absorbance of β-LG *vs* molar ratio of [DTAB]/[β-LG] in 50 mM glycine buffer, pH 2.0 and 50 mM phosphate buffer pH 6.7 and 8.0 in temperature of 298 K.

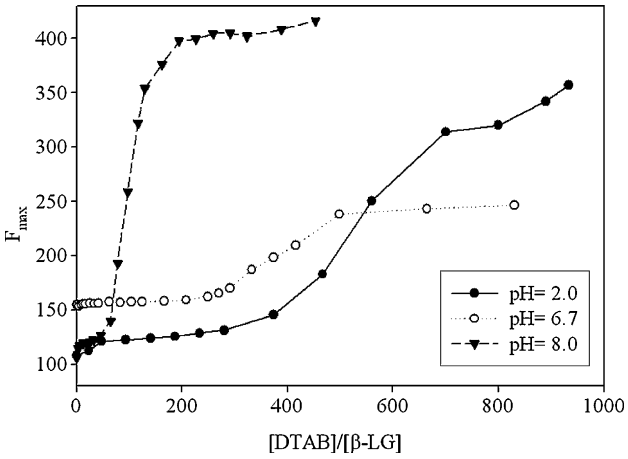

Fig. 3. Changes of fluorescence emission maximum intensity of β-LG *vs* molar ratio of [DTAB]/[β-LG] in 50 mM glycine pH 2.0 and 50 mM phosphate buffer pH 6.7 and 298 K.

precipitate that causes the increasing of scattering. However, after dissolving the precipitate the absorbance was going down due to removing of scattering. These points are corresponding to the first break in this figure. Second, precipitates dissolved at 360 and 490 molar ratio of [DTAB]/[β-LG] at pH 6.7 and 8.0, respectively.

The cooperative character of binding is obvious at pH 2.0 and two notable points that have been observed in case of pH 6.7 and 8.0 were absent at this pH.

3.3. Fluorescence spectra

The β-LG fluorescence depends on the microenvironment of the Trp residues of β-LG [38,39]. β-LG contains two Trp residues, Trp 19 and Trp 61 [18]. Fig. 2 shows Ribbon diagram of a single unit of bovine β-LG. As it shown in this figure, Trp 19 is in a non-polar environment in the main cavity of β-LG, whereas Trp 61 protrudes beyond the surface of the molecule and is quite close to the Cys 66–Cys 160 disulfide bridge [40,41]. Since the disulfide bridges are effective Trp fluorescence quenchers, the intrinsic fluorescence of β-LG is almost exclusively attributed to Trp 19 [23,42]. The surfactant ions would cluster around the oppositely charged side chains of aspartates and glutamates of β-LG, and anionic surfactants cluster around protonated His, Arg and Lys side chains [4].

Fig. 2. Ribbon diagram of a single unit of bovine β-LG. The locations of Trp 19 and Trp 61 are indicated.

Analysis of the 3D structure of β-LG shows that when DTAB molecules bind to the residues of Glu and Asp, Glu 62 which projects from the surface may becomes a most accessible binding site for DTAB (Fig. 2). Since Glu 62 is very close to Trp 61, the binding and clustering of DTAB molecules around Glu 62 might increase the distance between Trp 61 and the Cys 66–Cys 160 disulfide bridge, and weaken the quenching action effect of Cys 66–Cys 160 disulfide bridge on Trp 61 fluorescence, which could result in the increase of the β-LG fluorescence intensity.

Fig. 3 shows the changes of maximum of fluorescence intensity *vs* [DTAB]/[β-LG] molar ratio at various pH and 298 K. Despite the precipitation observed during UV–vis measurements, no precipitation of β-LG was observed at any of studied pH during fluorescence experiments. This is due to low concentrations of β-LG during fluorescence experiments. As shown in this figure, the cooperative character of binding is obvious at all studied pH. Analysis of these transition curves shows that the binding strength increases with the increase of the pH. This fact can be related to higher negative charge density of β-LG at higher pH.

It is known that the transfer of Trp to a hydrophilic environment and increase in polar interactions leads to a red shift in wavelength and to an increase in intensity of the emission maximum. This is the case of described here in our results. An exposure to increasing concentrations of DTAB at all studied pH induces slight red shift in the fluorescence emission maximum of Trp and a substantial increase of the fluorescence intensity (the graphs not shown). Similar behavior was observed also in case of UV–vis absorbance in presence of increasing amounts of this surfactant. The increase in the absorbance intensity indicates the perturbation of tertiary interactions quenching otherwise the β-LG fluorescence in the native conformation. As can be deduced from 3D structure of native β-LG, Trp 19, situated at the bottom of the calyx formed by eight anti-parallel β-strands, contributes probably about 80% to the total fluorescence [20] and a neighboring Cys 66–Cys 160 disulfide bond quenches Trp 61 emission [43]. Therefore, smaller quenching of Trp 61 by disulfide bond, when β-LG binds to DTAB, leads probably to an increase of the fluorescence intensity of β-LG. It indicates also that the protein unfolds partially during its interaction with DTAB. A red shift of the emission maxima indicates that at least part of the Trp residues is transferred/moved into more hydrophilic environment during the interaction of β-LG with the DTAB.

3.4. Circular dichroism study

Far-UV-CD measurements were performed in 50 mM phosphate buffer pH 6.7. The negative peaks of the ellipticity at 208 and 222 nm

Table 1
β-LG sequence secondary structure based on Fasman method (Dicroprot 2000) and its molar ellipticity at 222 nm.

Molar ratio of [DTAB]/[β-LG]	α-Helix	β-Sheet	Other	θ_{222} (degree cm^2 dmol^{-1})
0	13	38	49	−4936
10	14	36	50	−5124
30	19	28	53	−7512
50	21	26	53	−7784
100	27	19	54	−10141
X-ray crystallography	11	31	58	–

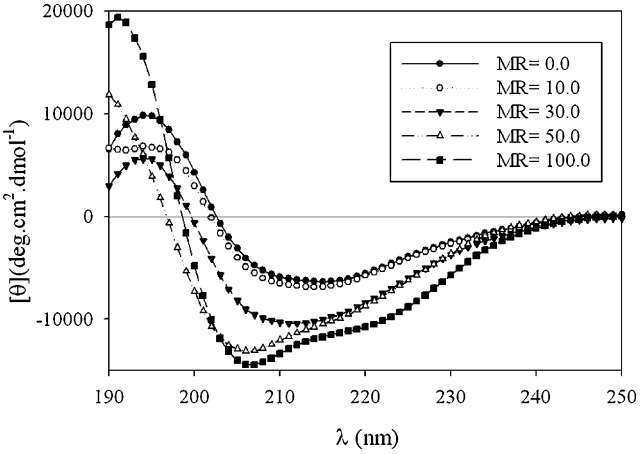

Fig. 4. Far-UV-CD of β-LG at different molar ratios of [DTAB]/[β-LG] in 50 mM phosphate buffer, pH 6.7. Spectra were averages of five accumulated scans with subtraction of the baseline, which were measured at 20 °C using a β-LG concentration of 1 mg/mL.

on circular dichroism spectrum are considered to be typical of the α-helical content of proteins. There is a weak but broad $n \rightarrow \pi^*$ transition centered around 210 nm and an intense $\pi \rightarrow \pi^*$ transition at about 190 nm. The analysis of β-LG structure by CD spectroscopy shows changes of β-LG secondary structure in various DTAB concentrations compared with native β-LG conformations. Computed according to the method of Fasman and Chou [44,45], a decrease in the β-sheet contents and subsequently an increase in α-helix contents by increasing surfactant concentration are obvious (Table 1). As shown in Fig. 4 and Table 1, the shifts in positions of the major minima and change in magnitude of ellipticity indicate two significant changes in structure of β-LG between 10–30 and 50–100 molar ratio of [DTAB]/[β-LG]. It looks that the binding of initial surfactant ions to positively charged sites on the surface of β-LG induces a conformational transition, which may be related to two different folding states. However, the second transition at higher molar ratio of DTAB can correspond to β-LG unfolding. Ultimately, the unfolded structures aggregate and precipitate. The following mechanism can be proposed for binding of DTAB to β-LG:

$$F_1 \rightarrow F_2 \rightarrow U$$

where F_1 and F_2 are corresponding to two different folded states and U corresponds to unfolded state.

3.5. Isothermal titration calorimetric study

One of the major factors influencing the electrostatic interactions of charged biopolymers in aqueous solutions is the pH, since it affects both the sign of the electrostatic charges and their magnitude. Therefore the effect of pH on the structure of β-LG was examined systematically in this study. The influence of surfactant concentrations at various pH on the enthalpy changes associated with protein–surfactant interactions was studied using ITC when DTAB solutions were added into β-LG solution (298 K, 50 mM phosphate buffer, pH 6.7 and 8.0). The dependence of ΔH_{int} *vs* molar ratio of [DTAB]/[β-LG] at pH 6.7 is complex. As we can see in Fig. 5, a relatively high endothermic enthalpy change was observed below 400 molar ratio of [DTAB]/[β-LG]. Its endothermicity grows up and falls rapidly to about zero and mutates into exothermic enthalpy change at higher surfactant concentrations. Such behavior is not observed at pH 8.0. The curve of ΔH_{int} *vs* [DTAB]/[β-LG] (Fig. 5) shows an increase of exothermicity with increasing surfactant concentrations until 430 molar ratio of [DTAB]/[β-LG] and after a decrease of exothermicity with increasing surfactant concentrations was observed.

It is usually difficult to assign precise molecular events to enthalpy changes measured in thermodynamic experiments because of the multiplicity of different physico-chemical phenomena contributing to the measured signal, *e.g.* various kinds of association–disassociation processes and conformational changes. However, the fact that the enthalpy changes observed during β-LG/DTAB interactions in the pH range 6.7–8.0 were endothermic but exothermic under other circumstances suggests that at least two different physico-chemical phenomena could occur in this pH range.

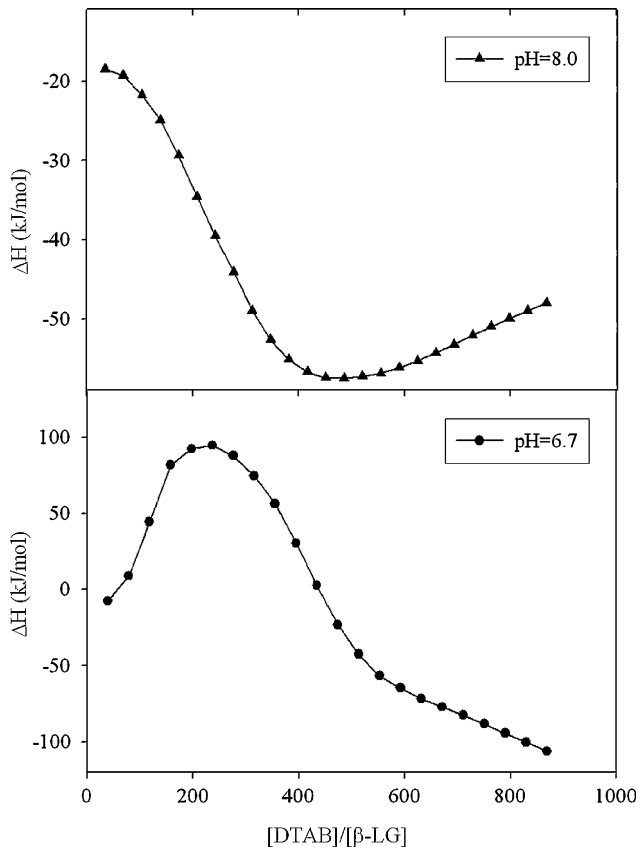

Fig. 5. Variation of enthalpy of interaction *vs* [DTAB]/[β-LG] in 50 mM phosphate buffer at pH 6.7 and 8.0 and 298 K.

Table 2
Apparent dissociation constants (K_d) and apparent molar ratio (n) of retinol/β-LG complexes in molar ratio of [DTAB]/[β-LG] at various pH and 298 K (results expressed per protein monomer).

pH	Molar ratio of [DTAB]/[β-LG]	K_d ($\times 10^{-8}$ M)	n
2.0	0.0	7.20	0.82
	10.0	8.14	0.84
	30.0	8.43	0.87
	50.0	9.67	0.89
	100.0	10.80	0.91
6.7	0.0	6.13	0.94
	10.0	7.62	0.99
	30.0	8.92	1.03
	50.0	9.01	1.04
	100.0	9.44	1.07
8.0	0.0	5.31	0.72
	10.0	7.48	0.78
	30.0	7.13	0.86
	50.0	8.56	0.94
	100.0	9.25	1.13

(1) The single well known conformational transition of β-LG at pH 6.7–8.0 range is Tanford transition [46].
(2) The dissociation of β-LG dimer to monomer in the studied pH range (β-LG is predominately dimeric at pH 6.7 and in monomeric form at pH 8.0) [47].

3.6. Influence of surfactant concentration on retinol-binding properties of β-LG

The apparent binding constants and the apparent molar ratios of retinol/β-LG complexes are reported in Table 2. The β-LG/retinol complex at pH 8.0 displayed the smallest apparent dissociation constant ($K_d = 5.31 \times 10^{-8}$ M) and the association of retinol with β-LG was slightly weaker at pH 2.0 ($K_d = 7.21 \times 10^{-8}$ M) and pH 6.7 ($K_d = 6.13 \times 10^{-8}$ M).

The obtained results show that the fluorescence intensity of β-LG tryptophan decreases significantly when retinol is bound to β-LG and that the β-LG/retinol binding is pH-dependent. Bigger quenching by retinol of tryptophan fluorescence at pH 6.7 and 8.0 as compared with what is observed at acidic pH could indicate (if tryptophan–retinol excitation energy transfer contributes to the quenching phenomenon) that distances between tryptophan and retinol become shorter at neutral pH. All the obtained data suggest that β-LG conformation changes in the studied pH range and that its binding properties change with its ionization status [21].

The results of retinol-binding studies in the presence of various DTAB concentrations at pH 2.0 show slight increase of both n and K_d with the increase of DTAB concentrations. The same is observed at other pH in case of β-LG/retinol binding in the presence of various concentrations of DTAB (Table 2). This can be related to the increase of the hydrophobic surfaces on the β-LG/DTAB complexes and insignificant changes in conformation of β-LG, especially around the calyx formed by the β-barrel at these molar ratios of [DTAB]/[β-LG]. In such case, the significant variation of fluorescence emission spectra of β-LG due to its interactions with DTAB could be related to changes of the polarity of microenvironment around indol moiety of Trp 61 protruding from the surface of the β-LG molecule.

4. Conclusions

The study of interactions of DTAB with β-LG at various pH show the significant changes in tertiary structure of β-LG due to its interactions with DTAB. Also as shown by spectrometric studies that the secondary structure of β-LG changes slightly during these interactions. The results of UV–vis and fluorescence studies showed a red shift in wavelength and an increase in absorbance and enhancement in the intensity of the quantum yield of protein during its interaction with DTAB. The results of UV–vis also showed two distinct conformational changes corresponding first to precipitation and second to solubilization of the precipitated β-LG at pH 6.7 and 8.0. The results indicate the cooperative character of binding at pH 2.0. The results of fluorescence studies showed that the binding strength of β-LG/DTAB complex increases with the increase of the pH. CD results showed the shifts in positions of the major minima and change in magnitude of ellipticity and subsequently signified two significant changes in structure of β-LG between 10–30 and 50–100 molar ratio of [DTAB]/[β-LG]. ITC measurements indicated the endothermic nature of β-LG/DTAB interactions at pH 6.7 and the exothermic nature of β-LG/DTAB interactions at pH 8.0. Surprisingly, the retinol-binding studies do not show considerable changes in the retinol-binding properties of β-LG during its simultaneous interactions with DTAB. This could be explained by the stability of the conformation of the calyx or by the stability of retinol-binding site. Insignificant effect of DTAB on retinol binding of β-LG represents the lack of competition of this surfactant with retinol for the occupancy of the hydrophobic calyx. The obtained information about the binding of this popular surfactant can be useful in their application in dairy food industry and in formulating surfactant complexes with other proteins of interest.

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References

[1] C. Tanford, Y. Nozaki, J.A. Reynolds, S. Makino, Molecular characterization of proteins in detergent solutions, Biochemistry 13 (1974) 2369–2376.
[2] M.N. Jones, A. Brass, Interactions between small amphipathic molecules and proteins, in: E. Dickinson (Ed.), Food Polymers, Gels and Colloids, The Royal Society of Chemistry, Cambridge, UK, 1991, pp. 65–80.
[3] M.N. Jones, Surfactant interactions with biomembranes and proteins, Chem. Soc. Rev. 21 (1992) 127–136.
[4] K.P. Ananthapadmanabhan, Protein–surfactant interactions, in: E.D. Goddard, K.P. Ananthapadmanabhan (Eds.), Interactions of Surfactants with Polymers and Proteins, CRC Press, Boca Raton, 1993, pp. 319–365.
[5] A.K. Bordbar, A.A. Moosavi-Movahedi, A.A. Saboury, Comparative thermo dynamical stability of bovine and pigeon haemoglobin by interaction with sodium *n*-dodecyl sulphate, Thermochim. Acta 287 (1996) 343–349.
[6] A.K. Bordbar, A.A. Moosavi-Movahedi, Binding data analysis of the interaction of bovine haemoglobin with dodecyl trimethyl ammonium bromide, Bull. Chem. Soc. Jpn. 69 (1996) 2231–2234.
[7] A.K. Bordbar, A.A. Saboury, M.R. Hosaindokht, A.A. Moosavi-Movahedi, Statistical effect to the binding of the ionic surfactant to protein, J. Colloid Interface Sci. 192 (1997) 415–419.
[8] A.K. Bordbar, A. Taheri-Kafrani, Binding and fluorescence study on interaction of human serum albumin (HSA) with cetylpyridinium chloride (CPC), Colloids Surf. B: Biointerfaces 55 (2007) 84–89.
[9] A.K. Bordbar, A. Taheri-Kafrani, H. Mousavi, T. Haertlé, Energetics of the interactions of human serum albumin with cationic surfactant, Arch. Biochem. Biophys. 470 (2008) 103–110.
[10] A. Taheri-Kafrani, A.K. Bordbar, H. Mousavi, T. Haertlé, β-Lactoglobulin structure and retinol binding changes in presence of anionic and neutral detergents, J. Agric. Food Chem. 56 (2008) 7528–7534.
[11] E.D. Goddard, K.P. Ananthapadmanabhan, Applications of polymer–surfactant systems, in: J.C.T. Kwak (Ed.), Polymer-Surfactant Systems, vol. 77, Marcel Dekker, New York, 1998, pp. 22–64.
[12] Y.X. Huang, R.C. Tan, Y.L. Li, Y.Q. Yang, L. Yu, Q.C. He, Effect of salts on the formation of C8-lecithin micelles in aqueous solution, J. Colloid Interface Sci. 236 (2001) 28–34.
[13] P.R. Majhi, A. Blume, Thermodynamic characterization of temperature-induced micellization and demicellization of detergents studied by differential scanning calorimetry, Langmuir 17 (2001) 3844–3851.
[14] A.K. Bordbar, N. Sohrabi, E. Hojjati, The estimation of the hydrophobic and electrostatic contributions to the free energy change upon cationic surfactants binding to Jack-Bean urease, Colloids Surf. B: Biointerfaces 39 (2004) 171–175.
[15] A.K. Bordbar, R. Hossainzadeh, K. Omidiyan, Study on interaction of α-amylase from *Bacillus subtilis* with cetyl trimethyl ammonium bromide, Colloids Surf. B: Biointerfaces 40 (2005) 67–71.

[16] A.K. Bordbar, R. Hossainzadeh, Binding of cetylpyridinum chloride to glucose oxidase, Colloids Surf. B: Biointerfaces 53 (2006) 288–295.
[17] A.K. Bordbar, R. Hossainzadeh, H. Norozi, Interaction of a homologous series of *n*-alkyl trimethyl ammonium bromides with egg white lysozyme: micro calorimetric and spectroscopic study, J. Thermal. Anal. Cal. 87 (2007) 453–456.
[18] D.W.S. Wong, W.M. Camirand, A.E. Pavlath, Structures and functionalities of milk proteins, Crit. Rev. Food Sci. Nutr. 36 (1996) 807–844.
[19] R.D. Fugate, P. Song, Spectroscopic characterization of β-lactoglobulin-retinol complex, Biochim. Biophys. Acta 625 (1980) 28–42.
[20] Y. Cho, C.A. Batt, L. Sawyer, Probing the retinol-binding site of bovine beta-lactoglobulin, J. Biol. Chem. 269 (1994) 11102–11107.
[21] E. Dufour, C. Genot, T. Haertlé, Beta-lactoglobulin binding properties during its folding changes studied by fluorescence spectroscopy, Biochim. Biophys. Acta 1205 (1994) 105–112.
[22] Q. Wang, J.C. Allen, H.E. Swaisgood, Binding of retinoids to β-lactoglobulin isolated by bioselective adsorption, J. Dairy Sci. 80 (1997) 1047–1053.
[23] D. Frapin, E. Dufour, T. Haertlé, Probing the fatty acid binding site of beta-lactoglobulins, J. Protein Chem. 12 (1993) 443–449.
[24] P. Busti, S. Scarpeci, C.A. Gatti, N.J. Delorenzi, Interaction of alkylsulfonate ligands with beta-lactoglobulin AB from bovine milk, J. Agric. Food Chem. 47 (1999) 3628–3631.
[25] H. Mousavi, J.M. Chobert, A.K. Bordbar, T. Haertlé, Ethanol effect on the structure of β-lactoglobulin B and its ligand binding, J. Agric. Food Chem. 56 (2008) 8680–8684.
[26] S.G. Hambling, A.S. McAlpine, L. Sawyer, in: P.F. Fox (Ed.), Advanced Dairy Chemistry 1: Proteins, Elsevier Applied Science, London, NY, 1992, pp. 141–190.
[27] H.L. Monaco, G. Zanotti, P. Spadon, M. Bolognesi, L. Sawyer, E.E. Eliopoulos, Crystal structure of the trigonal form of bovine beta-lactoglobulin and of its complex with retinol at 2.5 A resolution, J. Mol. Biol. 197 (1987) 695–706.
[28] M.Z. Papiz, L. Sawyer, E.E. Eliopoulos, A.C.T. North, J.B.C. Findlay, R. Sivaprasadarao, T.A. Jones, M.E. Newcomer, P.J. Kraulis, The structure of β-lactoglobulin and its similarity to plasma retinol-binding protein, Nature 324 (1986) 383–385.
[29] J.M. Chobert, T. Haertlé, in: S. Damodaran, A. Paraf (Eds.), Food proteins and their applications, Marcel Dekker Inc., NY, Basel, Hong Kong, 1997, pp. 143–170.
[30] R. Waning, M. Paulsson, T. Nylander, B. Ninham, P. Sellers, Binding of sodium dodecyl sulphate and dodecyl trimethyl ammonium chloride to β-lactoglobulin: a calorimetric study, Int. Dairy J. 8 (1998) 141–148.
[31] R.C. Lu, A.N. Cao, L.H. Lai, J.X. Xiao, Interactions of β-lactoglobulin with sodium decylsulfonate, decyltriethylammonium bromide, and their mixtures, J. Colloid Interface Sci. 299 (2006) 617–625.
[32] P. Maillart, B. Ribadeau-Dumas, Preparation of β-lactoglobulin and β-lactoglobulin-free proteins from whey retentate by NaCl salting out at low pH, J. Food Sci. 33 (1988) 743–752.
[33] T. Takakuwa, T. Konno, H. Meguro, A new standard substance for calibration of circular dichroism: Ammonium d-10-camphorsulfonate, Anal. Sci. 1 (1985) 215–218.
[34] M.R. Eftink, The use of fluorescence methods to monitor unfolding transitions in proteins, Biophys. J. 66 (1994) 482–501.
[35] U. Cogan, M. Kopelman, S. Mokady, M. Shinitzky, Binding affinities of retinol and related compounds to retinol binding proteins, Eur. J. Biochem. 65 (1976) 71–78.
[36] A.K. Moren, K. Eskilsson, A. Khan, Phase behaviour of oppositely charged protein and surfactant mixtures: DOTAC-BLG-Water, Colloids Surf. B: Biointerfaces 9 (1997) 305–314.
[37] A.K. Morén, A. Khan, Phase behavior and phase structure of protein–surfactant–water systems, J. Colloid Interface Sci. 218 (1999) 397–403.
[38] R.M. Hill, D.R. Briggs, A study of the interaction of *n*-octylbenzene-p-sulfonate with β-lactoglobulin, J. Am. Chem. Soc. 78 (1956) 1590–1597.
[39] H.C. Cheung, Resonance energy transfer, in: J.R. Lakowicz (Ed.), Topics in Fluorescence Spectroscopy, vol. 2, Plenum, New York, 1991, pp. 128–171.
[40] B.Y. Qin, M.C. Bewley, L.K. Creamer, H.M. Baker, E.N. Baker, G.B. Jameson, Structural basis of the Tanford transition of bovine β-lactoglobulin, Biochemistry 37 (1998) 14014–14023.
[41] B.Y. Qin, L.K. Creamer, E.N. Baker, G.B. Jameson, 12-Bromododecanoic acid binds inside the calyx of β-lactoglobulin, FEBS Lett. 438 (1998) 272–278.
[42] G.A. Manderson, M.J. Hardman, L.K. Creamer, Effect of heat treatment on bovine β-lactoglobulin A, B and C. Explored using thiol availability and fluorescence, J. Agric. Food Chem. 47 (1999) 3617–3627.
[43] G. Palazolo, F. Rodriguez, B. Farruggia, G. Pico, N. Delorenzi, Heat treatment of β-lactoglobulin: structural changes studied by partitioning and fluorescence, J. Agric. Food Chem. 48 (2000) 3817–3822.
[44] P.Y. Chou, G.D. Fasman, Empirical predictions of protein conformation, Annu. Rev. Biochem. 47 (1978) 251–276.
[45] P.Y. Chou, G.D. Fasman, Prediction of the secondary structure of proteins from their amino acid sequence, Adv. Enzymol. Relat. Areas Mol. Biol. 47 (1978) 45–148.
[46] C. Tanford, L.G. Bunville, Y. Nozaki, The reversible transformation of β-lactoglobulin at pH 7.5, J. Am. Chem. Soc. 81 (1959) 4032–4036.
[47] M.P. Thompson, H.M. Farrell, in: Larson, Smith (Eds.), Genetic Variants of the Milk Proteins: Lactation, Nutrition and Biochemistry of Milk/maintenance, Academic Press, New York, 1974.

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ARTICLE

Effects of Heating and Glycation of β-Lactoglobulin on Its Recognition by IgE of Sera from Cow Milk Allergy Patients

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β-Lactoglobulin (β-LG) is one of the cow's major milk proteins and the most abundant whey protein. This globular protein of about 18 kDa is folded, forming a β-barrel (or calyx) structure. This structure is stabilized by two disulfide bonds and can be altered by heating above 65 °C. β-LG is also one of the major allergens in milk. Heating is one of the most common technologic treatments applied during many milk transformations. During heating in the presence of reducing sugars, β-LG is also submitted to the Maillard reaction, which at the first stage consists of the covalent fixation of sugars on the ε-amino groups of lysyl residues. The following steps are condensation and polymerization reactions leading to the formation of melanoidins (brown pigments). Despite the frequency of use of heating during milk transformation, the effects of heat-induced denaturation and of glycation of β-LG on its recognition by IgE from cow's milk allergy (CMA) patients are not fully understood. The objectives of our work were to evaluate the effect of heat-induced denaturation of bovine β-LG on binding of IgE from CMA patients and to determine the effect of moderate glycation on the degree of recognition by IgE. We showed that heat-induced denaturation (loss of tertiary and secondary structures) of β-LG is associated with weaker binding of IgE from CMA patients. It was also shown that moderate glycation of β-LG in early stages of Maillard reaction has only a small effect on its recognition by IgE, whereas a high degree of glycation has a clear "masking" effect on the recognition of epitopes. This demonstrates the importance of ε-amino groups of lysines in the definition of epitopes recognized by IgE.

KEYWORDS: Allergy; milk; heating; Maillard reaction; glycation; β-lactoglobulin; protein denaturation; IgE

INTRODUCTION

Food allergies are ranked by the World Health Organization (WHO) as the sixth problem of human health. It is estimated that in the United States, between 4.5 and 8% of the children below 2 years old suffer from food allergies. The number of people with symptoms of allergic reactions to food and the severity of these symptoms have increased continuously during the past several years (*1*−*4*). Food allergy is an adverse reaction to an otherwise harmless food or food component that involves an abnormal response of the immune system to specific protein(s) in foods. The majority of food allergies are mediated by allergen-specific IgE antibodies that cause an immediate type of reaction with symptoms occurring from minutes to a few hours after ingestion of the untolerated foods (*5*). Allergenicity to certain foods or food ingredients affects both children and adults. The most serious sensitivities are those that affect children, particularly infants and babies because of the influence on their growth. Milk being the first food for newborns, cow's milk allergy (CMA), defined as an immunologically mediated reaction against cow's milk antigens (*6*), is an important problem in pediatrics. Childhood CMA is the third most prevalent food allergy in France, with approximately 9% of the total allergies diagnosed (*7*). The most abundant milk proteins are α_{S1}-, α_{S2}-, β-, and κ-caseins, α-lactalbumin, and β-lactoglobulin (β-LG). Caseins, β-LG, and α-lactalbumin are the main allergens in milk (*8*−*10*). Other proteins present in milk in lower amounts such as bovine serum albumin, lactotoferrin, and IgG-heavy chain are also recognized by CMA patients (*9*, *10*).

Nowadays, milk is quite exceptionally consumed in its raw state. Heating (pasteurization), one of the most commonly applied treatments of milk, induces sometimes important protein structural changes. Heating also induces chemical modifications of proteins, which can influence IgE binding and the allergenicity (*11*). Because milk contains significant amounts of lactose and lower quantities of other reducing sugars, milk proteins are modified by so-called Maillard reaction or glycation during

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heating (*12*). Glycation is one of the most frequent chemical modifications during industrial processing (*13*, *14*). It occurs during the heating of proteins in the presence of reducing sugars. This is a complex reaction, leading to the formation of advanced Maillard reaction products (AMPs) or advanced glycated end products (AGEs). The first step of the reaction consists of the creation of Schiff type adducts of reducing sugars with primary amino groups of mainly lysyl residues of the proteins, leading to the formation of Amadori and Heyns products. The reaction does not stop there; the following steps include condensation and polymerization reactions, leading to the formation of brown pigments called melanoidins. In milk, Maillard reaction mainly leads to the Amadori products. The extent of glycation in commercial foods depends on many factors such as the heating temperature, the duration of it, and the concentrations of reducing sugars. The extent of lysine blockage of whey proteins in milk is the same as in other food systems and is well-documented (*12*−*15*). Typical AGEs such as pyrraline or carboxymethyllysine do not play a major role in heated milk when compared to the Amadori product lactuloselysine. Additionally, the conditions of glycation used were specifically set to reduce the amount of AGE or to eliminate them altogether.

Several studies attempted to evaluate the IgE-binding ability of allergens modified by the Maillard reaction (*15*−*20*). In some cases, glycation of allergens increases their recognition by IgE (*15*, *18*), whereas in other cases, glycation reduces IgE binding (*16*, *19*) or has no effect (*16*, *17*). No general effect was observed; the effect seems to be allergen- and sugar-dependent.

β-LG is a small globular protein of 162 amino acid residues with a molecular mass of 18.36 kDa. Three-dimensional (3D) crystallographic studies have shown that the β-LG 3D structure consists of nine antiparallel β-sheet structures forming a so-called β-barrel (or calyx) stabilized by two disulfide bonds (one cysteinyl residue remaining free) (*21*−*23*). A short three-turn α-helix lying on the outer face of the barrel is present at the C-terminal part of the molecule. The interior of the calyx contains a hydrophobic pocket, allowing the binding of small hydrophobic molecules such as retinoids, fatty acids, vitamins, and cholesterol (*24*−*26*). The quaternary structure of β-LG varies according to the temperature, concentration, ionic strength, and pH (*27*). In physiological conditions (pH 5.5−7.5 and concentration < 5 g.L^{-1}), β-LG is mainly a noncovalent dimer stabilized by electrostatic interactions (*28*). At pH > 8, Tanford transition occurs consisting of dimer dissociation and increased exposition to the solvent (*22*, *29*) of tyrosines and tryptophans. At a pH close to the pI (between 4.5 and 5.5), β-LG aggregates in tetramers (*27*), whereas it dissociates in monomers at pH < 3.5. pH variations have only a small effect on the tertiary and secondary structures of β-LG (*21*). Heating to temperatures > 60 °C causes destabilization of β-sheets, unfolding of the β-barrel, and exposition of disulfide bonds and the free cysteine to the solvent. Above 65 °C, denaturation becomes irreversible: Loss of secondary and tertiary structures is associated with new hydrophobic interactions and disulfide bond exchanges, leading to irreversible aggregation (*30*−*33*). All of these structural modifications are likely to induce changes in the antigenic character or immunoreactivity of this important protein (*34*). The antigenicity of β-LG is increased by heating at temperatures of 80−90 °C and decreased after heating above 100 °C (*35*). The denatured structure of the molecule has been shown to modulate the immunologic response of rat and mouse models of allergy (*36*, *37*). The objectives of the present work were (1) to evaluate the effect of heat-induced denaturation of bovine β-LG on binding of IgE from CMA patients and (2) to determine the effect of moderate glycation on the degree of recognition by IgE.

MATERIALS AND METHODS

Sera. A series of 18 sera from CMA patients presenting various symptoms were used. Total milk protein- and β-LG-specific IgE concentrations were determined with the Phadia ImmunoCAP System. All of the sera had total milk protein-specific IgE concentrations from < 0.35 to 57.8 kAU L^{-1}. Thirteen sera had β-LG-specific IgE values from 0.36 to 26.3 kAU L^{-1}, and four had β-LG-specific IgE concentrations < 0.35 kAU L^{-1}. For competitive enzyme-linked immunosorbent assay (ELISA), a pool containing the 14 sera was constituted. Sera were collected in the Laboratory of Immuno-Allergology of Academic Hospital (Angers, France), and their use was approved by the internal Ethical Committee of the hospital.

Preparation of Heated Proteins. Native bovine β-LG (variant A) was purified in Nantes INRA laboratory according to the method of Mailliart and Ribadeau Dumas (*38*) from the milk of cows confirmed as homozygote for β-LG variant A allele. Lyophilized purified native bovine β-LG was diluted in phosphate-buffered saline (PBS), and its concentration was adjusted to 2.7 mg mL^{-1}, corresponding to its concentration in milk, after verification of its protein content with the BCA assay kit (Sigma, St. Quentin-Fallavier, France).

β-LG was heated with a Touchgene gradient thermocycler (Techne Inc., Princeton, NJ) at the rate of 1 °C min^{-1} and incubated for 20 min at 65, 75, 85, and 95 °C. After it was heated, the protein was rapidly cooled (about 1 °C s^{-1}) to 4 °C and used immediately for studies of its recognition by IgE.

Preparation of Glycated Proteins. Purified native bovine β-LG (variant A) was glycated with lactose (β-LG-Lac), ribose (β-LG-Rib), glucose (β-LG-Glu), galactose (β-LG-Gal), arabinose (β-LG-Ara), and rhamnose (β-LG-Rha) (Sigma) as described by Chevalier et al. (*39*, *40*). Briefly, β-LG (0.217 mM) and the different sugars (0.217 M) were dissolved in 0.1 M phosphate buffer, pH 6.5. After filtration on 0.22 μM acetate cellulose filters (Millipore, Bedford, MA), mixtures of protein and sugar were put in well-capped flasks and heated in a water bath at 60 °C for 72 h. This mild heat treatment limited the self-aggregation of β-LG. All experiments were performed under strictly anaerobic and sterile conditions; all media were purged and saturated with N_2. After they were heated, the different fractions were dialyzed against distilled water, freeze-dried, and stored at −20 °C. β-LG heated at 60 °C for 72 h without sugar (β-LG-60 °C) was used as a control. The quantity of available amino groups was determined by the modified *ortho*-phthaldialdehyde (OPA) method (*41*). The OPA reagent was prepared daily by mixing 40 mg of OPA (dissolved in 1 mL of methanol), 50 mL of 0.1 M sodium borate buffer, pH 9.3, 100 mg of *N*-dimethyl-2-mercaptoethylammonium chloride (DMMAC), and 1.25 mL of 20% w/w sodium dodecyl sulfate (SDS) in water. Fifty microliters of protein solution (2 g L^{-1} in 50 mM sodium phosphate buffer, pH 7.8) was added to 1 mL of OPA reagent. The absorbency was read at 340 nm after a minimal delay of 5 min. A calibration curve was obtained by using 0.25−2.00 mM L-leucine as a standard. The degrees of modification of the proteins were as follows: β-LG, 60 °C, 6.2%; β-LG-Lac, 34.4%; β-LG-Rha, 40.6%; β-LG-Glu, 41.2%; β-LG-Gal, 41.9%; β-LG-Ara, 55.0%; and β-LG-Rib, 69.4% (*40*). Lyophilized glycated proteins were diluted in PBS before use (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, and 8 mM Na_2HPO_4, pH 7.4), and their concentrations were adjusted to 1 mg mL^{-1}.

Electrophoresis. Proteins were analyzed by 12% SDS-polyacrylamide gel electrophoresis (PAGE). Briefly, proteins were diluted in loading buffer [60 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, and 0.025% (w/v) bromophenol blue] and loaded on 12% polyacrylamide gel containing 0.1% (w/v) SDS. Migration was performed for about 1 h in a buffer containing 0.3% (w/v) Tris and 1.44% (w/v) glycine. Gels were stained with an aqueous solution containing 50% (v/v) ethanol, 10% (v/v) glacial acetic acid, and 0.25% (w/v) Coomassie brilliant blue and destained with a solution containing 10% (v/v) ethanol and 7% (v/v) glacial acetic acid. Electrophoresis was performed under reducing and nonreducing conditions. In reducing conditions, the sample buffer was added with 5% reducing agent β-mercaptoethanol (β-ME).

Circular Dichroism (CD). CD spectra were recorded on a CD6 dichrograph (Jobin Yvon, Longjumeau, France), using cells of the appropriate lengths and a scan time of 1 nm s^{-1}. All measurements were carried out at 20 °C with thermostatically controlled cell holders. The instruments were calibrated with ammonium d-10-camphorsulfonic acid.

The data were expressed as molar residue ellipticity [θ], which is defined as $[\theta] = 100\ \theta_{obs}/cl$, where θ_{obs} is the observed ellipticity in degrees, c is the concentration in residue mol cm^{-3}, and l is the length of the light path in cm. For measuring CD spectra in the far-UV region, the sample concentration was 1 mg mL^{-1} in 100 mM PBS buffer, pH 7.4, and the spectra were recorded between 190 and 260 nm using a quartz cuvette with a 0.1 mm path length. Each spectrum was the accumulation of five successive measurements, and baseline was corrected by subtracting the buffer spectrum.

A protein concentration of 0.5 mg mL^{-1} was used for near-UV CD spectra, and the spectra were recorded between 260 and 350 nm using a quartz cuvette with a 1 cm path length. Four scans were averaged per spectrum. The baseline was corrected by subtracting the corresponding solvent spectrum from the sample spectrum.

Colorimetric ELISA (C-ELISA). Maxisorp bottom flat transparent 96 microtitration plates (Nunc, Roskilde, Denmark) were coated overnight with 100 μL per well native, heated, and sugar-modified β-LG diluted to 5 μg mL^{-1} in PBS, with native β-LG diluted to 5 μg mL^{-1} in 100 mM carbonate buffer, pH 9.6, or with PBS in wells used as negative controls. After they were coated, they were washed three times with PBS containing 0.1% (v/v) Tween-20 (PBS/T) and saturated for 1 h with 250 μL of a solution of PBS/T containing 1% (w/v) polyvinyl alcohol (Sigma) (PBS/T/PVA). Plates were washed three times with PBS/T and incubated for 90 min at room temperature with 100 μL of mouse monoclonal anti-β-LG antibody (IgG) diluted to 1:100 in PBS/T/PVA from hybridoma supernatant. Mouse monoclonal anti-β-LG antibody mAb37 and mAb96, which bind β-LG on two different epitopes independently of secondary and tertiary structures of β-LG, were produced and characterized in Nantes INRA laboratory (*42*). Plates were washed three times with PBS/T and incubated for 1 h at room temperature with a peroxidase-conjugated antimouse IgG (Bio-Rad Laboratories, Hercules, CA) diluted 1:3000 in PBS/T/PVA. The secondary antibody binding was revealed after three washes with PBS/T by addition of 100 μL of OPD (Sigma, 0.4 mg mL^{-1} in 50 mM citrate buffer, pH 5.5). The staining reaction was stopped after 20 min of incubation at room temperature by the addition of 100 μL of 2 M H_2SO_4. The absorbance was measured at 492 nm with the EL_X800 plate reader (BioTek Instruments, Inc., Winooski, VT).

Fluorescent ELISA (F-ELISA). Maxisorp bottom flat white 96 microtitration plates (Nunc) were coated overnight with either 100 μL per well of a mouse monoclonal antihuman IgE antibody ($IgG_{2\beta}$) (Fitzgerald, Concord, United States) diluted to 1:2500 (1.6 μg mL^{-1}) in PBS or with native, heated, and sugar-modified β-LG diluted to 5 μg mL^{-1} in PBS or with native β-LG diluted to 5 μg mL^{-1} in 100 mM carbonate buffer, pH 9.6. After they were coated, they were washed three times with PBS/T and saturated for 1 h with 250 μL of a solution of PBS/T/PVA. Plates were washed three times with PBS/T. Wells coated with the antihuman IgE antibody were incubated with 100 μL of serial dilutions to 1/2 from 160 to 0.08 ng mL^{-1}, plus one dilution at 1 ng mL^{-1}, of the second WHO international reference preparation of human IgE (prepared in PBS/T/PVA). In the wells coated with allergens and in those coated with PBS as a negative control, 100 μL of 1:25 dilutions of sera from patients was added. After an overnight incubation at 4 °C, the plates were washed three times with PBS/T and incubated for 2 h at room temperature with an alkaline-phosphatase-conjugated polyclonal antihuman IgE (Sigma) diluted 1:1000 in PBS/PVA. The secondary antibody binding was revealed, after three washings with PBS/T, by the addition of 4-methylumbelliferyl phosphate (4-MUP) substrate diluted 1:5 in 1 M Tris-HCl, pH 9.8. The fluorescence emission was measured after 90 min of incubation at 37 °C with the FL_X800 plate reader (BioTek Instruments) fitted with a 360 nm excitation filter and a 440 nm emission filter. The reading parameters were adjusted to 45 in sensitivity for a reading from the top. Controls included secondary antibody on capture antibody (no IgE), secondary antibody on IgE (no capture antibody), secondary antibody on allergens (no patient serum), and secondary antibody on serum (no allergen).

To relate the fluorescence intensity to IgE concentrations, a sigmoid four-parameter model [$y = d + (a - d)/1 + (x/c)^b$] was adjusted to standard curve data by nonlinear regression using the Solver option from Microsoft Excel 2000 by minimizing the sum of the squared differences. Limits of detection [mean + three standard deviations (SD)] and quantification (mean + 10 SDs) were calculated from fluorescence data of the eight wells with no IgE. Fluorescence data measured for each antigen and serum were corrected by subtracting the fluorescence of the control with no antigen, and corresponding specific IgE concentrations were calculated from the adjusted standard curve provided that the fluorescence exceeded the quantification limit.

Competitive ELISA. Maxisorp bottom flat white 96 microtitration plates (Nunc) were coated overnight with 100 μL per well of native β-LG diluted to 5 μg mL^{-1} in PBS. F-ELISA was performed as previously described except that sera of patients were replaced by a pool of sera preincubated for 1 h at 37 °C in the presence of increasing concentrations of competitor. The final dilution of the serum was 1:20, and the final concentration of the inhibitor ranged from 0 to 100 μg mL^{-1} (1:10 serum pool dilution and inhibitor solution were mixed v/v to a final volume of 100 μL). Because of the low amount of serum available, the experiment was realized once in triplicate. The concentration of protein (inhibitor) needed to inhibit 50% of IgE binding (IC_{50}) was calculated from the inhibition curves by relating fluorescence intensity to inhibitor concentrations. A sigmoid four-parameter model [$y = d + (a - d)/1 + (x/c)^b$] was adjusted to standard curve data by nonlinear regression using the Solver option from Microsoft Excel 2000.

RESULTS

Characterization of Heated β-LG. Analysis of SDS-PAGE results of heated β-LG (**Figure 1A**) in nonreducing conditions showed that until 65 °C, β-LG is present mostly in a monomeric form in PBS solution at pH 7.4 (band at about 14.4 kDa). A small proportion of β-LG was also dimeric (band slightly higher than 31 kDa). After they were heated at higher temperatures, the proportion of dimers increased, and additional bands corresponding to larger polymers (trimers, tetramers, etc.) appeared. Under reducing conditions (**Figure 1B**), β-LG migrated as a unique band, indicating that heat-induced polymerization involves disulfide bond formation. Those data are in accordance with what is already well-known. Heat-induced denaturation and aggregation of β-LG by the formation of disulfide bonds occur when it is heated at temperatures higher than 70−72 °C (transition temperature) (*43*). However, this allowed checking that in the samples used for ELISA, β-LG structure has been effectively changed. A more accurate characterization of β-LG samples was

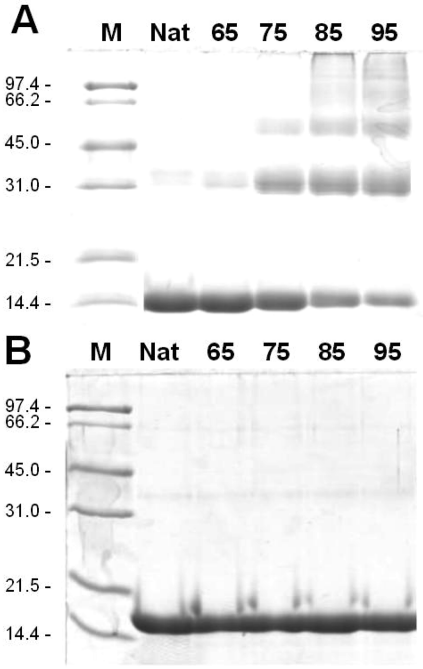

Figure 1. Heat-induced polymerization of β-LG: Native β-LG (line Nat) and β-LG heated for 20 min at 65, 75, 85, and 95 °C (lines 65−95) in PBS buffer were analyzed on 12% SDS-PAGE in nonreducing (**A**) or in reducing (**B**) conditions; M, molecular mass marker (kDa).

achieved by CD spectra measurements, which allowed monitoring of the folding changes of heated β-LG.

The main chromophores in the near-UV CD are tryptophan, tyrosine, and phenylalanine (*44*). Near-UV CD spectra allow us to characterize the tertiary structure of proteins mainly due to the asymmetries in the environment of the aromatic amino acids and to characterize the stability of their conformers. β-LG contains two tryptophans (Trp 19 and Trp 61). It is known that Trp 61 is on the surface of the protein and has considerable rotational freedom, which together with Trp19 is the source of the near-UV CD signals at 286 and 293 nm (*22*, *24*, *45*). As shown in **Figure 2A**, heat treatment of β-LG induces a decrease in the intensity of the deep troughs observed at 286 and 293 nm, confirming other recent findings (*30*, *46*). The changes in [θ] at 286 and 293 nm, which were most probably due to the changes in the environment of Trp 19, were likely to reflect irreversible structural changes that occurred within the calyx of the β-LG molecule as a result of heat treatment.

Far-UV CD spectra have been used to follow the evolution of the secondary structure of β-LG when submitted to heat treatment at pH 7.4 (**Figure 2B**). The trough, with a minimum at 216 nm, gradually broadened and deepened so that the minimum shifted to lower wavelengths, indicating the loss of secondary structure between 65 and 75 °C, as already observed (*33*, *47*); α-helix and β-sheet structures were progressively converted to aperiodic structure. These results confirm that the tertiary and secondary structures of heated β-LG used for assaying IgE binding were progressively altered by heating.

Characterization of Glycated β-LG. Figure 3 illustrates the strong effect of glycation on β-LG. On SDS-PAGE, in nonreducing conditions, a band at about 31 kDa corresponding to β-LG dimers was present. In reducing conditions, this band disappeared in the case of nonglycated β-LG but persisted in the case of glycated β-LG. Consequently, as compared to native β-LG, glycated β-LG is also able to form covalent dimers that do not involve disulfide bond formation. In the case of substitution by ribose and arabinose (sugars inducing the highest degree of modification), a smear was observed in both conditions, indicating that substituted proteins form polymers stabilized by sugar-induced covalent bonds (*39*). Full modifications induced by glycation, including conformational changes, have been analyzed in detail by Chevalier et al. (*39*, *40*).

Response of Patient Sera to Heated β-LG. The binding of IgE from patients having CMA on native and heated β-LG was studied by F-ELISA. Because heating of β-LG causes its polymerization and aggregation, it was essential to check that heated β-LG was able to coat the wells as well as native β-LG. Consequently, in parallel to F-ELISA, the adsorption of heated β-LG on microtitration plates was checked by C-ELISA using two β-LG-specific monoclonal antibodies mAb37 and mAb96, which bind β-LG on two different epitopes independently of secondary and tertiary structures of β-LG (*42*). The result showed that coating of microtitration plate wells with heated β-LG, whatever the temperatures, was identical to what was observed with native β-LG (**Figure 4A**).

For each patient, the specific IgE titer was determined for native and heated β-LG. The experiment was repeated three times. Three of the patients with [IgE] > 0.35 kAU L^{-1} generated values above the detection limit but below the quantification limit; they were eliminated from the study. In contrast, three out of the four patients with [IgE] < 0.35 kAU L^{-1} generated well quantifiable IgE concentration values. For each treatment, specific IgE concentration values were compared to that obtained with native β-LG. The results are expressed as a percentage of the native β-LG-specific IgE titer (**Figure 4B**). Significance of

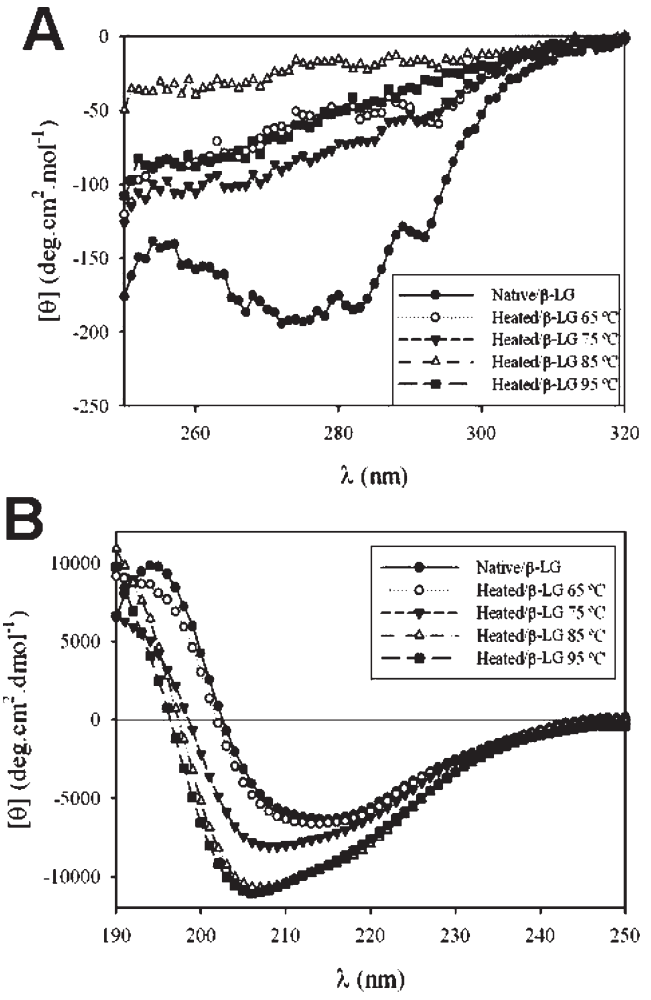

Figure 2. Far-UV (**A**) and near-UV (**B**) circular dichroism spectra of native and heated β-LG.

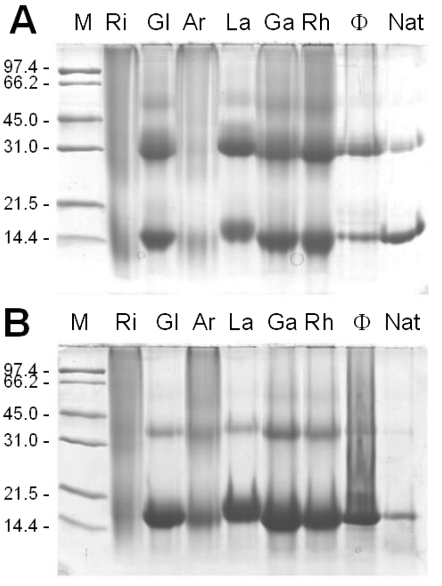

Figure 3. Polymerization of glycated β-LG: β-LG glycated with ribose (Ri), glucose (Gl), arabinose (Ar), lactose (La), galactose (Ga), and rhamnose (Rh) as compared with native β-LG (Nat) and β-LG heated for 72 h at 60 °C in the absence of sugar (Φ) were analyzed on 12% SDS-PAGE in nonreducing (**A**) or in reducing (**B**) conditions; M, molecular mass marker (kDa).

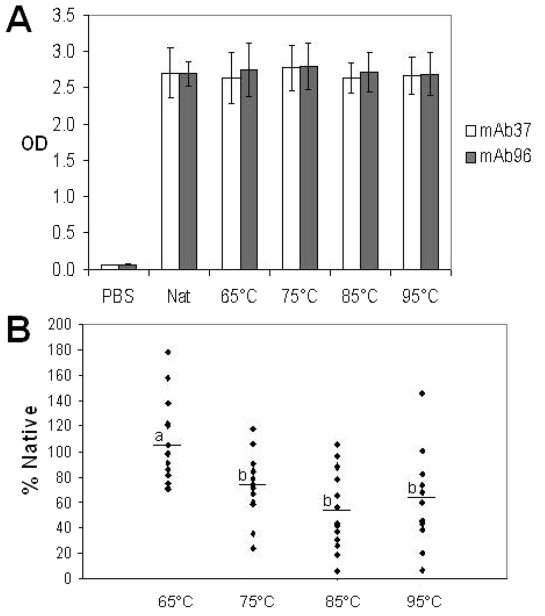

Figure 4. (**A**) Adsorption of heated β-LG on ELISA plate: signal from the binding (OD) of two anti-β-LG monoclonal antibodies in wells coated with native (Nat) and β-LG heated at 65, 75, 85, and 95 °C. (**B**) IgE binding on heated β-LG: binding of IgE from 17 CMA patients on native (Nat) and β-LG heated at 65, 75, 85, and 95 °C. Results are expressed as the percentage of the signal obtained by ELISA on native β-LG. The mean is indicated by an horizontal black bar. Identical letters indicate that means are not significantly different at a level of 5%.

differences between medians was tested by the Kruskal−Wallis test. The result indicated that the differences of means observed between native and β-LG heated at 65 °C (mean 106%) and β-LG heated at 75, 85, or 95 °C (means of 73, 55 and 64%, respectively) were significant ($\alpha = 0.05$). At the opposite, the slight differences observed between means obtained with β-LG heated at 75, 85, and 95 °C were not significant.

A relative heterogeneity of patient responses was observed. This heterogeneity is illustrated in **Figure 5**, which shows the different individual responses of three patients. Patient M95 reacted as the average. Patient M86 had a β-LG-specific IgE response strongly affected by β-LG heating. In contrast, the binding of IgE from patient M88 was greatly reduced on β-LG heated to temperatures higher than 65 °C. Ten out of fourteen patients (70%) had a similar response to patient M95, 6/14 patients (43%) had a similar response to patient M89, and only patient M88 (7%) had a strong decrease in IgE binding to heated β-LG.

Lower binding of β-LG-specific IgE to heated β-LG was confirmed by IgE binding inhibition experiments using a pool of sera constituted by the 17 tested sera (**Figure 6**). The standard deviation was not reported on the graph because it was always < 10%. The calculated IC_{50} value was 0.34 μg mL^{-1} for native β-LG and increased progressively with the heating to reach a maximum of 11.74 and 11.63 μg mL^{-1} for 85 and 95 °C heated β-LG, respectively (**Table 1**), indicating that heated β-LG is less recognized than native β-LG by IgE from CMA patients and that the effect increases progressively with temperature.

Response of Patient Sera to Glycated β-LG. As well as in the case of heated β-LG, the adsorption of glycated β-LG on ELISA plates was checked using monoclonal antibodies mAb37 and mAb96. The results showed that coating of microtitration plate wells was identical with either glycated β-LG or with β-LG incubated at 60 °C for 72 h in the absence of sugars (**Figure 7A**).

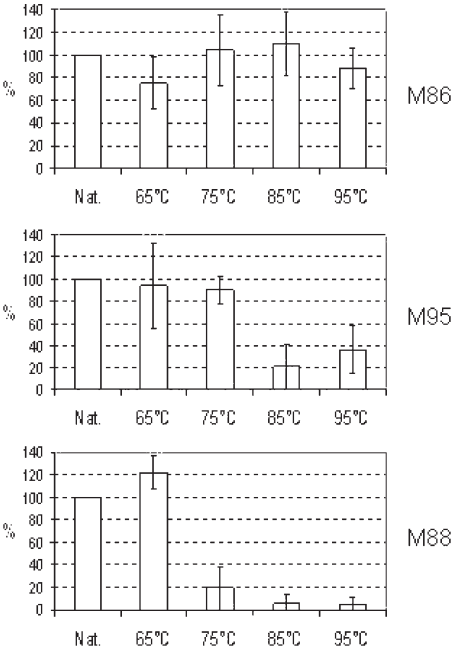

Figure 5. Individual IgE response of CMA patients to heated β-LG: average of the binding of IgE from 17 CMA patients on native (Nat) and β-LG heated at 65, 75, 85, and 95 °C. Results are expressed as the percentage of the signal obtained by ELISA on native β-LG.

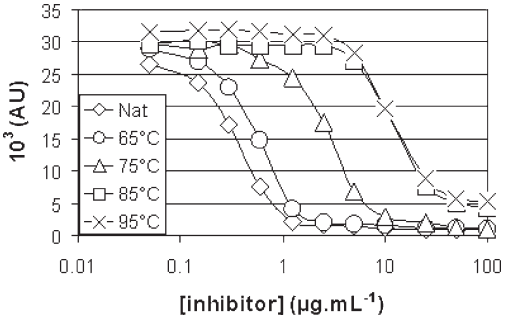

Figure 6. Inhibition of anti-β-LG IgE in serum pool. Comparison between native (Nat) and heat-treated β-LG at 65, 75, 85, and 95 °C. The signal (AU) resulting from the binding of IgE on native β-LG coated plates is shown.

Table 1. Inhibition of IgE Binding to Native β-LG by Native and Heated β-LG[a]

	β-LG treatment				
	native	65 °C	75 °C	85 °C	95 °C
IC_{50} (μg mL^{-1})	0.34	0.52	2.44	11.74	11.63

[a] IC_{50} values are presented.

To evaluate the effect of glycation of β-LG on IgE binding, the mean IgE binding to glycated β-LG was compared with IgE binding to β-LG incubated at 60 °C for 72 h in the absence of sugars (no glycation) (**Figure 7B**). The significance of differences between medians was tested by the Kruskal−Wallis test ($\alpha = 0.05$). The binding of patient's IgE on β-LG-Lac, β-LG-Gal, β-LG-Glu, and β-LG-Rha (means of 99, 98, 95, and 74%, respectively) was not significantly different from the IgE binding to β-LG heated at 60 °C for 72 h. IgE binding to β-LG-Ara and β-LG-Rib was significantly lower. The obtained values represent 50 and 24%, respectively, of the value obtained with control β-LG. The difference between these two values was statistically irrelevant.

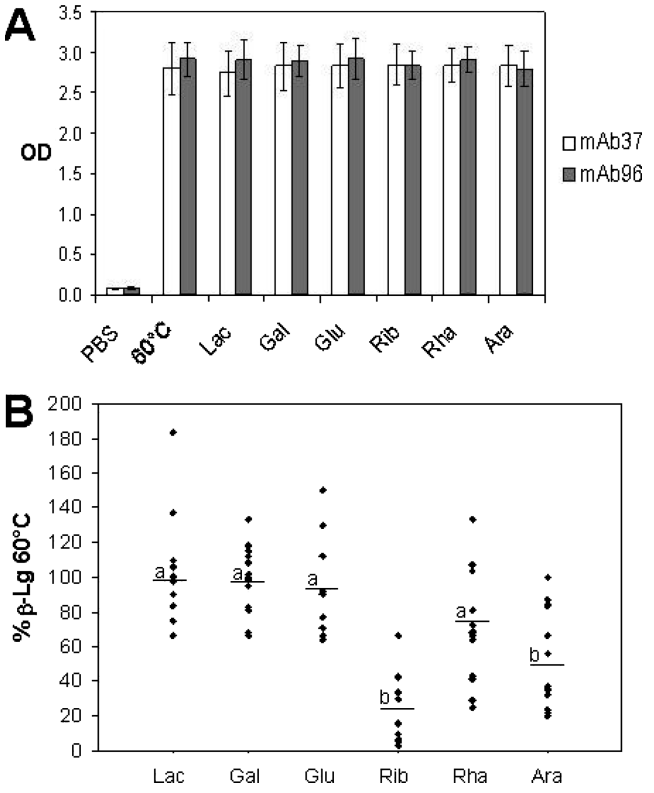

Figure 7. (**A**) Adsorption of glycated β-LG on ELISA plate: signal from the binding (OD) of two anti-β-LG monoclonal antibodies in wells coated with β-LG heated for 72 h at 60 °C and β-LG glycated with lactose (Lac), galactose (Gal), glucose (Glu), ribose (Rib), rhamnose (Rha), and arabinose (Ara). (**B**) IgE binding on glycated β-LG: binding of IgE from 14 CMA patients on β-LG glycated with lactose (Lac), galactose (Gal), glucose (Glu), ribose (Rib), rhamnose (Rha), and arabinose (Ara). Results are expressed as the percentage of the signal obtained by ELISA on β-LG heated for 72 h at 60 °C in the absence of sugar (control). The mean is indicated by an horizontal black bar. Identical letters indicate that means are not significantly different at a level of 5%.

Results obtained by indirect ELISA were confirmed by IgE-binding inhibition experiments using the pool of sera previously described (**Figure 8**). The standard deviation was not reported on the graph because it was always < 10%. The calculated IC_{50} value was 2.12 μg mL^{-1} for β-LG heated for 72 h at 60 °C and 2.50 μg mL^{-1} for lactosylated β-LG (**Table 2**). This difference was not significant. A considerable increase of the IC_{50} ($\sim$100 μg mL^{-1}) was observed in the case of ribosylated β-LG, indicating that the recognition of β-LG by IgE from CMA patients is strongly impaired by a high degree of glycation, especially with ribose. Because of the too low amount of available sera, the other glycated β-LGs were not assayed.

DISCUSSION

The binding of IgE from CMA patients to heat-modified β-LG is presented in this study. For nearly 70% of CMA patients sensitized to β-LG, it was observed that moderate heating of β-LG at 75 °C caused a decrease in its recognition by IgE. The maximal effect was observed between 85 and 95 °C (**Figure 4B** and **Figure 6**). This is consistent with the observation of Ehn et al. (*48*) who found, using pools of sera but not individual sera, that heating of β-LG at 74 °C caused a significant decrease in IgE binding, more evident after heating at 90 °C (*48*). Because the denaturation (modification of secondary and tertiary structures) of β-LG begins at 70−72 °C, it seems that its consecutive

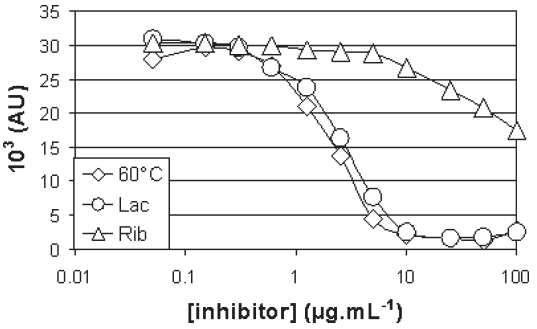

Figure 8. Inhibition of anti-β-LG IgE in serum pool. Comparison between β-LG heated for 72 h at 60 °C in the absence of sugar and in the presence of lactose (Lac) or ribose (Rib). The signal (AU) resulting from the binding of IgE on native β-LG coated plates is shown.

Table 2. Inhibition of IgE Binding to Native β-LG by β-LG Heated for 72 h at 60 °C without the Presence of Sugar or with Lactose or Ribose[a]

	β-LG treatment		
	60 °C	lactose	ribose
IC_{50} (μg mL^{-1})	2.12	2.50	~100

[a] IC_{50} values are presented.

aggregation is responsible for the disappearance of conformational epitopes and, consequently, of reduced binding. Reorganization of protein structure and/or aggregation of β-LG may have a masking effect on the recognition of sequential (or linear) epitopes. This should explain, at least partially, why some CMA patients are more tolerant to boiled milk than to raw milk. Our results are also consistent with the data obtained by Rytkönen et al. (*36*) who observed that rats sensitized with heat-denatured β-LG produce less specific IgE than rats sensitized with native β-LG, despite the fact that it induces a more intense inflammatory response. According to these results, it is clear that heat-induced denaturation of β-LG and/or aggregates generated by heat treatments at moderately high temperatures induced a more or less pronounced decrease of β-LG recognition by IgE from CMA patients. None of the studied sera from allergic patients showed a stronger IgE response against denatured β-LG, indicating that this protein does not contain internal masked linear epitopes becoming exposed only after denaturation.

We found that all of the patients do not react identically to heated β-LG. Mild reduction of IgE binding associated with β-LG heating was observed for the majority (60%) of the patients studied (**Figures 4B** and **Figure 5**). Nevertheless, it was found that one serum among 17 (6% of patients) recognizes only native β-LG and β-LG heated to 65 °C. This patient serum recognizes most likely conformational epitope(s), which are damaged by heating. It appears that in the case of a few sera, heating of β-LG to temperatures higher than 75 °C abrogate near totally its recognition by IgE. Surprisingly, the measured IgE concentration by the β-LG-specific Phadia ImmunoCAP System (F77) was < 0.35 kIA L^{-1}, suggesting that patients sensitized only with native β-LG are not well-detected by this assay. It should be underlined that β-LG adsorbed on the surface of the ImmunoCAP System is probably not in its native state any more. Consequently, for a better diagnosis, it would be important to ensure the use of native β-LG or execute them in solution and not adsorbed on any surface.

Glycation of proteins by the Maillard reaction occurred when they were incubated with the sugar at 60 °C for 72 h. This reaction is slow at room temperature, but its yield increases with temperature. The resulting products are responsible for the browning of

Table 3. Comparison between the Glycation Degree of β-LG and the IgE Binding[a]

sugar	glycation degree (%)	modified amino groups	IgE binding (%)
no	6.2	1.0	100
lactose	34.4	5.5	98
galactose	41.9	6.7	99
glucose	41.2	6.6	95
ribose	69.4	11.1	24
rhamnose	40.6	6.5	74
arabinose	55.0	8.8	50

[a] One hundred percent of binding corresponds to the IgE binding on β-LG heated for 72 h at 60°C in the absence of sugar.

cooked food. Because milk contains a high amount of lactose and because milk proteins are also present in multiple food preparations containing free reducing sugars, milk proteins, including β-LG, are susceptible to glycations. The results presented in this study show that low or moderate glycation of β-LG has no effect on its recognition by IgE, whereas the strongest substitution rates are associated with a decreased recognition of β-LG by IgE (**Figures 6A** and **8** and **Table 3**). This result can be explained by a "masking" effect due to sugars. Modification of β-LG occurs mainly on lysyl residues. All major and minor β-LG epitopes (*49*) contain one or more lysyl residues, and some of them have been identified as critical for IgE binding (e.g., K_{75}, K_{83}, K_{135}, K_{138}, and K_{141}) (*50*). It is clear that substitution of lysyl residues contained in the epitopes weakens or prevents IgE binding. When β-LG is substituted at a low rate, binding to epitopes is not much affected since remaining in the standard error range; hence, they are hardly detectable (**Figures 6** and **8** and **Table 3**). However, when β-LG is highly substituted (with important percentages of lysyl ε-amino groups glycated), IgE encounters less unhindered epitopes, and their binding to the protein is either decreased or nonexistent. In the early 1980s, Otani and Tokita (*51*) found that antigenicity (IgG binding) of heated β-LG decreased when the temperature increased and that this effect was less in the presence of lactose, suggesting that lactosylation by the Maillard reaction increased the antigenicity of denatured β-LG. They also showed that the sugar moiety linked to β-LG can act as a neo-epitope generated during the browning reaction (*52*). The fact that glycated β-LG did not show any increase of IgE binding as compared to the control β-LG indicates that, in the conditions used, the glycation does not lead to the formation of neo-epitopes even stronger recognized by the studied IgE. This apparent discrepancy can be explained by the fact that Otani and Tokita (*51*) used to work with immunized animal, whereas we worked with human sera. This induced two kinds of important sources of variability: (1) Otani and Tokita studied only β-LG-specific IgG epitopes, whereas we studied IgE epitopes, which are different, and their differences in the case of β-LG are well-known (*49*); (2) additionally, the epitopes recognized by animal immune systems are often different from those recognized by the humans. Moreover, the experimental conditions used by Otani and Tokita (*52*) for glycosylation lead to a more AMPs since they refer to browning products. In the present study, lactosylated β-LG was not brown. There are several investigations of the effect of Maillard reaction on protein allergenicity (*15*−*20*). The effects of allergen glycation are variable according to the allergen tested, the sugars used, and the stage of the Maillard reaction. No general effect on IgE binding could be seen. However, only scarce data about the effect of Maillard reaction are available. Consequently, it is not surprising that glycated β-LG shows a lower allergenicity than nonglycated β-LG as it was observed in the case of Pru av 1, the major allergen from cherry (*16*) and squid tropomyosin (*19*).

Despite of these in vitro allergenicity data, Bleumink and Berrens (*52*) observed in the 1960s that incubation of β-LG at 50 °C in the presence of lactose increases 100 times its skin reactivity. In this work, β-LG was heated from 48 to 216 h. The authors did not describe precisely what preparation was used for the intradermal reaction. They probably used β-LG samples more intensively modified than those used in our study, quite probably containing AGE. Additionally, in vivo skin reactivity involves other complex mechanisms than IgE binding on the allergen. It is possible that AGE present in the reaction medium affects these mechanisms, increasing the overall inflammation and allergic reactions without affecting IgE binding. Otani and Tokita (*52*) have reported contradictory results since they found that lactosylated β-LG was able to strongly inhibit the passive coetaneous anaphylaxis test. This result suggests lower allergenicity of glycated β-LG as compared to native β-LG. The human in vivo allergenicity of different glycated β-LG remains to be carefully evaluated.

In conclusion, for most studied sera of the CMA patients, a moderate heat treatment and glycation of β-LG during the moderate stages of the Maillard reaction do not have a drastic influence on the recognition of the protein by IgE. Nevertheless, in the case of rare individuals, heat treatment of β-LG can neutralize near totally its recognition by IgE.

ABBREVIATIONS USED

AGEs, advanced glycated end products; AMPs, advanced Maillard reaction products; AUs, arbitrary units; β-LG, β-lactoglobulin; β-LG-Lac, β-LG-lactose; β-LG-Rib, β-LG-ribose; β-LG-Glu, β-LG-glucose; β-LG-Gal, β-LG-galactose; β-LG-Ara, β-LG-arabinose; β-LG-Rha, β-LG-rhamnose; CMA, cow's milk allergy; CD, circular dichroism; C-ELISA, colorimetric enzyme-linked immunosorbent assay; F-ELISA, fluorescent enzyme-linked immunosorbent assay; OPD, *ortho*-phenylene diamine; 4-MUP, 4-methylumbelliferyl phosphate; SD, standard deviation; WHO, World Health Organization.

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LITERATURE CITED

(1) Vieths, S. Allergenic cross-reactivity, food allergy and pollen. *ETAP-Environ. Toxicol. Pharmacol.* **1997**, *4*, 61–70.
(2) Jäer, L.; Wüthrich, B. *Food Allergies and Intolerances* (in German); 1st ed.; Gustav Fischer Verlag: Ulm, Germany, 1998.
(3) Caballero, T.; Martin-Esteban, M. Association between pollen hypersensivity and edible vegetable allergy: A review. *J. Invest. Allergol. Clin. Immunol.* **1998**, *1*, 6–16.
(4) Vieths, S.; Scheurer, S.; Ballmer-Weber, B. K. Current understanding of cross-reactivity of food allergens and pollen. *Ann. N. Y. Acad. Sci.* **2002**, *964*, 47–68.
(5) FAO. Evaluation of allergenicity of genetically modified foods. Report from FAO/WHO expert consultation on foods derived from biotechnology, Rome, January 22−25, 2001.
(6) Sampson, H. A. Food allergy: Immunopathogenesis and clinical disorders. *J. Allergy Clin. Immunol.* **1999**, *103*, 717–728.
(7) Dubuisson, C.; La Vielle, S.; Martin, A. Allergies alimentaires: État des lieux et propositions; *Agence Française de Sécurité Sanitaire des Aliments Report*, France, 2002.
(8) Docena, G. H.; Fernandez, R.; Chirdo, F. G.; Fossati, C. A. Identification of casein as the major allergenic and antigenic protein of cow's milk. *Allergy* **1996**, *51*, 412–416.

(9) Natale, M.; Bisson, C.; Monti, G.; Peltran, A.; Garoffo, L. P.; Valentini, S.; Fabris, C.; Bertino, E.; Coscia, A.; Conti, A. Cow's milk allergens identification by two-dimensional immunoblotting and mass spectrometry. *Mol. Nutr. Food Res.* **2004**, *48*, 363–369.
(10) Gaudin, J.-C.; Rabesona, H.; Choiset, Y.; Yeretssian, G.; Chobert, J.-M.; Sakanyan, V.; Drouet, M.; Haertlé, T. Assessment of the immunoglobulin E-mediated immune response to milk-specific proteins in allergic patients using microarrays. *Clin. Exp. Allergy* **2008**, *38*, 686–693.
(11) Davis, P. J.; Smales, C. M.; James, D. C. How can thermal processing modify the antigenicity of proteins?. *Allergy* **2001**, *56*, 56–60.
(12) Maillard, L. C. Action des amines sur les sucres: formation des mélanoïdines par voie méthodique. *C. R. Acad. Sci.* **1912**, *154*, 66–68.
(13) Ames, J. M. Control of the Maillard reaction in food systems. *Trends Food Sci. Technol.* **1990**, *1*, 150–154.
(14) Chuyen, N. V. Maillard reaction and food processing. Application aspects. *Adv. Exp. Med. Biol.* **1998**, *434*, 213–235.
(15) Chung, S. Y.; Champagne, E. T. Association of end-product adducts with increased IgE-binding of roasted peanuts. *J. Agric. Food Chem.* **2001**, *49*, 3911–3916.
(16) Gruber, P.; Vieths, S; Wangorsch, A.; Nerkamp, J.; Hofmann, T. Maillard reaction and enzymatic browning affect the allergenicity of Pru av 1, the major allergen from cherry (*Prunus avium*). *J. Agric. Food Chem.* **2004**, *52*, 4002–4007.
(17) Mondoulet, L.; Paty, E.; Drumare, M. F.; Ah-Leung, S.; Scheinmann, P.; Willemot, R. M.; Wal, J. M.; Bernard, H. Influence of thermal processing on the allergenicity of peanut proteins. *J. Agric. Food Chem.* **2005**, *53*, 4547–4553.
(18) Nakamura, A.; Watanabe, K.; Ojima, T.; Ahn, D. H.; Saeki, H. Effect of Maillard reaction on allergenicity of scallop Tropomyosin. *J. Agric. Food Chem.* **2005**, *53*, 7559–7564.
(19) Nakamura, A.; Sazaki, F.; Watanabe, K.; Ojima, T.; Ahn, D. H.; Saeki, H. Changes in allergenicity and digestibility of squid tropomyosin during Maillard reaction with ribose. *J. Agric. Food Chem.* **2006**, *54*, 9529–9534.
(20) Nakamura, S.; Suzuki, Y.; Ishikawa, E.; Yakushi, T.; Jing, H.; Miyamoto, T.; Hashizume, K. Reduction of *in vitro* allergenicity of buckwheat Fag e 1 through the Maillard-type glycosylation with polysaccharides. *Food Chem.* **2008**, *109*, 538–545.
(21) Fogolari, F.; Ragona, L.; Zetta, L.; Romagnoli, S.; De Kruif, K. G.; Molinari, H. Monomeric bovine beta-lactoglobulin adopts a beta-barrel fold at pH 2. *FEBS Lett.* **1998**, *436*, 149–154.
(22) Qin, B. Y.; Bewley, M. C.; Creamer, L. K.; Baker, H. M.; Baker, E. N.; Jameson, G. B. Structural basis of the Tanford transition of bovine β-lactoglobulin. *Biochemistry* **1998**, *37*, 14014–14023.
(23) Forge, V.; Hoshino, M.; Kuwata, K.; Arai, M.; Kuwajima, K.; Batt, C. A.; Goto, Y. Is folding of beta-lactoglobulin non-hierarchic? Intermediate with native-like beta-sheet and non-native alpha-helix. *J. Mol. Biol.* **2000**, *296*, 1039–1051.
(24) Qin, B. Y.; Creamer, L. K.; Baker, E. N.; Jameson, G. B. 12-Bromododecanoic acid binds inside the calyx of bovine beta-lactoglobulin. *FEBS Lett.* **1998**, *438*, 272–278.
(25) Wu, S.-Y.; Perez, M. D.; Puyol, P.; Sawyer, L. β-Lactoglobulin binds palmitate within its central cavity. *J. Biol. Chem.* **1999**, *274*, 170–174.
(26) Kontopidis, G.; Holt, C.; Sawyer, L. The ligand-binding site of bovine beta-lactoglobulin: Evidence for a function?. *J. Mol. Biol.* **2002**, *318*, 1043–1055.
(27) McKenzie, H. A.; Sawyer, W. H. Effect of pH on β-lactoglobulins. *Nature (London)* **1967**, *214*, 1101–1104.
(28) Sakurai, K.; Goto, Y. Manipulating monomer-dimer equilibrium of bovine β lactoglobulin by amino acid substitution. *J. Biol. Chem.* **2002**, *277*, 25735–25740.
(29) Tanford, C.; Nozaki, Y. Physico-chemical comparison of β-lactoglobulins A and B. *J. Biol. Chem.* **1959**, *234*, 2874–2877.
(30) Iametti, S.; Gregori, B.; Vecchio, G.; Bonomi, F. Modifications occur at different structural levels during the heat denaturation of β-lactoglobulin. *Eur. J. Biochem.* **1996**, *237*, 106–112.
(31) de la Fuente, M. A.; Singh, H.; Hemar, Y. Recent advances in the characterisation of heat-induced aggregates and intermediates of whey proteins. *Trends Food Sci. Technol.* **2002**, *13*, 262–274.
(32) Hoffmann, M. A. M.; van Mil, P. J. J. M. Heat-induced aggregation of β-lactoglobulin: Role of the free thiol group and disulfide bonds. *J. Agric. Food Chem.* **1997**, *45*, 2942–2948.
(33) Prabakaran, S.; Damodaran, S. Thermal unfolding of β-lactoglobulin: Characterization of initial unfolding events responsible for heat-induced aggregation. *J. Agric. Food Chem.* **1997**, *45*, 4303–4308.
(34) Chen, W. L.; Liu, W. T.; Yang, M. C.; Hwang, M. T.; Tsao, J. H.; Mao, S. J. T. A novel conformation-dependent monoclonal antibody specific to the native structure of β-lactoglobulin and its application. *J. Dairy Sci.* **2006**, *89*, 912–921.
(35) Kleber, N.; Hinrichs, J. Antigenic response of beta-lactoglobulin in thermally treated bovine skim milk and sweet whey. *Milchwissenschaft* **2007**, *62*, 121–124.
(36) Rytkonen, J.; Karttunen, T. J.; Karttunen, R.; Valkonen, K. H.; Jenmalm, M. C.; Alatossava, T.; Bjorksten, B.; Kokkonen, J. Effect of heat denaturation on beta-lactoglobulin-induced gastrointestinal sensitization in rats: Denatured beta-LG induces a more intensive local immunologic response than native β-LG. *Pediatr. Allergy Immunol.* **2002**, *13*, 269–277.
(37) Adel-Patient, K.; Nahori, M.-A.; Proust, B.; Lapa e Silva, J. R.; Creminon, C.; Wal, J.-M.; Vargaftig, B. B. Elicitation of the allergic reaction in beta-lactoglobulin-sensitized Balb/c mice: Biochemical and clinical manifestations differ according to the structure of the allergen used for challenge. *Clin. Exp. Allergy* **2003**, *33*, 376–385.
(38) Mailliart, P.; Ribadeau-Dumas, B. Preparation of β-lactoglobulin and β-lactoglobulin-free proteins from whey retentate by NaCl salting out at low pH. *J. Food Sci.* **1988**, *53*, 743–745.
(39) Chevalier, F.; Chobert, J. M.; Mollé, D.; Haertlé, T. Maillard glycation of β-lactoglobulin with several sugars: Comparative study of the properties of the obtained polymers and of the substituted sites. *Lait* **2001**, *81*, 651–666.
(40) Chevalier, F.; Chobert, J. M.; Dalgalarrondo, M.; Choiset, Y.; Haertlé, T. Maillard glycation of beta-lactoglobulin induces conformation changes. *Nahrung* **2002**, *46*, 58–63.
(41) Frister, H.; Meisel, H.; Schlimme, E. OPA method modified by use of *N*,*N*-dimethyl-2-mercaptoethylammonium chloride as thiol component. *Fresenius Z. Anal. Chem.* **1988**, *330*, 631–633.
(42) Venien, A.; Levieux, D.; Astier, C.; Briand, L.; Chobert, J.-M.; Haertlé, T. Production and epitopic characterization of monoclonal antibodies against bovine β-lactoglobulin. *J. Dairy Sci.* **1997**, *80*, 1977–1987.
(43) Manderson, G. A.; Creamer, L. K.; Hardman, M. J. Effect of heat treatment on the circular dichroism spectra of bovine β-lactoglobulin A, B, and C. J. Agric. *Food Chem.* **1999**, *47*, 4557–4567.
(44) Strickland, E. H. Aromatic contributions to circular dichroism spectra of proteins. *CRC Crit. Rev. Biochem.* **1974**, *2*, 113–175.
(45) Qin, B.; Bewley, M.; Creamer, L.; Baker, E.; Jameson, G. Functional implications of structural differences between variants A and B of bovine β-lactoglobulin. *Protein Sci.* **1999**, *8*, 75–83.
(46) Matsuura, J. E.; Manning, M. C. Heat-induced gel formation of β-lactoglobulin: A study on the secondary and tertiary structure as followed by circular dichroism spectroscopy. *J. Agric. Food Chem.* **1994**, *42*, 1650–1656.
(47) Qi, X. L.; Holt, C.; McNulty, D.; Clarke, D. T.; Brownlow, S.; Jones, G. R. Effect of temperature on the secondary structure of beta-lactoglobulin at pH 6.7, as determined by CD and IR spectroscopy: A test of the molten globule hypothesis. *Biochem. J.* **1997**, *324*, 341–346.
(48) Ehn, B.-M.; Ekstrand, B.; Bengtsson, U.; Ahlstedt, S. Modification of IgE binding during heat processing of the cow's milk allergen β-lactoglobulin. *J. Agric. Food Chem.* **2004**, *52*, 1398–1403.
(49) Järvinen, K.-M.; Chatchatee, P.; Bardina, L.; Beyer, K.; Sampson, H. A. IgE and IgG binding epitopes on alpha-lactalbumin and beta-lactoglobulin in cow's milk allergy. *Int. Arch. Allergy Immunol.* **2001**, *126*, 111–118.
(50) Cocco, R. R.; Jarvinen, K.-M.; Han, N.; Beyer, K.; Sampson, H. A. Mutational analysis of immunoglobulin E-binding epitopes of beta-casein and beta-lactoglobulin showed a heterogeneous pattern of critical amino acids between individual patients and pooled sera. *Clin. Exp. Allergy* **2007**, *37*, 831–838.

(51) Otani, H.; Tokita, F. Natural and secondary antigenicity of beta-lactoglobulin heated in the presence of lactose. *Jpn. J. Zootechnical Sci.* **1980**, *51*, 711–716.
(52) Otani, H.; Tokita, F. Contribution of the sugar moiety in the browning product between beta-lactoglobulin and lactose as an antigenic determinant. *Jpn. J. Zootechnical Sci.* **1982**, *53*, 344–350.

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β-Lactoglobulin Structure and Retinol Binding Changes in Presence of Anionic and Neutral Detergents

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Bovine β-lactoglobulin (β-LG) in vivo (in milks) has been found in complexes with lipids such as butyric and oleic acids. To elucidate the still unknown structure−function relationship in this protein, the structural changes of β-lactoglobulin variant A (β-LG A) in the presence of anionic surfactant such as sodium *n*-dodecyl sulfate (SDS) and in the presence of nonionic surfactant such as Triton X-100 have been investigated. Subsequently, the retinol binding by β-LG has been investigated in the presence of various amounts of these surfactants as its binding indicator. The results of UV−vis and fluorescence studies show a higher denaturating effect of SDS at acid pH that can be due to greater positive charges of β-LG at this pH indicating also the nonspecific hydrophobic interactions of Triton X-100 with β-LG at all studied pHs. Isothermal titration calorimetry (ITC) measurements indicate the endothermic nature of β-LG/SDS interactions and the exothermic nature of Triton X-100/β-LG interactions. The analysis of the binding data demonstrates the absence of considerable changes in retinol binding properties of β-LG in the presence of various amounts of these surfactants. This implies that surfactant binding does not change the conformation of β-LG in the regions defining the retinol-binding site.

KEYWORDS: β-Lactoglobulin; sodium *n*-dodecyl sulfate; Triton X-100; retinol; isothermal titration calorimetry; fluorimetry

INTRODUCTION

The interactions of proteins with surfactants have been studied extensively, since they are of great importance in a wide variety of industrial, biological, pharmaceutical, and cosmetic systems (*1*−*5*). The interactions between biopolymers and surfactants depend strongly on the type of biopolymer and surfactant as well as on medium and its physicochemical properties such as pH, ionic strength, and temperature (*6*−*14*). Protein−surfactant interactions often alter the stabilities of many proteins. An understanding of the mechanisms involved in protein−surfactant interactions provides a basis for the evaluation of protein stability and for rational strategies to optimize the applications of surfactants. In these studies, the globular protein β-lactoglobulin (β-LG), which is the major protein in the whey of ruminant milk (*15*) and binds retinol and its derivatives, was used. Its interactions with amphiphilic and hydrophobic ligands such as retinoids, long chain fatty acids, and surfactants were investigated by different methods. Although there was no evidence for sequential loosening of the protein structure (e.g., the α-helix displacement or unfolding exposing the hydrophobic, external face of the β-barrel), all the parameters examined in these studies were unlikely to be linked with such structural changes. Thus, partial structural unfolding of β-LG could not be excluded (*16*−*23*).

Bovine β-lactoglobulin (β-LG) is a major whey protein of bovine milk with known primary, secondary, and three-dimensional structures but with still unknown biological function(s) (*24*). All the structural data concerning β-LG suggest that this protein shall be classified in the superfamily of hydrophobic molecule transporters termed lipocalins (*25, 26*). Its polypeptide chain is composed of 162 amino acid residues including two disulfide bonds (Cys66−Cys160 and Cys109−Cys119) and one free cysteine (Cys121) (*24*). The molecule of β-LG is constituted by nine antiparallel β-strands and one α-helix (*25, 26*). The core of the β-LG molecule includes a structural motif similar to that found in retinol binding protein

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(RBP) (*26*). Like RBP, β-LG is able to bind a wide variety of hydrophobic molecules (*24, 27*).

When the retinol binding by β-lactoglobulin is considered, there is some circumstantial evidence of protrusion of a retinol hydroxyl group out of the binding site deduced from its susceptibility to an attack by dehydrogenase (*28*). This could also indicate the external placement of the retinol binding site on the β-LG molecule (*25*). β-LG is known to bind tightly, in vitro, one retinol molecule per monomer (*28*). Developments in structural studies of β-LG (*25, 26, 29*), retinol binding protein (*30*), and bilin binding protein (*31*) show that these hydrophobic molecule transporters share a three-dimensional structural pattern termed β-barrel. Unambiguous crystallographic data analysis (*30, 31*) indicates that the ligands of retinol binding protein and bilin binding protein are bound inside the calyx formed by the β-barrel. The exact placement of the binding sites in other proteins from this super family is less clear.

It has been suggested that in the case of β-LG and some other proteins close to their isoelectric points, cooperative binding with sodium *n*-dodecyl sulfate (SDS) can induce an association of the protein to form *n*-dodecyl sulfate-complexed aggregates (*32*). Electrophoretic measurements indicate that the binding of *n*-octylbenzene-*p*-sulfonate anions to β-LG occurs in three stages (*33*). Additional studies show that, at low concentrations of SDS, the β-LG complexes with amphiphilic ligands aggregate in solution, while this does not occur at higher SDS concentrations. Calorimetric measurements at pH below isoelectric pH represent an exothermic process. However, the extent of exothermicity decreased with increasing of pH (*34*).

It has been concluded from an unfolding study of β-LG by urea in the presence of SDS that stability of the β-LG increased in the presence of SDS suggesting that SDS may occupy the cavity of the β-barrel (binding pocket). However, this claim has not been proven by binding measurement of retinol (*23*).

The binding studies on a homologous series of *n*-alkylsulfonate ligands with β-LG were performed by Busti et al.; they conclude that these surfactants stabilize the monomeric structure of β-LG (*22*).

It has been shown that the interactions of anionic phospholipids with β-LG cause a structural reorganization of the elements of its structure accompanied by an increase in α-helical content and a loosening of the protein tertiary structure (*35*).

A study of the interactions of the mixtures of cationic−anionic surfactants with β-LG was also reported recently by Lu et al. (*36*). They concluded that the extent of interaction is dependent on the ratio of surfactants in the mixture. Despite these reports, there are no comprehensive studies on the structure−function relationship of β-LG in the presence of surfactants.

In the present study, the structural changes of β-LG have been investigated initially in the presence of SDS and Triton X-100 using various experimental techniques such as UV−vis, fluorescence, and isothermal titration calorimetry (ITC). Subsequently, the retinol binding properties of β-LG as its functional indicator was investigated in the presence of the various amounts of these surfactants using the spectrofluorimeter titration method. Comparison of the results allows for the determination of some aspects of the structure−function relationship of β-LG in the presence of these surfactants.

MATERIALS AND METHODS

Chemicals. β-Lactoglobulin variant A (β-LG A) was isolated from the milk of a homozygous cow by a salting out/in method according to Maillart and Ribadeau-Dumas precipitating the majority of whey protein when conserving uniquely β-LG in solution (*37*). Homogeneity of the protein preparation was assessed by high-performance gel permeation chromatography and SDS gel electrophoresis. The obtained preparations of β-lactoglobulin were over 98% pure. Sodium *n*-dodecyl sulfate (SDS), *N*-acetyl-L-tryptophanamide (NATA) and *trans*-retinol palmitate were purchased from Sigma Chemical Co. Triton X-100, glycine, ethanol, Na_2HPO_4, and NaH_2PO_4 were obtained from Merck Chemical Co. All the used reagents were of the highest degree of purity. All of the solutions were prepared using double-distilled water. The 50 mM glycine pH 2.0 and the 50 mM phosphate pHs 6.7 and 8.0 were used as buffers. All of solutions were used fresh after preparation. The concentrations of β-LG and retinol were determined from the optical density of prepared solutions using the extinction coefficients of 17 600 M^{-1} cm^{-1} at 280 nm and of 48 000 M^{-1} cm^{-1} at 330 nm, respectively (*18*).

Apparatus. Fluorescence measurements were performed using a RF-5000 Shimadzu spectrofluorimeter with a thermostatted cell compartment at 298 K. Isothermal titration calorimetry (ITC) was performed at 298 K on a thermal activity monitor calorimeter (Thermometrics AB, Järfälla, Sweden) equipped with a high performance titration unit and a nanowatt amplifier. The absorbance measurements were carried out using a Carry-500 Scan UV−vis-NIR double beam spectrophotometer, which is well equipped with a thermostatted cell compartment at 298 K.

Fluorescence Spectroscopy Measurements. Fluorescence spectroscopy is used to study the binding and the conformational changes of proteins exploiting the intrinsic fluorescence of tryptophan (Trp) residues, which are particularly sensitive to the changes of their microenvironments (*38*). During fluorescence measurements, the samples were put in quartz cuvettes of 1 cm optical path length. In typical experiments, 3.0 mL of β-LG solution was placed into the cuvette. Emission spectra were recorded after each addition of SDS and Triton X-100 stock solutions (10 mM) at 298 K. The excitation was performed at 280 nm, and the emitted light was recorded between 300 and 400 nm for SDS and between 285 and 385 nm in the case of Triton X-100 binding studies. The observed fluorescence intensities were corrected for dilution. The band slits for excitation and emission were 3 and 5 nm for SDS and 1.5 and 3 nm for Triton X-100, respectively.

During binding experiments, fluorescence spectra were recorded at 298 K and between 300 and 400 nm (excitation: 280 nm). The binding of retinol was measured following the decrease of protein tryptophan fluorescence at 330 nm.

The following procedure was used for titration of β-LG solutions or various [surfactant]/[β-LG] solutions, with retinol: 3 mL of β-LG solutions or various [surfactant]/[β-LG] solutions were placed in a cuvette, and small increments (1−5 μL) of the ligand solution were injected in the cuvette with a Hamilton syringe. The experiments were performed in 50 mM glycine at pH 2.0 and in 50 mM phosphate buffer at pHs 6.7 and 8.0. In order to eliminate the dilution of β-LG solution by the added ligand solution and tryptophan fluorescence changes induced by alcohol, a blank containing *N*-acetyl-L-tryptophan amide, NATA, solution titrated with ligand was monitored as described above. The fluorescence intensity changes of the blank were subtracted from fluorescence intensity measurements of the ligand/protein complexes for every considered titration point. The β-LG solutions were freshly prepared just before the measurements, and their absorbencies at 280 nm did not exceeded 0.1; to avoid inner filter effect, β-LG concentration was around 3 μM. The concentration of NATA has been selected in the way that it had the same absorbance at 280 nm as the studied β-LG solution.

Isothermal Titration Calorimetric Measurements. The nanowatt isothermal titration microcalorimeter was a calorimeter supported by Thermometric 2277, thermal activity monitor (Thermometrics AB, Järfälla, Sweden), controlled by Digitam 4.1 software. The instrument had an electrical calibration with a precision better than ± 1%. The reaction and reference cell of the calorimeter were made from stainless steel. This isothermal titration calorimeter was used to measure enthalpies of mixing at 298 K for the interaction of SDS at pH 6.7 and Triton X-100 at pHs 6.7 and 8.0 with β-LG. Aliquots (250 μL) of SDS or Triton X-100 solution (10 mM) were injected sequentially (10 μL in each injection) by a 250 μL Hamilton syringe controlled by a Thermometric 612 Lund pump into a 2600 μL reaction cell containing initially either buffer solution or β-LG solution. The concentration of

β-LG in the cell was around 3 μM. Each injection took 5 min, and there was an interval of 20 min between every successive injection. The solution in the reaction cell was stirred at a speed of 60 rpm. All the solutions were degassed before the measurements. All the experiments were carried out at least twice using freshly prepared samples, and the results are reported as the averages. The reproducibility of the enthalpy changes measured on a particular sample by ITC was acceptable with less than 10% of error. Data were analyzed using the proprietary software Digitam 4.1 supplied by Thermometric AB (Järfälla, Sweden).

UV−Vis Spectroscopy Measurements. During UV−vis spectroscopy measurements, 800 μL of β-LG solution was placed into the 1 cm optical path quartz cuvettes. The absorbance spectra were recorded between 250 and 350 nm after each addition of SDS and Triton X-100 stock solutions at 298 K. The observed absorbances were corrected for dilution. The β-LG solutions were freshly prepared just before the measurements.

Determination of the Apparent Dissociation Constants. The following procedure was used for the titration of β-LG solutions or various [surfactant]/[β-LG] solutions with retinol: 3 mL of protein solution or various [surfactant]/[β-LG] solution was placed in a cuvette and small increments of retinol solution were injected with a micropipette. The ethanol added with retinol during titration never exceeded 3% (v/v). Differences in fluorescence intensity at 330 nm (excitation at 280 nm) were monitored in order to measure apparent dissociation constants of β-LG and various [surfactant]/[β-LG] molar ratios with retinol. It was assumed that the change in the fluorescence depends on the amount of protein/ligand complex. The apparent dissociation constants were determined according to Cogan et al. using the following equation (*39*):

$$P_0\alpha = (L_0/n)(\alpha/(1-\alpha)) - K_d/n \quad (1)$$

where α is the fraction of free binding sites, L_0 is the total ligand concentration, and P_0 is the total protein concentration.

By plotting $P_0\alpha$ versus $\alpha/(1-\alpha)$, a straight line is obtained with an intercept of K_d/n and a slope of L_0/n, where K_d is the apparent dissociation constant and n is the apparent molar ratio of β-LG/retinol at saturation. The α is defined as the fraction of unoccupied binding sites on the protein molecules. The value of α was calculated for every desired point on the titration curve of fluorescence quenching intensity using the relationship

$$\alpha = (F - F_{min})/(F_0 - F_{min}) \quad (2)$$

where F represents the fluorescence intensity (corrected for blank) at a certain L_0, F_{min} represents the fluorescence intensity upon saturation of β-LG molecules, and F_0 is the initial fluorescence intensity without retinol.

RESULTS AND DISCUSSION

Influence of Various Surfactant Concentrations on the Structure of β-LG. The addition of SDS and Triton X-100 resulted in the enhancement of the β-LG fluorescence emission maximum. Particularly, in the case of studies of the Triton X-100/β-LG complex, the slightly blue shift of the emission maximum was observed. When ionic surfactant are added into β-LG solution, the ionic surfactant monomers first bind electrostatically to charged residues at the β-LG surface, and this binding induces an expansion of the β-LG structure. This expansion opens for interactions of the surfactant hydrophobic tails with the β-LG nonpolar globulin interior (nonspecific, cooperative binding). This leads to β-LG unfolding or aggregation and loss of its secondary structure. It has been reported that nonionic surfactants interact with proteins weaker than ionic surfactants do (*1*). The nonionic surfactants interact with the β-LG via hydrophobic interactions.

The β-LG molecule contains 3 Arg residues, 14 Lys, and 1 His, and these residues could be the binding sites of anionic surfactants. When SDS (anionic surfactant) was added to β-LG solution at pH 2.0, they could form precipitates between 30 and 35 of [SDS]/[β-LG] molar ratios because β-LG is positively charged at pH 2.0 (*15*). Such a behavior was observed in β-LG/SDS mixed solution at pH 6.7 at about 10-fold higher molar ratios [SDS]/[β-LG] between 300 and 310 because both SDS and β-LG are negatively charged at pH 6.7. These results are similar to what was already observed for other positively charged protein−surfactant systems (*36, 40*). At pH 2.0, β-LG is positively charged (the isoelectric point of β-LG is 5.2 (*15*)), so it can form precipitates with negatively charged SDS because of the formation of electrostatically neutral complexes of β-LG/SDS. At pH 6.7, when anionic surfactant SDS binds to β-LG, the β-LG/SDS complex remains negatively charged, so it precipitates at higher molar ratios due to unfolding and aggregation (the precipitation has been observed).

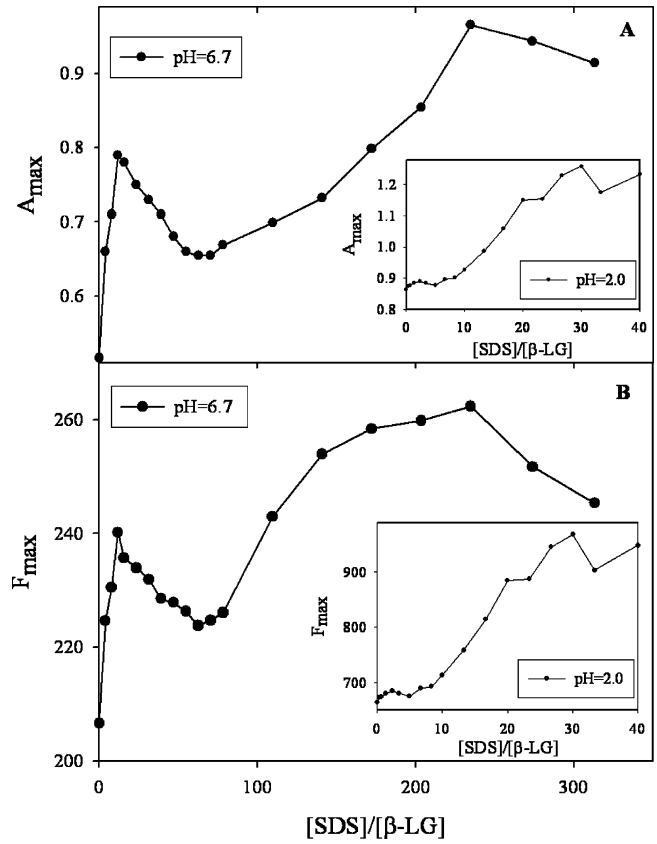

Figure 1. Variation in absorbance of β-LG at 280 nm (**A**) and changes in fluorescence emission maximum intensity of β-LG (**B**) vs molar ratio of [SDS]/[β-LG] in 50 mM glycine pH 2.0 and 50 mM phosphate buffer pH 6.7 at temperature of 298 K.

The nonionic surfactant Triton X-100 can bind to β-LG and change β-LG structure, with the effect weaker than anionic surfactant SDS do.

UV−Vis Absorption Spectra. UV−visible spectroscopy was also used to analyze the binding of SDS and Triton X-100 to β-LG. The absorption maximum of β-LG depends upon the microenvironment in which the probe is located albeit this dependence is significantly smaller than in the case of fluorescence measurements. The study of the absorbance of β-LG during its interactions with surfactants allows the determination of the micropolarity of the local environment surrounding the probe. **Figure 1A** shows the plot of the maximum of absorbance of β-LG versus molar ratio of [SDS]/[β-LG] in pHs 2.0 and 6.7 at 298 K. As shown in this figure, the overall structural changes are similar at both pHs. Two distinct conformational changes can be distinguished. First, transition is observed at

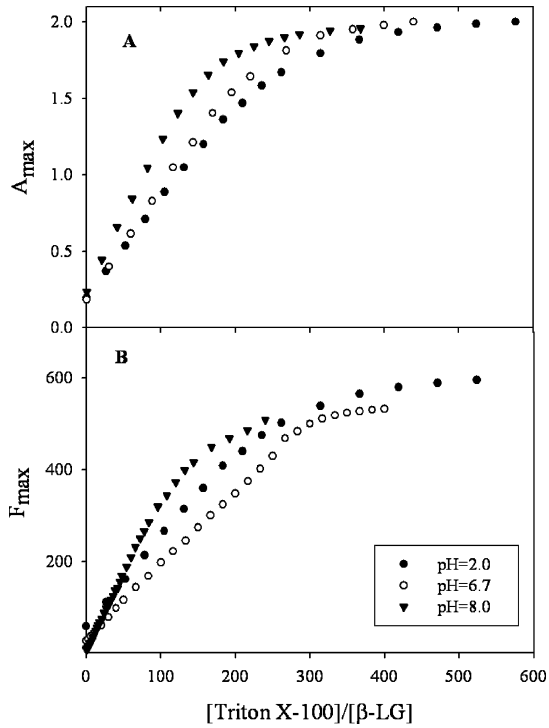

Figure 2. Changes in absorbance of β-LG at 280 nm (**A**) and changes in fluorescence emission maximum intensity of β-LG (**B**) vs molar ratio of [Triton X-100]/[β-LG] in 50 mM glycine pH 2.0 and 50 mM phosphate buffer pHs 6.7 and 8.0 at temperature of 298 K.

molar ratios of 4 and 10 at pHs 2.0 and 6.7, respectively. These points are corresponding to the first maximum in **Figure 1A**. The following conformational changes are beginning at molar ratios of 8 and 60 at pHs 2.0 and 6.7, respectively. It appears that the binding of initial surfactant ions to negatively charged sites on the surface of β-LG induces a conformational transition, which may be related to two different folding states. However, the second transition at higher molar ratio of SDS corresponds certainly to β-LG unfolding. Ultimately, the unfolded structures aggregate and precipitate. The following mechanism can be proposed for binding of SDS to β-LG:

$$F_1 \rightarrow F_2 \rightarrow U$$

where F_1 and F_2 are corresponding to two different folded states and U corresponds to the unfolded state.

Figure 2A shows the changes in maximum of absorbency of β-LG solution versus [Triton X-100]/[β-LG] molar ratios at various pH and at temperature of 298 K. No precipitation of β-LG was observed at any of the studied pHs. The cooperative character of binding is obvious at all studied pH. Analysis of the location of these transition curves shows that the binding strength decreases with increase of the pH. This fact can be related to the larger hydrophobic surface area of the β-LG/Triton X-100 complex at lower pH due to the predominant nature of hydrophobic interactions in the case of Triton X-100 binding. However, the two-step binding process that has been observed in the case of SDS binding was absent in the case of Triton X-100.

Fluorescence Spectra. The β-LG fluorescence depends on the microenvironment of the Trp residues of β-LG (*33, 41*). β-LG contains two Trp residues, Trp 19 and Trp 61 (*15*). **Figure 3** shows a ribbon diagram of a single unit of bovine β-LG. As it shown in this figure, Trp 19 is in an apolar environment in the main cavity of β-LG, whereas Trp 61 protrudes beyond the surface of the molecule and is quite close to the Cys 66−Cys

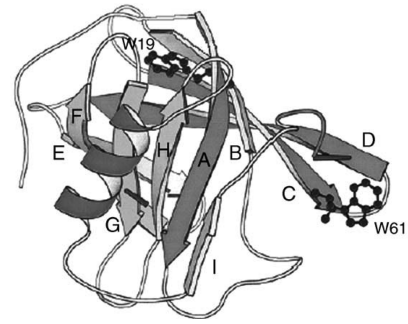

Figure 3. Ribbon diagram of a single unit of bovine β-LG. The locations of Trp19 and Trp61 are indicated.

160 disulfide bridge (*42, 43*). Since the disulfide bridges are effective Trp fluorescence quenchers, the intrinsic fluorescence of β-LG is almost exclusively attributed to Trp 19 (*21, 44*). The surfactant ions would cluster around the oppositely charged side chains of proteins; that is, anionic surfactants cluster around protonated Arg and Lys side chains, and cationic surfactants cluster around anionic Glu and Asp side chains (*1*). Analysis of the 3D structure of β-LG shows that, when SDS molecules bind to the residues of Arg and Lys, they are far away from Trp 61 and have little effect on Trp 61, so the fluorescence intensity changes are comparatively lower than in the case of Triton X-100 placed more evenly around the β-LG molecule.

Figure 1B shows the changes in maximum fluorescence intensity versus [SDS]/[β-LG] molar ratio at pHs 2.0 and 6.7 and 298 K. As shown in this figure, the variations of F_{max} versus the [SDS]/[β-LG] mole ratio are very similar to what is seen in case of absorbency measurements A_{max}. The maxima were observed at [SDS]/[β-LG] molar ratios of 3 and 9 at pHs 2.0 and 6.7, respectively. Nearly the same wavelength of maxima were observed for A_{max}. The precipitation of the complex was observed at molar ratios of 30 and 280 at pHs 2.0 and 6.7, respectively. These results agree well with UV−vis results of study of β-LG/SDS interactions.

It is known that the transfer of Trp from an aqueous to a hydrophobic environment leads to a blue shift in wavelength and to an increase in intensity of the emission maximum. This is the case of the results described here. An exposure to increasing concentrations of Triton X-100 in pH 2.0 induces a slight blue shift in the fluorescence emission maximum of Trp and a substantial increase in the fluorescence intensity (graphs are not shown). Similar behavior was observed also in the case of UV−vis absorbance in presence of this surfactant. The increase in the absorbance intensity indicates the perturbation of tertiary interactions quenching the otherwise β-LG fluorescence in the native conformation. As seen in the 3D structure of native β-LG, Trp 19, situated at the bottom of the calyx formed by eight antiparallel β-strands, contributes about 80% to the total fluorescence (*17*) and a disulfide bond quenches Trp 61 emission (*45*). Therefore, smaller quenching of Trp 61 by a disulfide bond, when β-LG binds to Triton X-100, leads probably to an increase in the fluorescence intensity of β-LG. It indicates also that the protein unfolds partially during its interaction with Triton X-100. A blue shift of the emission maxima indicates that at least part of the Trp residues is transferred/moved into a more hydrophobic environment during the interaction of β-LG with the Triton X-100. The same effect can be observed in other pHs. **Figure 2B** shows the changes in the maximum of the fluorescence intensity versus the [Triton X-100]/[β-LG] molar ratio at various pHs and 298 K. No precipitation of β-LG is observed at any of the studied pHs. Similarly to UV−vis measurements, the cooperative binding

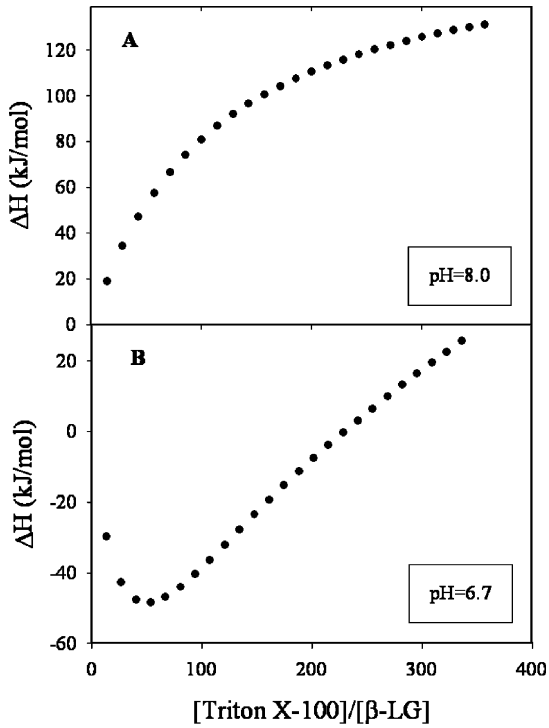

Figure 4. Variation of enthalpy of interaction vs [Triton X-100]/[β-LG] in 50 mM phosphate buffer at pHs 8.0 (**A**) and 6.7 (**B**) and at 298 K.

was observed that indicates the decrease of the strength of the binding with the increase of pH. Consequently, similar interpretation can be advanced in the case of the interpretation of obtained fluorescence data. Hence, all of these spectrophotometric results are coherent.

Isothermal Titration Calorimetric Study. One of the major factors influencing the electrostatic interactions of charged biopolymers in aqueous solutions is the pH, since it affects both the sign of the charge and its magnitude. Therefore, the effect of pH and surfactant type on the structure of β-LG was examined systematically in this study. The influence of surfactant concentrations at various pHs on the enthalpy changes associated with protein−surfactant interactions was studied using ITC when SDS or Triton X-100 solutions were added into β-LG solution (298 K, 50 mM phosphate buffer, pHs 6.7 and 8.0). We have investigated the changes in the enthalpy of the interactions of SDS with β-LG at pH 6.7 and at the temperature of 298 K (data not shown). The results indicate that the β-LG/SDS interaction is endothermic and shows a linear increase with increasing SDS concentrations starting about 10 kJ/mol at molar ratio 0 until about 100 kJ/mol at molar ratio of 350. This indicates the hydrophobic nature of interactions at this pH. β-LG at pH 6.7 is negatively charged, so its bulk electrostatic interactions are repulsive. In such a case, the hydrophobic interactions between the nonpolar tail of SDS and hydrophobic patches at the surface of β-LG should prevail and should be the major driving force of complex formation.

The dependence of ΔH_{int} versus the mole ratio of [Triton X-100]/[β-LG] at pH 6.7 is complex. As we can see in **Figure 4B**, a relatively high exothermic enthalpy change was observed below 50 molar ratio of [Triton X-100]/[β-LG]. Its exothermicity grows up and falls rapidly to about zero and mutates into endothermic enthalpy change at higher surfactant concentrations. Such behavior is not observed at pH 8.0. The curve of ΔH_{int} versus [Triton X-100]/[β-LG] (**Figure 4A**) shows an increase in endothermicity with increasing surfactant concentrations, and the saturation of complex formation at the surface of β-LG is obvious.

Table 1. Apparent Dissociation Constants (K_d) and Apparent Molar Ratio (n) of Retinol−β-LG Complexes in Molar Ratio of [Surfactant]/[β-LG] at Various pH and 298 K[a]

pH	surfactant	mole ratio of [surfactant]/[β-LG]	K_d ($\times 10^{-8}$ M)	n
2.0	SDS	0.0	7.24	0.79
		5.0	6.32	0.74
		10.0	6.04	0.69
		20.0	5.88	0.65
		30.0	4.95	0.63
	Triton X-100	4.6	9.73	0.81
		9.2	10.78	0.89
		13.7	11.61	0.93
		18.3	11.26	0.97
6.7	SDS	0.0	5.94	0.83
		5.0	6.28	0.85
		10.0	6.95	0.88
		20.0	7.20	0.92
		30.0	7.51	0.95
	Triton X-100	3.9	6.54	0.91
		7.9	7.21	0.94
		11.8	8.09	0.97
		15.7	8.94	0.99
8.0	Triton X-100	0.0	5.42	0.74
		3.6	6.98	0.77
		7.3	8.13	0.91
		17.2	9.86	0.96
		22.9	11.37	1.07

[a] Results expressed per protein monomer.

Although, it is usually difficult to assign precise molecular events to enthalpy changes measured in thermodynamic experiments because of the multiplicity of different physicochemical phenomena contributing to the measured signal (e.g., various kinds of association−disassociation processes and conformational changes), but the fact that the enthalpy changes observed during β-LG/Triton X-100 interactions in the pH range 6.7−8.0 were endothermic under some circumstances but exothermic under other circumstances suggests that at least two different physicochemical phenomena could occur in this pH range: (1) The single well-known conformational transition of β-LG at this pH range is a Tanford transition (*46*). (2) The dissociation of β-LG dimer to monomer at this pH range (β-LG is predominately dimeric at pH 6.7 and in monomer form at pH 8.0 (*47*).

Influence of Surfactant Concentration on Retinol Binding Properties of β-LG. The apparent binding constants and the apparent molar ratios of retinol/β-LG complexes are reported in **Table 1**. The β-LG/retinol complex at pH 8.0 displayed the smallest apparent dissociation constant ($K_d = 5.42 \times 10^{-8}$ M), and the association of retinol with β-LG was slightly weaker at pH 2.0 ($K_d = 7.24 \times 10^{-8}$ M) and pH 6.7 ($K_d = 5.94 \times 10^{-8}$ M).

The obtained results show that β-LG tryptophan fluorescence intensity decreases significantly when retinol is bound to β-LG. This agrees well with the previous results of Futterman and Heller (*28*) and Georghiou and Churchich (*48*). The apparent β-LG/retinol dissociation constants decrease between pHs 2.0 and 8.0. This observation does not support the suggestions of Fugate and Song (*16*) who claimed that the binding of retinol to β-LG is pH-independent in the pH range 2.0−7.5 but agrees with the results reported by Dufour et al. (*18*). In the case of this study, the obtained results show that the β-LG/retinol binding is pH-dependent. Bigger quenching by retinol of

tryptophan fluorescence at pHs 6.7 and 8.0 as compared with what is observed at acidic pH could indicate (if tryptophan−retinol excitation energy transfer contributes to the quenching phenomenon) that distances between tryptophan and retinol become shorter at neutral pH. All the obtained data suggest that β-LG conformation changes in the studied pH range and that its binding properties change with its ionization status (*18*).

The appearance of precipitation at a [SDS]/[β-LG] molar ratio of about 30 can be explained taking in to account the total net charge on β-LG at pH 2.0, which should be around +20. Hence, about 20 SDS anions would bind to β-LG at a molar ratio of 30. Such binding stoichiometry would cause the neutralization of charges on the protein and its precipitation. The analysis of retinol binding constants (n, K_d) as a function of SDS concentrations (**Table 1**) shows only a small increase in binding affinity. This can be related to the increase of the hydrophobic area of β-LG/SDS complexes and insignificant changes in the conformation of β-LG, especially around the calyx formed by the β-barrel. In such a case, the significant variation of the fluorescence emission spectra of β-LG due to interaction with SDS could be related to changes of the polarity of the microenvironment around the indol moiety of Trp 61 protruding on the surface of the protein. The binding of SDS to Arg^+ and Lys^+ residues has a greater influence on fluorescence quantum yield of this Trp. However, when taking in to account the results presented in **Figure 1B**, it seems that this fluorescence enhancement effect occurs in two distinct steps. Consequently, the occurrence of two local conformational changes due to binding of SDS is proposed. These local changes do not have great influence on the retinol binding site and consequently on retinol binding properties. The results of retinol binding studies in the presence of various SDS concentrations at pH 6.7 show slight increase in both n and K_d with the increase in SDS concentration. β-LG is dimeric at pH 6.7 while it is in predominately monomeric form at pH 2.0. Additionally, the binding affinity of SDS decreases with the rise of pH because of the increase of negative charge density and the decrease of electrostatic attraction between SDS anions and β-LG. These phenomena are probably the main causes of these observations.

The binding of Triton X-100 to hydrophobic patches on the β-LG surface is nonspecific. The hydrophobic interactions are the major driving forces of these interactions. Consequently, the increase of surfactant binding affinity with growth of pH can be related to increase of the area of hydrophobic clusters on β-LG at higher pH. The retinol binding affinity increases slightly with [Triton X-100]/[β-LG] molar ratio. This documents the inefficency of this surfactant as denaturant of β-LG. Additionally, it is clear that Triton X-100 cannot compete with retinol for binding site, due to its big size.

In the case of Triton X-100, at pH 2.0, both n and K_d increase with the increase in concentration of Triton X-100. The same is observed at other pHs in the case of β-LG retinol binding in the presence of various concentrations of this surfactant (**Table 1**). While Triton X-100 concentrations increase in solution, the hydrophobicity of β-LG medium increases, and the induced folding changes of β-LG in the presence of this surfactant increase the values of n and K_d. Comparison between n and K_d in the presence of SDS and Triton X-100 at various pHs shows that the number of observed independent binding sites for retinol (n) in the present of Triton X-100 is greater than in the presence of SDS. This may due to the increase of hydrophobicity of medium with rise of Triton X-100 molar ratio affecting hydrophobic interactions, which may cause the expansion of the β-LG molecule. The study of interactions of SDS and Triton X-100 with β-LG at various pHs shows the significant changes in tertiary structure of β-LG due to its interactions with SDS, which follows a two-step mechanism. Surprisingly, the retinol binding studies do not show considerable changes in the binding properties of β-LG during its simultaneous interactions with SDS. This could be explained by the stability of the conformation of the calyx or by the stability of the retinol binding site. Also, as shown by spectrometric studies, secondary structure of β-LG remains stable during these interactions.

The obtained results attest smaller denaturing activity of Triton X-100 in respect to tertiary structure of β-LG. However, despite all that, high affinity for Triton X-100 of β-LG reduces with pH increase. Insignificant effect of Triton X-100 on retinol binding of β-LG attests the noncompetitor rule of this surfactant with retinol for occupation of hydrophobic calyx. The obtained information about the binding of these popular surfactants can be useful in their application in the dairy food industry and in the formulation of functional surfactant complexes with other proteins of interest. This may also give some guidance as to what could be expected during interactions of charged and neutral lipids with β-LG and other food proteins.

ABBREVIATIONS USED

β-LG, β-lactoglobulin; SDS, sodium *n*-dodecyl sulfate; ITC, isothermal titration calorimetry; Trp, tryptophan; Cys, cysteine; Arg, arginine; Lys, lysine; Glu, glutamic acid; Asp, aspartic acid.

LITERATURE CITED

(1) Ananthapadmanabhan, K. P. In *Interactions of Surfactants with Polymers and Proteins*; Goddard, E. D., Ananthapadmanabhan, K. P., Eds.; CRC Press: Boca Raton, FL, 1993; p 319.

(2) Tanford, C.; Nozaki, Y.; Reynolds, J. A.; Makino, S. Molecular characterization of proteins in detergent solutions. *Biochemistry* **1974**, *13*, 2369–2376.

(3) Schwuger, M. J.; Bartnik, F. G. Interaction of anionic surfactants with proteins, enzymes and membranes. In *Anionic Surfactants−Biochemistry, Toxicology, Dermatology*; Gloxhuber, C., Ed.; Marcel Dekker Inc.: New York, 1980; pp 1−49.

(4) Jones, M. N.; Brass, A. Interactions between small amphipathic molecules and proteins. In *Food Polymers, Gels and Colloids*; Dickinson, E., Ed.; The Royal Society of Chemistry: Cambridge, U.K., 1991; pp 65−80.

(5) Jones, M. N. Surfactant interactions with biomembranes and proteins. *Chem. Soc. Rev.* **1992**, *21*, 127–136.

(6) Goddard, E. D.; Ananthapadmanabhan, K. P. Applications of polymer−surfactant systems. In *Polymer−Surfactant Systems*; Kwak, J. C. T., Ed.; Marcel Dekker Inc.: New York, 1998; Vol. 77, pp 22−64.

(7) Huang, Y. X.; Tan, R. C.; Li, Y. L.; Yang, Y. Q.; Yu, L.; He, Q. C. Effect of salts on the formation of C8-Lecithin micelles in aqueous solution. *J. Colloid Interface Sci.* **2001**, *236*, 28–34.

(8) Majhi, P. R.; Blume, A. Thermodynamic characterization of temperature-induced micellization and demicellization of detergents studied by differential scanning calorimetry. *Langmuir* **2001**, *17*, 3844–3851.

(9) Landeira, P. M.; Ruso, J. M.; Prieto, G.; Sarmiento, F. Surface tensions, critical micelle concentrations, and standard free energies of micellization of C_8-Lecithin at different pHs and electrolyte concentrations. *J. Chem. Eng. Data* **2002**, *47*, 1017–1021.

(10) Bordbar, A. K.; Sohrabi, N.; Hojjati, E. The estimation of the hydrophobic and electrostatic contributions to the free energy change upon cationic surfactants binding to Jack-bean urease. *Colloids Surf., B* **2004**, *39*, 171–175.

(11) Bordbar, A. K.; Hossainzadeh, R.; Omidiyan, K. Study on interaction of α-amylase from *Bacillus subtilis* with cetyl trimethyl ammonium bromide. *Coloids Surf., B* **2005**, *40*, 67–71.

(12) Bordbar, A. K.; Hossainzadeh, R. Binding of cetylpyridinum chloride to glucose oxidase. *Colloids Surf., B* **2006**, *53*, 288–295.
(13) Bordbar, A. K.; Taheri-Kafrani, A. Binding and fluorescence study on interaction of human serum albumin (HSA) with cetylpyridinium chloride (CPC). *Colloids Surf., B* **2007**, *55*, 84–89.
(14) Bordbar, A. K.; Taheri-Kafrani, A.; Mousavi, H.; Haertlé, T. Energetics of the interactions of human serum albumin with cationic surfactant. *Arch. Biochem. Biophys.* **2008**, *470*, 103–110.
(15) Wong, D. W. S.; Camirand, W. M.; Pavlath, A. E. Structures and functionalities of milk proteins. *Crit. Rev. Food Sci. Nutr.* **1996**, *36*, 807–844.
(16) Fugate, R. D.; Song, P. Spectroscopic characterization of β-lactoglobulin-retinol complex. *Biochim. Biophys. Acta* **1980**, *625*, 28–42.
(17) Cho, Y.; Batt, C. A.; Sawyer, L. Probing the retinol-binding site of bovine beta-lactoglobulin. *J. Biol. Chem.* **1994**, *269*, 11102–11107.
(18) Dufour, E.; Genot, C.; Haertlé, T. Beta-Lactoglobulin binding properties during its folding changes studied by fluorescence spectroscopy. *Biochim. Biophys. Acta* **1994**, *1205*, 105–112.
(19) Wang, Q.; Allen, J. C.; Swaisgood, H. E. Binding of retinoids to β-lactoglobulin isolated by bio selective adsorption. *J. Dairy Sci.* **1997**, *80*, 1047–1053.
(20) Pérez, M. D.; Dı´´az de Villegas, C.; Sanchez, L.; Aranda, P.; Ena, J. M.; Calvo, M. Interaction of fatty acids with beta-lactoglobulin and albumin from ruminant milks. *J. Biochem.* **1989**, *106*, 1094–1097.
(21) Frapin, D.; Dufour, E.; Haertlé, T. Probing the fatty acid binding site of beta-lactoglobulins. *J. Protein Chem.* **1993**, *12*, 443–449.
(22) Busti, P.; Scarpeci, S.; Gatti, C. A.; Delorenzi, N. J. Interaction of alkylsulfonate ligands with beta-lactoglobulin AB from bovine milk. *J. Agric. Food Chem.* **1999**, *47*, 3628–3631.
(23) Creamer, L. K. Effect of sodium dodecyl sulphate and palmitic acid on the equilibrium unfolding of bovine β-lactoglobulin. *Biochemistry* **1995**, *34*, 7170–7176.
(24) Hambling, S. G.; McAlpine, A. S.; Sawyer, L. In *Advanced Dairy Chemistry 1: Proteins*; Fox, P. F., Ed.; Elsevier Applied Science: London, 1992; pp 141−190.
(25) Monaco, H. L.; Zanotti, G.; Spadon, P.; Bolognesi, M.; Sawyer, L.; Eliopoulos, E. E. Crystal structure of the trigonal form of bovine beta-lactoglobulin and of its complex with retinol at 2.5 Å resolution. *J. Mol. Biol.* **1987**, *197*, 695–706.
(26) Papiz, M. Z.; Sawyer, L.; Eliopoulos, E. E.; North, A. C. T.; Findlay, J. B. C.; Sivaprasadarao, R.; Jones, T. A.; Newcomer, M. E.; Kraulis, P. J. The structure of β-lactoglobulin and its similarity to plasma retinol-binding protein. *Nature* **1986**, *324*, 383–385.
(27) Chobert, J. M.; Haertlé, T. In *Food Proteins and Their Applications*; Damodaran, S., Paraf, A., Eds.; Marcel Dekker Inc.: New York, 1997; pp 143−170.
(28) Futterman, S.; Heller, J. The enhancement of fluorescence and the decreased susceptibility to enzymatic oxidation of retinol complexed with bovine serum albumin, beta-lactoglobulin, and the retinol-binding protein of human plasma. *J. Biol. Chem.* **1972**, *247*, 5168–5172.
(29) Jayat, D.; Gaudin, J. C.; Chobert, J. M.; Burova, T.; Holt, C.; Mcnae, I.; Sawyer, L.; Haertlé, T. A recombinant C121S mutant of bovine beta-lactoglobulin is more susceptible to peptic digestion and to denaturation by reducing agents and heating. *Biochemistry* **2004**, *43*, 6312–6321.
(30) Newcomer, M. E.; Jones, T. A.; Aqvist, J.; Sundelin, J.; Eriksson, U.; Rask, L.; Peterson, P. A. The three-dimensional structure of retinol-binding protein. *EMBO J.* **1984**, *3*, 1451–1454.
(31) Huber, R.; Schneider, M.; Epp, O.; Mayr, I.; Messerschmidt, A.; Pflugrath, J.; Kayser, H. Crystallization, crystal structure analysis and preliminary molecular model of the bilin binding protein from the insect *Pieris brassicae*. *J. Mol. Biol.* 1987*195*, 423–434.
(32) Ray, A.; Chatterjoe, R. In *Conformation of Biopolymers*; Ramachandran, G. N., Ed.; Academic Press: London, 1967; pp 235−251.
(33) Hill, R. M.; Briggs, D. R. A study of the interaction of *n*-octylbenzene-*p*-sulfonate with β-lactoglobulin. *J. Am. Chem. Soc.* **1956**, *78*, 1590–1597.
(34) Jones, M. N.; Wilkinson, A. The interaction between β-lactoglobulin and sodium *n*-dodecyl sulphate. *Biochemistry* **1976**, *15*, 713–718.
(35) Liu, X.; Shang, L.; Jiang, X.; Dong, S.; Wang, E. Conformational changes of β-lactoglobulin induced by anionic phospholipids. *Biophys. Chem.* **2006**, *121*, 218–223.
(36) Lu, R. C.; Cao, A. N.; Lai, L. H.; Xiao, J. X. Interactions of β-lactoglobulin with sodium decylsulfonate, decyltriethylammonium bromide, and their mixtures. *J. Colloid Interface Sci.* **2006**, *299*, 617–625.
(37) Maillart, P.; Ribadeau-Dumas, B. Preparation of β-lactoglobulin and β-lactoglobulin-free proteins from whey retentate by NaCl salting out at low pH. *J. Food Sci.* **1988**, *33*, 743–752.
(38) Eftink, M. R. The use of fluorescence methods to monitor unfolding transitions in proteins. *Biophys. J.* **1994**, *66*, 482–501.
(39) Cogan, U.; Kopelman, M.; Mokady, S.; Shinitzky, M. Binding affinities of retinol and related compounds to retinol binding proteins. *Eur. J. Biochem.* **1976**, *65*, 71–78.
(40) Morén, A. K.; Khan, A. Phase behaviour and phase structure of protein-surfactant-water systems. *J. Colloid Interface Sci.* **1999**, *218*, 397–403.
(41) Cheung, H. C. Resonance energy transfer. In *Topics in Fluorescence Spectroscopy*; Lakowicz, J. R., Ed.; Plenum: New York, 1991; Vol. 2, pp 128−171.
(42) Qin, B. Y.; Bewley, M. C.; Creamer, L. K.; Baker, H. M.; Baker, E. N.; Jameson, G. B. Structural basis of the Tanford transition of bovine β-lactoglobulin. *Biochemistry* **1998**, *37*, 14014–14023.
(43) Qin, B. Y.; Creamer, L. K.; Baker, E. N.; Jameson, G. B. 12-Bromododecanoic acid binds inside the calyx of β-lactoglobulin. *FEBS Lett.* **1998**, *438*, 272–278.
(44) Manderson, G. A.; Hardman, M. J.; Creamer, L. K. Effect of heat treatment on bovine β-lactoglobulin A, B and C. Explored using thiol availability and fluorescence. *J. Agric. Food Chem.* **1999**, *47*, 3617–3627.
(45) Palazolo, G.; Rodriguez, F.; Farruggia, B.; Pico, G.; Delorenzi, N. Heat treatment of β-lactoglobulin: structural changes studied by partitioning and fluorescence. *J. Agric. Food Chem.* **2000**, *48*, 3817–3822.
(46) Tanford, C.; Bunville, L. G.; Nozaki, Y. The reversible transformation of β-lactoglobulin at pH 7.5. *J. Am. Chem. Soc.* **1959**, *81*, 4032–4036.
(47) Thompson, M. P.; Farrell, H. M. In *Genetic Variants of the Milk Proteins, Lactation: Nutrition and Biochemistry of Milk/Maintenance*; Larson, Smith, Eds.; Academic Press: New York, 1974.
(48) Georghiou, S.; Churchich, J. E. Nanosecond spectroscopy of retinol. *J. Biol. Chem.* **1975**, *250*, 1149–1151.

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Energetics of the interactions of human serum albumin with cationic surfactant

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Abstract

The heat capacity changes for interaction of human serum albumin (HSA) and a cationic surfactant—cetylpyridinium chloride (CPC), were studied at conditions close to physiological (50 mM HEPES or phosphate buffer, pH 7.4 and 160 mM NaCl) carrying out isothermal calorimetric titrations (ITC) at various temperatures (20–40 °C). ITC measurements indicated that the small endothermic changes associated with CPC demicellization were temperature independent at these conditions. Surprisingly, important enthalpy changes associated with binding of CPC to HSA were exothermic and temperature independent at lower concentrations (below 0.022 mM) of CPC and endothermic and temperature dependent at higher concentrations of CPC. The values of heat capacity changes were obtained for each studied concentration of CPC from the plot of enthalpy changes vs temperature. The obtained results demonstrate the temperature independence of heat capacity changes at entire range of studied CPC concentrations. Both enthalpograms and heat capacity curves indicate the two-step mechanism of HSA folding changes due to its interactions with CPC. The first step corresponds to transition from native state to partially unfolded state and the second to unfolding and to the loss of tertiary structure. The analysis of the results indicates that predominant cooperative unfolding occurs at CPC/HSA molar ratio region between 25 and 30. Such information could not be extracted from thermograms and describes the role of heat capacity as a major thermodynamic quantity giving insight on physical, mechanistic and even atomic-level into how HSA may unfold and interact with CPC. The effect of CPC binding on HSA intrinsic fluorescence, UV-Vis and CD spectra were also examined. Hence, the analysis of spectral data confirms the ITC results about the biphasic mechanism of HSA folding changes induced by CPC. The CD measurement also represents the conservation of considerable secondary structure of HSA due to interaction with CPC.

Keywords: Human serum albumin; Cetylpyridinium chloride; Isothermal titration calorimetry; Cationic surfactant; Unfolding; Heat capacity

Interactions of proteins with surfactants have been studied extensively for decades because of their importance in many biological, pharmaceutical and industrial systems [1–10]. The interactions between biopolymers and surfactants depend strongly on biopolymer and surfactant type, as well as on medium and its physico–chemical properties such as pH, ionic strength and temperature [11–19]. Protein–surfactant interactions often alter the stabilities of many proteins. An understanding of the mechanisms involved in protein–surfactant interactions provides a basis for the evaluation of protein stability and for rational strategies to optimize the applications of surfactants.

Several methods have been used to characterize protein–surfactant interactions. Isothermal titration calorimetry (ITC)[1] is one of valuable tools useful for this purpose.

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[1] *Abbreviations used:* HSA, human serum albumin; CPC, cetylpyridinium chloride; ITC, isothermal titration calorimetry; CTAB, cetyltrimethylammonium bromide; SDS, sodium dodecyl sulfate.

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Quite recently, high sensitive ITC has been used to obtain the complete ''interaction isotherms'' of protein–surfactant interactions. This implies titrations of the protein with high surfactant concentrations [19–25]. Studied proteins showed complex interactions isotherms, or enthalpograms, which were rich in information, but their analysis, were difficult. Each process involved will generate a characteristic thermodynamic ''fingerprint'' and this may include contributions from one or several equilibria-like binding, conformational changes in the proteins and the micellisation. Although, several different analytic methods were assayed to overcome this so complex problem [26], there is no a unified treatment proposed for the analysis of such systems. Hence, the introduction of new and more informative analytic method could be very useful.

Human serum albumin (HSA) is frequently used in biophysical and biochemical studies since it has a well-known primary structure and it binds different categories of small molecules. The role of HSA as the main carrier of free (non-esterified) fatty acids in the plasma has prompted a great interest in the studies of its structure and stability [27–30]. Human serum albumin is highly homologous to bovine serum albumin, known to be one of the allergenic proteins present in the milk. The interaction of HSA with surfactants has been studied by several authors [31–35]. The binding of cetyltrimethylammonium bromide (CTAB) to HSA was recently studied at neutral pH and at various ionic strengths using ion-selective membrane electrodes [36]. The existence of two and three binding sites on HSA for CTAB at various ionic strengths was reported in this study. A fluorescence study of interaction of HSA at pH 7.0 and 9.0 with anionic sodium dodecyl sulfate (SDS), zwitterionic *N*-hexadecyl-*N*,*N*-dimethyl-3-ammonium-1-propanesulfonate (HPS) and cationic cetyltrimethylammonium chloride (CTAC) was also performed and the first associative binding constants of these surfactants to HSA were determined [34]. The obtained results showed an enhancement of fluorescence efficiency of HSA at low concentration of the surfactant and partial loss of its secondary structure upon surfactant binding indicating high stability of HSA during such interactions. Recently, the binding of cetylpyridinium chloride (CPC) to HSA at various pHs has been studied using ion-selective membrane electrodes and fluorescence spectroscopy [37]. Cetylpyridinium chloride (CPC) is a mild antibacterial used often in the antibacterial protection in buccal treatments and also in food conservation [29,38]. The obtained results suggest the existence of two distinct classes of binding sites on HSA for CPC and the predominant role of hydrophobic interactions in occupation of both binding sites. This should be also compared with the available crystallographic data on the binding of lauric and myristic acids reminding a cetyl tail of CPC. In their cases up to 8 binding sites were detected.

In the present study, the binding of CPC to HSA at physiological conditions has been investigated by ITC at various temperatures in order to determine enthalpy and heat capacity changes. The obtained results can be very informative due to importance of heat capacity change as a major thermodynamic quantity that is one of the richest potential sources of information in physical terms. According to our knowledge, this report is one of the first on heat capacity changes for ionic surfactant–globular protein interactions using ITC. The obtained precise thermograms and heat capacity curves were interpreted in terms of molecular events such as specific and non-specific binding and the unfolding process. The conclusions of ITC experiments were confirmed by the results of fluorescence, UV-Vis and CD measurements.

Materials and methods

Materials

HSA (free fatty acid fraction V, purity > 97%) and cetylpyridinium chloride were purchased from Sigma Chemical Co and used without further purification. Sodium chloride and *N*-(2-hydroxyethyl) piperazine-*N*-(2-ethanesulfonic acid) (HEPES) buffer were obtained from Merck Chemical Co. All the used reagents were of highest degree of purity. All of the solutions were prepared using double distilled water. The 50 mM HEPES or 50 mM phosphate, pH 7.4, 160 mM NaCl was used as buffer. The HSA solutions were used freshly after preparation. The HSA concentrations were determined from the optical density of appropriate solutions using the extinction coefficient at 280 nm of 35,700 M^{-1} cm^{-1} [31]. The concentrations of CPC in reaction cell were below its cmc during all measurements.

Apparatus

The calorimetric measurements were carried out using MCS-ITC (MicroCal Inc., Northampton, MA, USA) isothermal titration calorimeter. Fluorescence measurements were performed using RF-5000 Shimadzu spectrofluorimeter with a thermostated cell compartment at 25 °C. The absorbance measurements were carried out using UV–Vis, Carry-500 double beam spectrophotometer. CD spectra were recorded from 190 to 350 nm on a Jobin Yvon CD Mark 6 spectropolarimeter in using a 0.01 cm cell at 0.5 nm intervals with three scans averaged for each CD spectra.

Isothermal titration calorimetric (ITC) experiments

An isothermal titration calorimeter (VP-ITC, Microcal, Inc., Northampton, MA) was used to measure enthalpies of mixing at 20.0, 25.0, 30.0, 35.0, and 40.0 °C. 210 μL aliquots of CPC solution (10 mM) were injected sequentially (5 μL in each injection) into a 1460 μL reaction cell initially containing either buffer solution or HSA solution. The concentration of HSA in the cell was 22.83 μM. Each injection lasted 5 s and there was an interval of 180 s between every successive injection. The solution in the reaction cell was stirred at a speed of 315 rpm throughout the experiments. All the solutions were degassed before the measurements. All the experiments were carried out at least twice using freshly prepared samples and the results are reported as the average. Typically, the reproducibility of the enthalpy changes measured on a particular sample by ITC was acceptable with less than 10% of error.

Fluorescence spectroscopy

In fluorescence measurements, the samples were put in quartz cuvettes of 1 cm optical path. In typical experiments 2.0 mL of HSA solution were placed into the cuvette. Emission spectra were recorded after each addition

of CPC stock solutions (1 mM) in the same buffer at pH 7.4 and 25 °C. The excitation was performed at 280 nm and the emitted light was recorded between 300 and 400 nm. The observed fluorescence intensities were corrected for dilution. The band slits for excitation and emission were 3 and 5 nm, respectively. The HSA solutions were freshly prepared just before the measurements and their absorbances at 280 nm did not exceeded 0.1, HSA concentration around 3 μM, to avoid inner filter effect.

Assessment of secondary structure chances by circular dichroism

The molar ratio of HSA to CPC was 1:0, 1:4, 1:10, and 1:30 were used for recording of CD spectra. The concentration of HSA in all CD experiments was 27.7 μM. All CD spectra were corrected for the blank, which consisted in the recording of CD spectra of the same concentration of CPC in buffer in absence of HSA [39].

UV–Vis absorption measurements

UV–Vis spectra were recorded between 250 and 350 nm on Carry-500 double beam spectrophotometer at 25 °C in a rectangular cuvette with 1 cm path-length. Temperature control was provided by a Le Peltier thermostat equipped with magnetic stirring. The concentration of HSA in UV–Vis experiments was 27.7 μM and all absorbance spectra were corrected for the blank, which consisted in the recording of UV–Vis spectra of the same concentration of CPC in buffer in absence of HSA.

Results and discussion

Influence of temperature on micellization of CPC

The cmc about 0.07 mM of the CPC in the buffer solution used in this study (pH 7.4, 160 mM NaCl, 50 mM HEPES) was determined using ITC at various temperatures (20–40 °C). Profiles of heat flow vs time resulting from sequential injections of 5 μL aliquots of surfactant solution (10 mM CPC) into a 1460 μL reaction cell initially containing the buffer solution were endothermic at all studied temperatures, however, the heights of the peaks grew with the temperature increase. The cause of this behavior is discussed further. In this case, initially, a series of relatively large endothermic peaks was observed when the CPC solutions were injected into the reaction cell. These enthalpy changes are the result of micelle dissociation because the surfactant concentration in the reaction cell was initially below the cmc. The endothermic nature of these peaks ($\Delta H > 0$) indicates that the simultaneous demicellization process must lead to an increase in the overall entropy of the system, because micelle dissociation is thermodynamically favorable below the cmc ($\Delta G < 0$); therefore, $T\Delta S > \Delta H$. This entropy increase has been attributed to the release of counter ions associated with the surfactant head groups and also to the formation of water cage around hydrocarbon tail of surfactant (hydrophobic effect) when micelles dissociate into monomers [40].

Fig. 1 shows the dependence of the enthalpy change per mole of CPC (ΔH/Δ[CPC]) on surfactant concentration in the reaction cell, which was calculated by integration of the heat flow vs time profiles at different temperatures. At relatively low CPC concentration, the enthalpy change per mole of CPC changed from relatively low endothermic

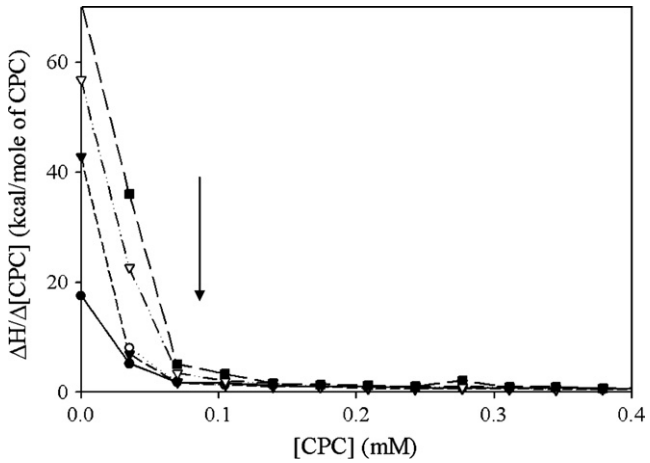

Fig. 1. Dependence of enthalpy change per mole of surfactant on the surfactant concentration in the reaction cell for 10 mM CPC injected into buffer at 20 °C (●), 25 °C (○), 30 °C (▼), 35 °C (▽) and 40 °C (■). The obvious distinct break corresponds to cmc.

(~70 kcal/mol) to about zero. A break is obvious in Fig. 1 what corresponds to cmc. In fact, the relatively large enthalpy changes observed at low CPC concentrations are due to demicellization of the surfactant injected to the reaction cell, but the relatively very small enthalpy changes at high CPC concentrations are due to micelle dilution effect which is negligible. The cmc was estimated from the results presented in Fig. 1 to be about 0.07 mM of CPC. In contrast with other literature reports the obtained cmc value is temperature independent within experimental error. This may be due to coincidence of the data, which are close to the extreme of plotted cmc temperature.

The enthalpy changes associated with the micelle dissociation were temperature dependent, but the cmc was temperature independent. At temperatures <~25 °C, the hydrophobic effect is exothermic, but at higher temperatures it is endothermic [41]. Hence demicellization involves exposure of non-polar groups to water. The increase of endothermic effect during demicellization with the temperature raise can be attributed to the hydrophobic effect. It appears that the enthalpy changes associated with micelle dilution are negligible and are independent of temperature, probably because of permanent exposure of non-polar groups to water.

Enthalpy of CPC binding to HSA at different temperatures

ITC experiments were carried out using surfactant solution in the injector and the HSA solution in the cell. The enthalpy change per mole of surfactant at each temperature was determined from sequential injections of 5 μL aliquots of CPC solution (10 mM) into 1460 μL reaction cell containing initially stock buffer solution (pH 7.4, 160 mM NaCl, 50 mM HEPES) or HSA solution (0.023 mM, pH 7.4, 160 mM NaCl, 50 mM HEPES). The plots of heat flow vs time profiles resulting from sequential injections of 5 μL aliquots of surfactant solution into reaction cell containing

HSA solution at 20 and 40 °C are shown in Fig. 2a and b, respectively. A similar trend is observed at both limiting temperatures but the endothermicity increased with temperature. The enthalpy change per mole of CPC in the absence and in the presence of HSA was calculated. The difference between these two calculated enthalpies should be equal to enthalpy of interaction between CPC ions and HSA (ΔH_{int}). The variation of ΔH_{int} vs molar ratio of CPC/HSA shows similar trends at all temperatures (Fig. 3). This suggests that interaction of CPC to HSA was highly exothermic at low molar ratio (about 4) and sharply becoming endothermic until molar ratios between 9 and 11 and then gradually becoming less endothermic. In the other words, an inflection point has been observed around this molar ratio at all studied temperatures. The endothermicity increased with the temperature. It is possible to interpret the observed profile of ΔH_{int} vs temperature in terms of enthalpy changes associated with the various physicochemical phenomena occurring, e.g., binding and folding changes of HSA. It has been reported that the binding of ionic surfactants to globular proteins begins initially

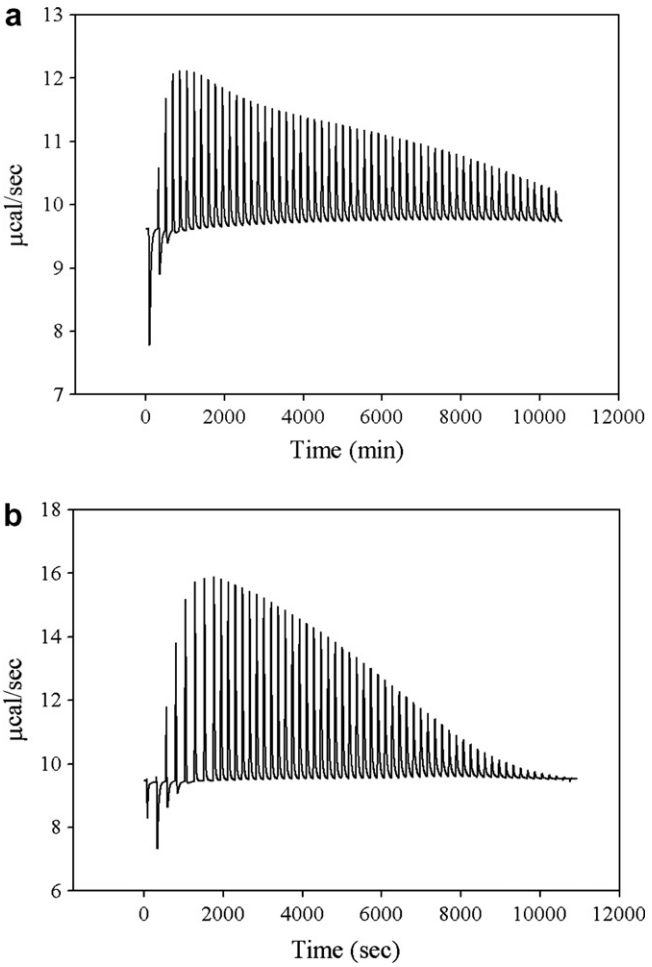

Fig. 2. Heat flow vs time profiles resulting from sequential injections of 5 μL aliquots of surfactant solution (10 mM CPC) into a 1460 μL reaction cell containing HSA solution at (a) 20 and (b) 40 °C. The concentration of HSA in the cell was 0.023 mM.

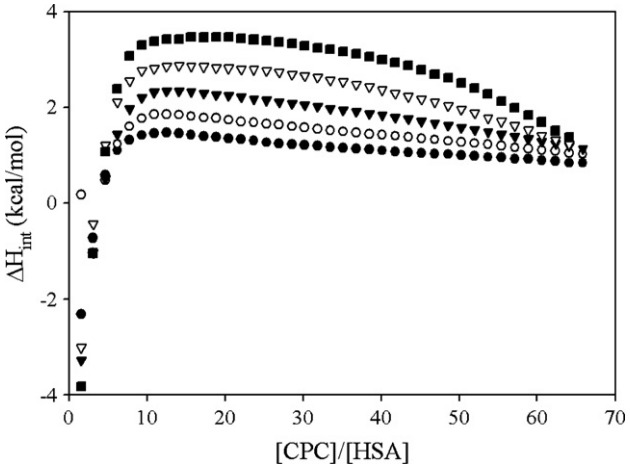

Fig. 3. Dependence of interaction enthalpy change (ΔH_{int}) vs mol ratio of CPC/HSA in the reaction cell for 10 mM CPC injected into HSA solution (0.023 mM) at pH 7.4, 50 mM HEPES, 160 mM NaCl and 20 °C (●), 25 °C (○), 30 °C (▼), 35 °C (▽) and 40 °C (■).

with columbic interactions of ionized fragments of surfactant with opposite charges on the protein surface. These strong electrostatic interactions are usually followed by interactions of non-polar group of surfactant tail with hydrophobic patches at protein surfaces or hydrophobic clefts. These initial interactions should be exothermic. The subsequent folding changes of protein in the larger ratios of surfactant, modify the character of the subsequent interactions into endothermic. At larger surfactant/protein ratios, the density of positive charge of HSA–CPC complex increases. This makes electrostatic interactions less favorite and the growing repulsive forces cause the separation of domains of the molecule what is an endothermic process. The increase of endothermicity of interaction enthalpy with temperature can be interpreted in this light. However, it looks that after the mentioned inflection point, the non-specific binding starts to predominate. Hence, the smooth behavior of enthalpy curve after this point may be related to more hydrophobic binding. The interaction enthalpy converged to approximately the same value at CPC/HSA about 65, what may be related to binding saturation. The point of inflection may represent the start of protein folding changes. However, this is not so clear, because of both ΔH_{int} and the molar ratio correspond to this inflection points with temperature increase. It may be related also to the decrease of binding affinity of CPC with temperature. The opposite effect would be observed if another factors were linked with the stability of HSA.

Heat capacity changes of binding of CPC to HSA

In order to get better insight of interaction of CPC with HSA, the values of interaction heat capacity changes ($\Delta C_{P,int}$) were calculated, using the standard approach for determining $\Delta C_{P,int}$ by ITC, which requires the acquisition of binding isotherms at different temperature. Fig. 4 shows

the variation of ΔH_{int} vs T at any specified CPC concentration. Various plots in this Figure relate to various concentrations of CPC and their slope should be equal to $\Delta C_{P,\text{int}}$ as is implied by the definition of heat capacity, $\Delta C_{P,\text{int}} = d\Delta H_{\text{int}}/dT$, i.e., the increase in energy (heat) with temperature. The linearity of plots in Fig. 4 indicates the invariability of $\Delta C_{P,\text{int}}$ with temperature. The changes of calculated $\Delta C_{P,\text{int}}$ with molar ratio of CPC/HSA are shown in Fig. 5. Two distinct extreme regions that can be identified in this Figure are indicated by arrows. These regions are observed approximately at CPC/HSA molar ratios of 10–14 and 25–30, respectively. The trend of variation of $\Delta C_{P,\text{int}}$ represents a sharp increase at initial stages of binding and a flat region at CPC/HSA ratio of about 10–14. This behavior can be explained by the change of the nature of interaction from electrostatic to non-polar (hydrophobic) interaction. The shift of $\Delta C_{P,\text{int}}$ to more positive values is a sign for the increasing role of hydrophobic interactions. After, this flat region, the $\Delta C_{P,\text{int}}$ increases to more positive values and reaches the maximum CPC/HSA molar ratio in the region of 25–30. It is useful to interpret this observation in terms of heat capacity changes associated with the various physiochemical phenomena occurring, e.g., binding and conformational changes. The nature of binding after flat region is more hydrophobic that has positive sign on $\Delta C_{P,\text{int}}$. The conformational change in HSA due to its interactions with CPC could have also influence on the sign of $\Delta C_{P,\text{int}}$ because of possible unfolding of the protein and the exposure of non-polar regions to water. The increase of $\Delta C_{P,\text{int}}$ after flat region can be related to unfolding. It may be expected that the unfolding will increase at higher during surfactant binding ratios and reaching maximum at region of molar ratios 25–30 when HSA will be predominately unfolded and the contribution of conformational changes in $\Delta C_{P,\text{int}}$ will decrease after this point. Therefore,

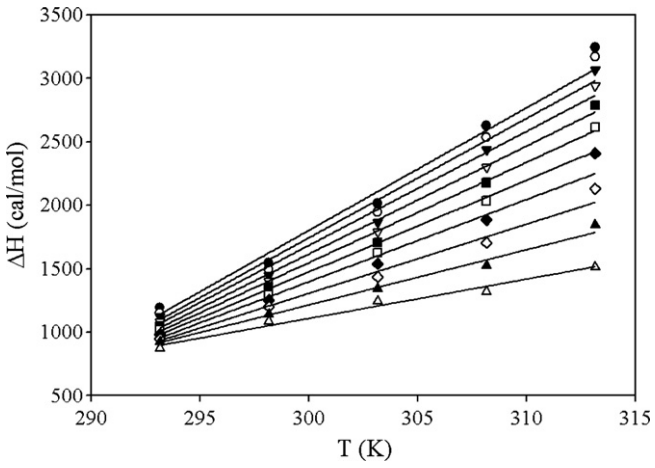

Fig. 4. Dependence of enthalpy change per mole of surfactant with absolute temperature at any specified CPC concentration. The slope of each curve equals to heat capacity change due to interaction. The molar ratios of CPC/HSA equal to 31.84 (●), 35.14 (○), 38.46 (▼), 41.81 (▽), 45.17 (■), 48.56 (□), 51.97 (◊), 55.40 (♦), 58.86 (▲) and 62.33 (Δ). The similar curves were obtained at other molar ratios.

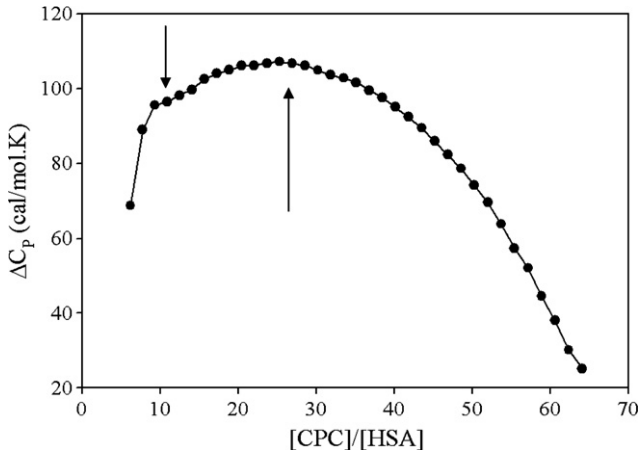

Fig. 5. Variation of heat capacity change corresponds to interaction of CPC with HSA, vs molar ratio of CPC/HSA, at pH 7.4, 50 mM HEPES and 160 mM NaCl.

other distinct conformational change is taking place in the range of 12–30 CPC/HSA molar ratios. Hence, the following mechanism can be proposed as interaction mechanism of CPC with HSA with respect to measured enthalpograms (Fig. 3) and heat capacity profiles (Fig. 5)—the initial binding of CPC ions causes the separation and probably unfolding of one domain of HSA molecule and its transition such as N → F, where F is the intermediate state. This process occurs until molar ratio of about 12. It is initially exothermic and is becoming an endothermic phenomenon at the end. This change can be only perceived from enthalpograms (Fig. 3). The subsequent CPC binding causes unfolding of domains and is leading to unfolded extended state of HSA (E) (F → E). This step can not be deduced from enthalpograms but can be distinguished from heat capacity profiles. The heat capacity changes for both hydrophobic binding of CPC tail and unfolding of HSA domains are positive but due to cooperative nature of unfolding, its contribution to overall binding capacity should pass by a maximum. This is the reason that a maximum is observed at region of CPC/HSA mole ratios of 25–30.

Intrinsic fluorescence spectroscopy

Steady state fluorescence was used in study of structural changes in HSA upon addition of CPC. The quenching of intrinsic fluorescence is indicating that the Trp residues of HSA become less exposed to the solvent upon the binding of CPC ions. The quenching of Trp emission in relation to the CPC/HSA molar ratio is shown in Fig. 6. Two extreme regions at molar ratios 10–14 and 25–45 can be identified from Fig. 6. This can be related to the existence of two forms of the complexed HSA with CPC. The first form would be associated with HSA–CPC intermediate in which the domains of HSA are separated (F state) and the second could be explained by the formation of complexes of CPC with unfolded HSA (E state). The variation of maximum of emission vs CPC/HSA molar ratio is shown in Fig. 7. The

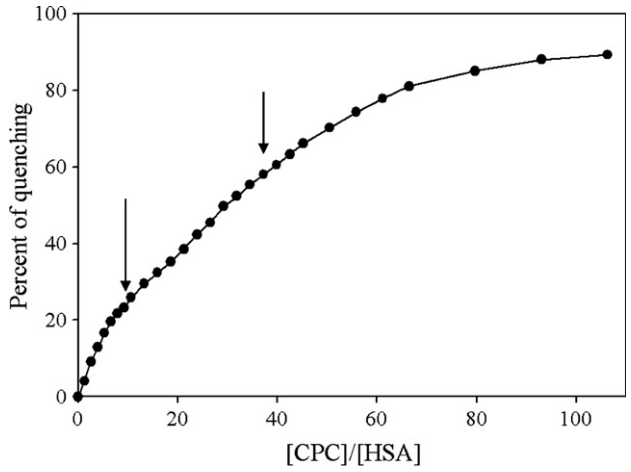

Fig. 6. Percent of quenching of HSA solution (about 3 μM), excited at 280 nm, after each addition of CPC stock solutions (1 mM) in the same buffer at pH 7.4, 50 mM HEPES, 160 mM NaCl, and 25 °C, vs mol ratio of CPC/HSA. The arrows indicate two extreme regions.

considerable blue shift is indicating the existence of various species in the solution. Two distinct minima in Fig. 7 indicated by arrows can be related to the existence of two different forms of complexes. The first can be correspond to first proposed intermediate (F state) and the second to unfolded extended state of HSA bound to CPC ions (E state). Therefore, the fluorescence data confirms the $N \rightarrow F \rightarrow E$ mechanism that has been supported by ITC results.

Changes of secondary structure of HSA measured by circular dichroism

The changes of chirality are good indication of ongoing folding changes and especially of tertiary and secondary structure modifications. The CD measurements were performed at specific mole ratios (0, 4, 10, and 30), which

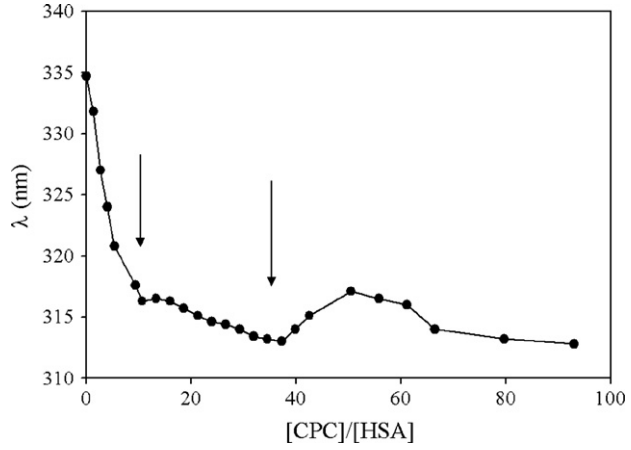

Fig. 7. Variation of maximum wavelengths of emissions of HSA vs molar ratio of CPC/HSA at pH 7.4, 50 mM HEPES, 160 mM NaCl, and 25 °C. The excitation wavelength was 280 nm and the band slits were 3 and 5 nm for excitation and emission wavelengths, respectively. The arrows indicate two minimum points.

are critical points on enthalpograms and on heat capacity curve. Some features attributed to α-helix and β-structure can be seen in the far-UV CD spectra of HSA titrated with CPC in the far ultraviolet region. The results of deconvolution of the CD spectra and calculation of α-helix and β-structure using Chou and Fasman method was judged to give the most close assessment of the α-helix content to what can be assessed from in the analysis of 3 D structure of the HSA defined by crystallographic measurements PDB entry 1ao6 [42]. Contents of secondary structure elements in HSA in presence and absence of CPC evaluated by deconvolution of their CD spectra are presented in Table 1. The molar ellipticity at 222 nm is also presented in this table at all studied molar ratios. This implies that interactions with CPC does not affect HSA folding at the molar ratio of CPC/HSA = 4. The changes of secondary structure become visible at the molar ratio of CPC/HSA = 10 and 30 (more than 30% in α-helix content). Some changes of fine structure of aromatic bands are observed in the near-UV CD spectrum (not shown). However, they are not radical and the chiral environment of the aromatic residues seems to be either unchanged or simply reestablished after refolding. The near-UV CD spectrum of CPC/HSA at various molar ratios shows negative band with fine structure due to the presence of chirally perturbed aromatic residues at 262 and 268 nm, which shifted to 260 and 269 nm at molar ratio 30 (Fig. 8). Peak intensities of CPC/HSA complexes in aromatic range increased at molar ratio 4, whereas they decrease at molar ratio 10. Even more significant decrease is observed at molar ratio 30 with a shift to 260 and 269 nm, what indicates that the tertiary structure of HSA was considerably changed. In order to examine the change in microenvironment of aromatic residues of HSA due to its interaction with CPC, the UV–Vis spectra of HSA were also recorded at these mole ratios and shown in Fig. 8. HSA has an intense absorbance peak at 277 nm. The intensity of the peak is decreased at molar ratio 4 while it increased at molar ratio 10. At molar ratio 30, even more significant increase of absorbance intensity accompanying with blue shift is observed. This confirms the near-UV CD results implying that HSA structure changes occurred at molar ratio of 30 CPC/HSA.

Conclusions

Heat capacity is one of the major thermodynamic quantities measured in proteins. This quantity provides

Table 1
HSA sequence secondary structure based on Chou and Fasman method (Dicroprot, 2000) and its molar ellipticity at 222 nm

Molar ratio	Helix	Sheet	Other	θ_{222} (deg cm^2 dmol^{-1})
0	80	0	20	2541
4	75	13	12	2137
10	54	24	22	2814
30	56	23	21	1825
X-ray crystalography	76	0	24	—

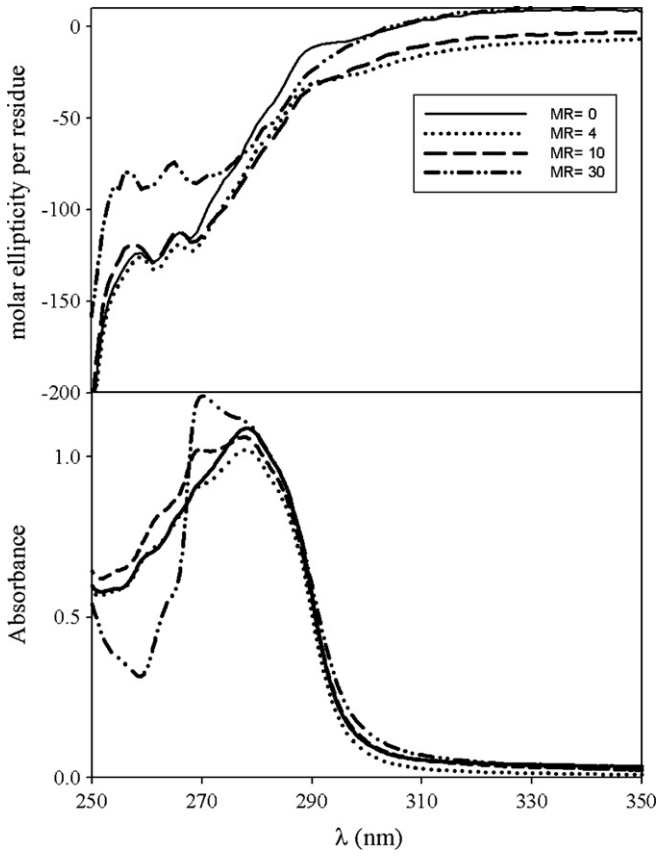

Fig. 8. Near-CD and UV spectra of CPC/HSA complexes at various molar ratios. The concentration of HSA was 27.7 μM in 50 mM HEPES buffer, pH 7.4, at 25 °C.

physical, mechanistic and atomic-level insight into how proteins fold and how they are interact with small molecules (ligand). It is the richest potential source of thermodynamic and structural information and the hardest of the thermodynamic quantities to understand, in physical terms. As it is known, there is no report in literature about measurement of heat capacity changes associated with interactions of ionic surfactants with globular proteins. The following mechanism is proposed on basis of ITC and fluorescence studies: $N \rightarrow F \rightarrow E$, where N, F, and E correspond to native, partially unfolded and extended states, respectively. The previous study of interactions of CPC with HSA using potentiometric and fluorimetric techniques showed the existence of two types of binding sites for CPC and indicated biphasic behavior of quenching process of HSA by CPC [37]. It can be concluded the F-state corresponds to the occupation of first binding set. The results of this study agree with the previous observations but also give more precise information about possible mechanisms of interactions based on reliable and precise calorimetric measurements. The number of binding sites of the first binding type at neutral pH is about 10 and the maximal value of ΔH_{int} is also found at molar ratio around 10. This confirms the role of both electrostatic and hydrophobic interactions in the sites of the first binding class. The variations of $\Delta C_{P,\text{int}}$ and of fluorescence quantum efficiency with molar ratios also confirm this conclusion. Moreover, the obtained results can depict the cooperative unfolding at the begging of binding of CPC to the sites of second class. The CD results indicate significant changes in secondary structure during $N \rightarrow F$ transition. However, the second transition, $F \rightarrow E$, which is seconded by the loss of tertiary structure, does not trigger significant changes of secondary structure. Hence, HSA does not loose all of secondary structure during its interactions with CPC. The ITC results, especially heat capacity changes, indicate that the major unfolding occur in region of CPC/HSA molar ratios of 25–30. The character of folding changes detected by thermodynamic analysis is confirmed by the analysis of HSA fluorescence and CD spectra in presence and absence of CPC. The similar conclusions were reported for pH induced denaturations of BSA and HSA. With decreasing pH, BSA/HSA undergoes two unfolding transitions from the folded state (F). The first to the so-called F-form intermediate where initially one domain of the molecule separates from the other two, then eventually to the so-called E-form (i.e., "extended" form) where all three domains are now separated from each other. This is a well-known phenomenon induced by the changes of pH [43]. All recently small-angle neutron scattering studies confirmed the presence of $N \rightarrow F \rightarrow E$ transition of BSA in the presence of a cationic surfactant [44]. This agrees well with our conclusions obtained on basis of calorimetric measurements. However, it is clear that further analysis is needed before the complete amount of information could be mined from general spectral and thermodynamic data of folding changes. It is certain that this type of information would be very hard to obtain from fluorescence quenching study of protein–ligand interactions, only.

Acknowledgments

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References

[1] C. Tanford, Y. Nozaki, J.A. Reynolds, S. Makino, Biochemistry 13 (1974) 2369–2376.
[2] M.J. Schwuger, F.G. Bartnik, in: C. Gloxhuber (Ed.), Anionic Surfactants—Biochemistry, Toxicology, Dermatology, Marcel Dekker Inc., 1980, pp. 1–49.
[3] M.N. Jones, A. Brass, in: E. Dickinson (Ed.), Food polymers, Gels and Colloids, The Royal Society of Chemistry, Cambridge, UK, 1991, pp. 65–80.
[4] M.N. Jones, Chem. Soc. Rev. 21 (1992) 127–136.
[5] E. Dickinson, in: E.D. Goddard, K.P. Ananthapadmanabhan (Eds.), Interactions of Surfactants with Polymers and Proteins, CRC Press, Boca Raton, 1993, pp. 295–317.
[6] K.P. Ananthapadmanabhan, in: E.D. Goddard, K.P. Ananthapadmanabhan (Eds.), Interactions of Surfactants with Polymers and Proteins, CRC Press, Boca Raton, 1993, pp. 319–365.

[7] A.A. Saboury, A.K. Bordbar, A.A. Moosavi-Movahedi, J. Chem. Therm. 28 (1996) 1077–1082.
[8] A.K. Bordbar, A.A. Moosavi-Movahedi, A.A. Saboury, Thermochim. Acta 287 (1996) 343–349.
[9] A.K. Bordbar, A.A. Moosavi-Movahedi, Bull. Chem. Soc. Jpn. 69 (1996) 2231–2234.
[10] A.K. Bordbar, A.A. Saboury, M.R. Hosaindokht, A.A. Moosavi-Movahedi, J. Coll. Inter. Sci. 192 (1997) 415–419.
[11] E.D. Goddard, K.P. Ananthapadmanabhan, in: J.C.T. Kwak (Ed.), Polymer—Surfactant Systems, vol. 77, Marcel Dekker, New York, 1998, pp. 22–64.
[12] Y.X. Huang, R.C. Tan, Y.L. Li, Y.Q. Yang, L. Yu, Q.C. He, J. Coll. Inter. Sci. 236 (2001) 28–34.
[13] P.R. Majhi, A. Blume, Langmuir 17 (2001) 3844–3851.
[14] P.M. Landeira, J.M. Ruso, G. Prieto, F. Sarmiento, J. Chem. Eng. Data 47 (2002) 1017–1021.
[15] A.K. Bordbar, N. Sohrabi, E. Hojjati, Coll. Surf. B Biointerfaces 39 (2004) 171–175.
[16] A.K. Bordbar, R. Hossainzadeh, K. Omidiyan, Bull. Chem. Soc. Jpn. 77 (2004) 2027–2032.
[17] A.K. Bordbar, R. Hossainzadeh, K. Omidiyan, Coll. Surf. B Biointerfaces 40 (2005) 67–71.
[18] A.K. Bordbar, R. Hossainzadeh, Coll. Surf. B Biointerfaces 53 (2006) 288–295.
[19] A.K. Bordbar, R. Hossainzadeh, M.H. Norozi, J. Thermal. Anal. Cal. 87 (2007) 453–456.
[20] M.H. Ropers, G. Czichocki, G. Brezesinski, J. Phys. Chem. B 107 (2003) 5281–5288.
[21] A. Chatterjee, S.P. Moulik, P.R. Majhi, S.K. Sanyal, Biophys. Chem. 98 (2002) 313–327.
[22] J. Xiang, Y. Liang, N. Chen, Acta Chim. Sin. 61 (2003) 1949–1954.
[23] D. Kelley, D.J. McClements, Food Hydrocoll. 17 (2003) 73–85.
[24] M.D. Lad, V.M. Ledger, B. Briggs, R.J. Green, R.A. Frazier, Langmuir 19 (2003) 5098–5103.
[25] A.K. Bordbar, A.A. Moosavi-Movahedi, M.K. Amini, Thermochim. Acta 400 (2003) 95–100.
[26] A.D. Nielsen, L. Arleth, P. Westh, Biochim. Biophys. Acta 1725 (2005) 124–132.
[27] S. Curry, H. Mandelkow, P. Brick, N. Franks, Nat. Struct. Biol. 5 (1998) 827–835.
[28] S. Curry, P. Brick, N.P. Franks, Biochim. Biophys. Acta 1441 (1999) 131–140.
[29] A.A. Bhattacharya, T. Grune, S. Curry, J. Mol. Biol. 303 (2000) 721–732.
[30] I. Petitpas, T. Grune, A.A. Bhattacharya, S. Curry, J. Mol. Biol. 314 (2001) 955–960.
[31] J.R. Brown, P. ShockleyLipid–Protein Interactions, vol. 1, Wiley, New York, 1982.
[32] D. Carter, J.X. HoAdvances in Protein Chemistry, vol. 45, Academic Press, New York, 1994.
[33] Y. Moriyama, D. Ohta, K. Hachiya, Y. Mitsui, K. Takeda, J. Protein Chem. 15 (1996) 265–272.
[34] E.L. Gelamo, M. Tabak, Spectrochim. Acta A 56 (2000) 2225–2271.
[35] P. Martinez-Landeira, J.M. Ruso, G. Prieto, F. Sarmiento, M.N. Jones, Langmuir 18 (2002) 3300–3305.
[36] A.K. Bordbar, N. Sohrabi, H. Gharibi, Bull. Korean Chem. Soc. 25 (2004) 791–795.
[37] A.K. Bordbar, A. Taheri-Kafrani, Coll. Surf. B Biointerfaces 55 (2007) 84–89.
[38] M. Osman, M.E. Janes, R. Story, R. Nannapaneni, M.G. Johnson, J. Food Prot. 69 (2006) 1286–1291.
[39] R.F. Chen, J. Biol. Chem. 242 (1967) 173–181.
[40] S.P. Stodghill, A.E. Smith, J.H. O'Haver, Langmuir 20 (2004) 11387–11392.
[41] K. Bijma, J.B.F.N. Engberts, M.J. Blandamer, P.M. Cullis, P.M. Last, K.D. Irlam, L.G. Soldi, J. Chem. Soc. Faraday Trans. 93 (1997) 1579–1584.
[42] S. Sugio, S. Mochizuki, M. Noda, A. Kashima, Protein Eng. 12 (1999) 439–446.
[43] M.Y. Khan, Biochem. J. 236 (1986) 307–310.
[44] S.-C. Wang, C.T. Lee Jr., J. Phys. Chem. B 110 (2006) 16117–16123.

Abstract

β-Lactoglobulin (β-Lg) is a lipocalin, which is the major whey protein of cow milk and the milk of other mammals. However, it is absent from milk of primates. This globular protein of about 18 kDa is folded forming a β-barrel (or calyx) structure. Each monomer contains two disulphide bonds and one cysteine at position 121 (C121). This free thiol plays an important role in the heat-induced aggregation of β-Lg, and, possibly, in the maintenance of its conformational stability. The biological function of β-Lg is not clear, but its potential role in carrying fatty acids through the digestive tract has been suggested. β-Lg is also one of major allergens in milk.

Heating is one of the most common technologic treatments applied during many milk transformations. Despite the frequency of use of heating, the effects of heating β-Lg and its denaturation on its recognition by IgE of patients are not fully described. Binding of IgE from patients with cow milk allergy to native β-Lg and to heated β-Lg in the temperature range 65-95°C was measured by ELISA. Since β-Lg can be subjected to modifications during heating by the existing reducing sugars in the milk through Maillard reaction, binding of IgE of patients to glycated β-Lg was also measured. Loss of tertiary and secondary structures of β-Lg by heating above 75°C resulted in a decrease in its recognition by IgE, whose reactivities differ between the patients. The intensity of the decrease of IgE binding was also observed in case of intensively glycated β-Lg**.**

The expression in the yeast *Pichia pastoris* of a mutant bovine β-Lg, in which C121 was changed into Ser (C121S) was accomplished during this study. The C121S mutation blocks completely the irreversible aggregation induced by heat treatment. Binding of IgE from patients with cow milk allergy to native β-Lg, wild-type β-Lg and C121S mutant β-Lg was aslo measured by ELISA.

β-Lg has been found in complexes with lipids such as butyric and oleic acids and has a high affinity for a wide variety of compounds. Serotonin (5-hydroxytryptamine, 5-HT), an important compound found in animals and plants, has various functions, including the regulation of mood, appetite, sleep, muscle contraction, and some cognitive functions such as memory and learning. In the last part of this study, the interaction of serotonin and one of its derivatives, arachidonyl serotonin (AA-5HT), with β-Lg was investigated using circular dichroism (CD) and fluorescence intensity measurements. These two ligands interact with β-Lg forming equimolar complexes. The binding constant for the serotonin/β-Lg interaction is between 10^5 and 10^6 M^{-1}, while for the AA-5HT/β-Lg complex it is between 10^4 and 10^5 M^{-1} as determined by measurements of either protein or ligand fluorescence. The observed binding affinities were higher in hydroethanolic media (25 % EtOH). The interactions between serotonin/β-Lg and AA-5HT/β-Lg may compete with self-association (micellization) of both the ligand and the protein. According to far-and near-UV CD results, these ligands have no apparent influence on β-Lg secondary structure, however they partially destabilize its tertiary structure. Their binding by β-Lg may be one of the peripheral mechanisms of the regulation of the content of serotonin and its derivatives in the bowel of milk-fed animals.

Keywords : beta-lactoglobulin, interaction, allergy, modification, serotonin.

Résumé

La β-lactoglobuline (β-Lg) est la protéine la plus abondante du lactosérum du lait de vache et des autres mammifères, elle appartient à la famille des lipocalines. Toutefois, elle est absente dans le lait de primates. C'est une protéine globulaire d'environ 18 kDa formant une structure en feuillet β (appelé calice). Chaque monomère contient deux ponts disulfures et une cystéine en position 121 (C121). Ce groupement thiol libre joue un rôle important dans l'agrégation de la β-Lg induite par la chaleur et, éventuellement, dans le maintien de sa stabilité de conformation. La fonction biologique de la β-Lg n'est pas claire, mais son rôle potentiel dans le transport des acides gras dans le tube digestif a été suggéré. La β-Lg est connue comme l'un des allergènes majeurs dans le lait.

Parmi les traitements technologiques le chauffage est le plus couramment appliqué au cours des procédés de transformation du lait. Malgré la fréquence d'utilisation du chauffage, les effets des traitements thermiques de la β-Lg sur sa dénaturation et sa reconnaissance par les IgE de patients ne sont pas décrits. La fixation des IgE de patients allergiques au lait de vache à la β-Lg native, et à la β-Lg chauffée dans la gamme de température de 65-95 $^{\circ}$C, a été étudiée par ELISA. La β-Lg a été soumise à des modifications au cours du chauffage en présence des sucres réducteurs par la réaction de Maillard. La fixation des IgE de patients à la β-Lg glycosylée a également été mesurée. La perte de structures tertiaires et secondaires de β-Lg par chauffage au dessus de 75 $^{\circ}$C a entraîné une diminution de sa reconnaissance par les IgE, dont les réactivités différent entre les patients. L'intensité de la baisse de la liaison IgE a également été observée dans le cas des β-Lg fortement glycosylées.

L'expression au niveau la levure *Pichia pastoris* d'un mutant de l'espèce bovine β-Lg, dans lequel le groupement C121 a été changé en Ser (C121S) a été réalisée au cours de cette étude. La mutation C121S bloque complètement l'agrégation irréversible induite par un traitement thermique. La fixation des IgE, obtenus de patients allergiques au lait de vache, avec la β-Lg native, β-Lg type sauvage et la β-Lg mutée C121S a également été étudiée par ELISA.

La β-Lg peut complexer des lipides tels que les acides butyrique et l'acide oléique ainsi que d'autres variété de ligands. La sérotonine (5-hydroxytryptamine, 5-HT) est un composé important qu'on trouve chez les animaux et les plantes. Elle est connue par ses diverses fonctions, y compris la régulation de l'humeur, l'appétit, le sommeil, la contraction musculaire, et certaines fonctions cognitives comme la mémoire et l'apprentissage. Dans la dernière partie de cette étude, l'interaction de la sérotonine et la sérotonine arachidonyl (AA-5HT) avec β-Lg a été étudiée par dichroïsme circulaire (CD) et par fluorescence. Ces deux ligands interagissent avec β-Lg pour former des complexes équimolaires. La constante d'affinité du complexe sérotonine/β-Lg est compris entre 10^5 et 10^6 M^{-1}, tandis que celle du complexe AA-5HT/β-Lg est comprise entre 10^4 et 10^5 M^{-1}. Les constantes d'affinités observées sont plus élevés en milieu hydro-éthanolique (25 % EtOH). Selon les résultats des UV proche et lointain en CD, ces ligands n'ont aucune influence apparente sur la structure secondaire de la β-Lg, mais ils déstabilisent partiellement la structure tertiaire. La liaison avec la β-Lg pourrait expliquer l'un des mécanismes périphériques de la régulation de la sérotonine et de ses dérivés au niveau de l'intestin des animaux nourris au lait.

Mots-clés : beta-lactoglobulin, interaction, allergie, modification, serotonin.