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Recherche de facteurs affectant la transformation amyloïde et l'agrégation des protéines

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Résumé

Selon le dogme central de la biologie structurale, le repliement correct des protéines est nécessaire pour remplir leur fonction biologique. En conséquence, l'absence de conformation stable est un facteur négatif, car selon le dogme les protéines non structurées étaient considérées comme des composants instables dépourvus d'activité physiologique et susceptibles de s'agréger. Au fil du temps, on a découvert que les protéines fonctionnaient normalement dans une conformation non pliée, mais le problème du repliement inapproprié des protéines devenait de plus en plus évident avec le temps. L'agrégation pathologique de protéines est un problème important de la biochimie moderne. De nombreuses maladies sont considérées comme dues à un repliement incorrect des protéines et à leur agrégation ultérieure. Pour le moment, ces maladies restent incurables et toute thérapie contre elles est palliative.

Les neuropathies spongiformes transmissibles (NST), également appelées maladies à prions, sont des maladies neurodégénératives mortelles pour l'homme et les animaux. La protéine prion (PrP) est connue pour sa capacité à s'agréger en oligomères solubles qui s'associent à leur tour en fibrilles amyloïdes. L'une des approches viables pour la prophylaxie et le traitement des maladies à prions (ainsi que d'autres pathologies conformationnelles) repose sur les composés capables de se lier à la protéine prion (ou à ses agrégats), inhibant ainsi sa conversion en forme pathologique agrégation supplémentaire. Les maladies neurodégénératives sont associées à une accumulation de produits de repliement défectueux de protéines de type amyloïde. Empêcher la formation de ces entités infectieuses et neurotoxiques constitue une stratégie viable pour lutter contre les maladies à prions. De nombreuses tentatives pour trouver des composés diététiques possédant des propriétés anti-prion ont été effectuées, mais

l'agent le plus prometteur découvert à ce jour était la curcumine, qui est peu soluble et simplement biodisponible.

Pour le moment, les méthodes de traitement des maladies à prions et, en général, de l'amyloïdose n'ont pas été développées. Leur recherche s'effectue dans les directions suivantes:

1. L'nhibition de l'expression de protéines amyloïdogènes ou stabilisation de celle-ci sous forme native à l'aide de ligands organiques de bas poids moléculaire;
2. L'inhibition de la libération de protéines infectieuses à partir de la cellule hôte par l'activation de protéases intracellulaires;
3. Agrégation de protéines amyloïdogènes par inhibition directe de l'utilisation de ligands organiques ou de vaccins;
4. L'nhibition des effets de l'amyloidose, éventuellement non directement liée à celle-ci (stress oxydatif, par exemple) et atténuation des symptômes de la maladie sans suppression directe du processus pathogène.

La première stratégie est la plus efficace, mais elle conduit à la suppression de la synthèse de protéines infectieuses et natives aussi, qui peut remplir une fonction vitale dans l'organisme. Par exemple, il a été démontré que les souris ayant un gène PRNP désactivé se caractérisent par une tendance accrue aux explosions d'agression non motivée. L'activation des protéases peut également avoir un effet systémique négatif, par exemple sur la protéolyse concomitante des polypeptides natifs nécessaires. L'utilisation de cette dernière stratégie est difficile en raison de la connaissance insuffisante de l'effet cytotoxique des agrégats d'amyloïde. Peut-être que la direction d'activation du système anti-inflammatoire par la suppression de l'époxyde hydrolase et une augmentation de la concentration en acides gras époxygénés sera prometteuse. Cependant, cette voie augmente la survie cellulaire, mais n'élimine pas les causes profondes des agrégats d'amyloïde. Par conséquent, les principaux efforts

des scientifiques visent à trouver des composés capables d'inhiber directement l'amyloïdogenèse. On connaît déjà pas mal de telles substances: par exemple, il a été démontré que l'agrégation de peptides β-amyloïdes peut supprimer les ligands de faible poids moléculaire, tels que la rifampicine, l'hémine et les porphyrines proches, ainsi que les molécules cyclodextrine. Le prion lui-même lie également les porphyrines cationiques, ce qui empêche son agrégation, et il est connu que ce processus dépend du pH. La capacité des molécules de protéine à interagir avec la PrP^{Sc} a également été démontrée. L'amyloïdogenèse d'une autre protéine transthyrétine peut inhiber à la fois les petits ligands (déjà connus 18) et, par exemple, les polyphénols. Des substances capables de se lier in vitro aux molécules de PrP sont déjà connues, et, probablement, certaines suppriment même la formation par prion d'oligomères intermédiaires - le principal agent infectieux - et de fibrilles, qui sont attribués à la plupart des dommages causés aux cellules. De plus, pour supprimer l'agrégation, le ligand peut se lier non seulement avec le C-terminal, mais également avec le domaine non structuré N-terminal.

La glycation des protéines par divers sucres et composés dicarbonyle réactifs est considérée comme l'une des modifications post-traductionnelles les plus courantes et les plus importantes, qui entraîne de nombreuses conséquences. Celles-ci incluent, en premier, lieu des modifications du fonctionnement des protéines glyquées, ainsi que la formation d'agrégats de protéines stabilisés par des liaisons covalentes entre les produits de glycation finaux et les groupes amino des protéines. L'identification des protéines glyquées est largement utilisée pour diagnostiquer les maladies associées à l'hyperglycémie, la neurodégénérescence. La glycation des protéines joue un rôle important dans la formation de structures amyloïdes pathologiques car elle affecte à la fois la transformation de l'amyloïde et la dégradation des protéines modifiées avec des systèmes protéasomes impliquant le ubiquitylation.

La recherche sur les protéines alimentaires, dont la glycation se produit de manière intensive lors de la cuisson à des températures élevées en présence de sucres endogènes ou ajoutés de l'extérieur, est un domaine de recherche distinct et important. D'une part, l'étude de la glycation des protéines alimentaires est nécessaire pour déterminer l'effet d'une telle modification sur la digestion des protéines dans le tube digestif, sur l'allergénicité des protéines et sur leur effet sur le microbiote. Par ailleurs, il est commode d'utiliser les protéines alimentaires disponibles en grande quantité comme objets modèles dans l'étude du rôle de la glycation dans l'induction de l'agrégation et de la transformation amyloïde des protéines afin de comprendre les processus intervenant au cours de perturbations pathologiques du développement. de l'amyloidse.

Dans plusieurs travaux, il a été montré que la glycation des caséines du lait par les sucres et les aldéhydes provoque des modifications de leur structure, révélées par les méthodes traditionnelles (dichroïsme circulaire, fluorescence ThT, etc.) et stimule l'agrégation de protéines. La glycation de la caséine a généralement aggravé l'absorption dans le tractus gastro-intestinal et réduit l'allergénicité. Parallèlement, la glycation de la caséine kappa a stimulé la transformation amyloïde de cette protéine. La glycation a exercé un effet similaire sur l'agrégation des albumines sériques qui, dans certaines conditions, ont subi une transformation amyloïde. L'effet le plus détaillé de la glycation sur les propriétés de la caséine bêta a été étudié dans les travaux de Reza Yousefi. Une étude a été réalisée sur l'effet de la glycation de la bêta-caséine par le D-glucose sur les propriétés structurelles et l'agrégation de la protéine. Après la glycation, la protéine a été thermoagrégée et ensuite, en utilisant la méthode du dichroïsme circulaire, ainsi que des mesures de la fluorescence intrinsèque et de l'intensité de la fluorescence de ThT et de ANS, les modifications structurelles de la protéine ont été étudiées. Des modifications structurelles de la caséine glyquée ont été

identifiées, mais aucune donnée n'a été obtenue sur la stimulation de la conversion de la protéine amyloïde par la protéine amyloïde. Il a également été démontré que l'activité de la caséine bêta glyquée, comme à celle du chaperon, était accrue et que l'allergénicité demeurait pratiquement inchangée.

La question de l'influence pathologique de la glycation sur la PrP reste ouverte. Il est connu que dans les organismes affectés par diverses formes d'encéphalopathie spongiforme, le prion converti se lie aux produits de glycation tardive au niveau de 3 résidus de lysine et d'1 arginine. C'est pas clair que ces modifications sont la cause ou la conséquence de la conversion de l'amyloïde. Cependant, il a été démontré que ces modifications se produisent parallèlement à l'amyloïdisation et que cette voie de glycation conduit à la formation de liaisons croisées entre les molécules de PrP^{Sc}, ce qui contribue à dans la résistance des agrégats de protéines à la protéolyse. Il a été constaté que prion, en tant que support d'information, stockait en lui-même des informations sur la structure protéique secondaire, mais également sur le niveau de sa modification. Ainsi, après avoir infecté des animaux avec du prion, le niveau de glycation de la PrP dans leur corps a été donné par l'agent infectieux. Il existe également des informations sur la glycation du prion dans la composition des structures amyloïdes et dans la région non structurée qui subit la conversion de l'amyloïde et qui participe à la formation de plis β résistants à la dégradation par la protéinase K. avec la PrP recombinante in vitro ont montré que la glycation non enzymatique non contrôlée de la protéine prion supprime son agrégation amyloïde. La PrP glyquée subit une conversion de l'amyloïde beaucoup plus lente, quel que soit l'agent par lequel elle a été modifiée.

L'intention de nos travaux était d'identifier de nouveaux facteurs de protection amyloïdogènes et protecteurs de l'amyloïde qui affectent la capacité des protéines à

transmettre, provoquer et supprimer l'agrégation pathologique. Pour résoudre ce problème, nous avons formulé les tâches suivantes:

- isoler et purifier des protéines possédant soit des propriétés amyloïdogènes, soit la capacité de supprimer l'agrégation pathologique d'autres protéines;
- démontrer la capacité des protéines amyloïdogènes et protectrices de l'amyloïde à interagir et à agir sur d'autres polypeptides;
- identifier de nouveaux ligands anti-amyloïdes et étudier leur effet sur différents types d'agrégation de protéines;
- choisir les conditions de glycation des protéines des protéines étudiées et étudier l'évolution de leurs propriétés après la glycation;
- évaluer la capacité des ligands anti-amyloïdes à inhiber la conversion et l'agrégation des protéines amyloids

Dans nos recherche, nous avons utilisé une protéine prion recombinante produite par une souche de E. coli compétente, qui contient un plasmide avec un gène de la protéine cible. L'expression recPrP en eux a été provoquée par l'injection de solution IPTG. La présence de protéines aux stades d'expression et d'isolement a été vérifiée par électrophorèse sur SDS d'échantillons prélevés du milieu de E. coli avant l'induction de l'expression, de la culture d'expression finale et de la solution de corps d'inclusion dans le tampon à la guanidine chlorhydrate. En raison de la présence dans la séquence PrP d'octapeptides capables de se lier à des ions métalliques bivalents, l'isolement et la purification peuvent être effectués en une seule étape en utilisant la chromatographie par affinité sur une colonne de Ni2 + immobilisée. La solution de protéines obtenue par élution avec de l'imidazole a été dialysée avec une diminution séquentielle de la concentration en agent tampon en modifiant la solution de dialyse. Le dialysat a été lyophilisé.

La β-caséine native a été purifiée selon la procédure de purification en deux étapes avec une chromatographie en phase inverse supplémentaire. Le lait frais de bovin a été dégraissé par centrifugation et les caséines ont été précipités à pH 4,6. Ensuite, la fraction de caséine a été divisée en protéines isolées par chromatographie sur échangeur d'ions sur colonne Q Sepharose High Performance. L'élution a été effectuée par le tampon avec une concentration croissante de NaCl. Après détection des protéines par spectrométrie et BCA méthode, les fractions contenant de la β-caséine pure ont été regroupées et purifiées à partir de composés de faible masse moléculaire par chromatographie en phase inverse.

L'expression des protéines du complexe GroEL/GroES a été réalisée dans la culture de la souche W3110 de E. coli avec le plasmide pOF39. Dans ce cas, l'activation de l'expression des protéines cibles n'était pas nécessaire. Les protéines contenues dans le surnageant, que a été reçu par destruction de cellules par ultrasons. Les protéines du suspension a été centrifugée et était salé du surnageant pour la séparation initiale d'autres protéines et la précipitation ultérieure de chaperonines. Ensuite, les protéines ont été résolubilisées et purifiées par chromatographie sur échangeur d'ions sur un gel DEAE-Sepharose avec élution du sel. La solution de GroEL a été dialysée contre le même tampon sans NaCl, puis soumise à une autre chromatographie dans les mêmes conditions afin d'atteindre le degré maximum de purification. Les nouvelles fractions contenant GroEL ont été combinées et, à partir du volume résultant, la protéine a été relarguée par addition de $(NH_4)_2SO_4$. Les échantillons GroES ont également été soumis à une purification supplémentaire, mais par une méthode de traitement thermique avec précipitation de la protéine cible. Les deux composants du complexe de chaperonine ont été maintenus en précipitation sous la forme $(NH_4)_2SO_4$.

Les effets anti-agrégation et chaperonine ont été analysés en fonction de la capacité des protéines cibles à agir sur le repliement et la réactivation de la glycéraldéhyde 3-phosphate déshydrogénase. Le GAPDH purifié de lapin a été utilisé.

Quelques substances de bas poids moléculaire ont été choisies pour la recherche. Il a été démontré que la curcumine alcaloïde végétale se liait à une molécule de protéine prion et supprimait sa conversion en amyloïde et son agrégation. Ce polyphénol végétal, extrait du curcuma aux épices, très répandu en Inde, a démontré son efficacité contre la conversion amyloïde de protéines in vitro et in vivo et c'est pourquoi il a été choisi comme ligand antiamyloïde confirmé. Par la méthode d'amarrage moléculaire, nous avons prédit que le site de liaison de la curcumine est disponible pour l'acide 3,4-diméthoxycinnamique, qui est structurellement très similaire à la moitié de la molécule de curcumine. Également pour l'acide diméthoxycinamique, une recherche de sites de liaison alternatifs avec la molécule de protéine prion a été effectuée, au cours de laquelle un site énergétiquement plus favorable que pour la curcumine a été trouvé. On s'attend à ce que le 3,4-DMCA ait une meilleure solubilité dans la phase aqueuse que la curcumine: la solubilité apparente de la 3,4-DMCA déterminée par nous dépasse la solubilité de la curcumine de 4 à 5 fois en moyenne sous la forme acide, et de 10 fois si l'acide cinnamique est présent sous forme de sel de sodium. Afin de déterminer expérimentalement l'affinité de liaison du 3,4-DMCA avec une protéine prion, nous avons utilisé la méthodes de calorimétrie à balayage différentiel et calorimétrie par titrage isotherme. Les paramètres de liaison de ce ligand se sont révélés supérieurs à ceux de la curcumine. En outre, nous avons décidé de tester 2 autres ligands, pour lesquels il n'existe aucune information complète sur les propriétés anti-amyloïdes, mais ils ont démontré un effet cytoprotecteur sur les cultures cellulaires et ont également montré la présence de propriétés antioxydantes, comme la curcumine. Le pentamidine-iséthionate est utilisé comme agent antimicrobien dans le traitement de la

trypanosomiase, de la leishmaniose et dans la prévention de la pneumonie chez les personnes à faible immunité. Le resvératrol est un composant du vin rouge et est connu comme un antioxydant. Il a été démontré que le resvératrol avait un effet positif mais indirect sur l'organisme lors de maladies neurodégénératives.

La glycation des protéines a été réalisée par D-glucose. La β-caséine a été incubée dans un tampon à pH 8,0, contenant 0,01% d'azide de sodium en présence de glucose à 37 ° C pendant 2-3 jours sous agitation douce. Après l'incubation pendant des intervalles de temps spécifiés, les solutions de protéines ont été purifiées à partir de glucose et de composés de faible poids moléculaire en utilisant une chromatographie en phase inverse, une évaporation après la chromatographie et une dialyse supplémentaire. Par rapport à la glycation de la caséine, la modification de la PrP a été réalisée dans des conditions plus douces en raison de sa forte tendance à l'agrégation: la concentration en protéines était inférieure (1 mg / ml au lieu de 5 pour la β-caséine) et le temps d'incubation a été réduit à 24 h. La chromatographie en phase inversée a été exclu de la purification pour éviter l'agrégation de protéines. Les produits de la modification ont été analysés par électrophorèse sur SDS pour déterminer le degré d'agrégation possible, en colorant les échantillons avec du NBT, en fonction du nombre de groupes amino libres restants selon la méthode de l'ortho-phtalaldéhyde. Le nombre de produits de glycation précoce a été déterminé par la méthode à la fructosamine, le nombre de produits tardifs a été déterminé par la fluorescence intrinsèque de de les produits de glycation mature.

Pour l'analyse de la concentration des protéines, nous avons utilisé une spectrométrie à la lumière UV, le test de Bradford et une mesure avec l'acide bicinchoninique. La composition des mélanges de protéines a été analysée par électrophorèse sur gel natif ou le même avec des agents dénaturants. Cette méthode a également été utilisée pour estimer l'état d'agrégation des protéines. Un processus

d'agrégation a été analysé plus en détail par diffusion dynamique de la lumière, mesure par turbidimétrie et microscopie optique ou par microscopie transmission électronique. La présence de structures amyloïdes a été détectée par la coloration du thioflavine T ou du congo rouge et du estimation de la résistance à la protéinase K. La structure des molécules de protéines a été analysée par mesure du dichroïsme circulaire. La microscopie à fluorescence avec la coloration ThT était la méthode d'analyse la plus informative. Les tests de coloration immunitaire ont également été utilisés pour la détection de protéines. L'activité de GAPDH a été estimée par la capacité de cette enzyme à produire une forme réduite de NADH.

Tout d'abord, nous avons étudié l'interaction entre les protéines amyloïdogènes et les protéines capables de supprimer l'agrégation pathologique. Ensuite, nous avons étudié l'influence des ligands anti-amyloïdes connus et en perspective sur ce processus pour évaluer leur effet sur l'amyloidisation et l'agrégation. Enfin, nous avons étudié l'effet d'une telle modification répandue de la protéine in vivo en tant que glycation sur les propriétés et l'interaction de ces protéines amyloïdogéniques et protégeant contre l'agrégation.

Au cours de la première étape, nous avons testé la co-agrégation de PrP avec certaines protéines alimentaires: la β-lactoglobuline, les κ- et β-caséines et la albumine des serum bovine. La protéine prion a subi la conversion et l'agrégation amyloïde elle-même, ainsi que la capacité d'activer des processus similaires dans d'autres proteins, que est habituellement stables: par exemple, PrP et BSA ont formé d'énormes agrégats de type gel avec des pourcentages de structure amyloïde élevés. La curcumine supprime efficacement l'amylose des protéines prions dans toutes les combinaisons testées avec d'autres protéines. L'acide 3,4-diméthoxycinnamique a montré moins de polyvalence, par conséquent, dans certaines combinaisons de protéines, son effet antiamyloïde a été réduit. Dans le même temps, dans les combinaisons avec effet anti-

amyloïde confirmé, l'acide 3,4-diméthoxycinnamique supprime plus significativement l'amylose et l'agrégation des protéines que la curcumine. De plus, l'acide 3,4-diméthoxycinnamique a montré une plus grande efficacité en tant que ligand supprimant l'agrégation des protéines, mais est moins efficace pour le démontage d'anciens agrégats denses contenant des structures amyloïdes. Ceci est probablement dû à une plus grande hydrophilie de l'acide 3,4-diméthoxycinnamique, qui, d'une part, permet une suppression plus efficace de l'agrégation, mais, d'autre part, ne lui permet pas de diffuser dans des agrégats hydrophobes denses enrichis par des structures amyloïdes.

Les expériences ont montré que la glycation de la protéine prion entraîne une diminution de son amyloïdogénicité, ce qui confirme les résultats obtenus précédemment par d'autres chercheurs. En même temps, cette modification facilite légèrement l'agrégation amorphe de la protéine prion. En conséquence, l'effet antiagrégant total de la curcumine et de l'acide 3,4-diméthoxycinnamique a été affaibli, car après la modification, l'agrégation s'est faite davantage par la voie non spécifique, qui n'est pas efficacement supprimée par le DMCA et surtout par la curcumine.

L'effet de la bêta-caséine native sur l'agrégation de la protéine prion était estimé. La présence de cette protéine avec une activité semblable à celle du chaperon supprime significative tout type d'agrégation des protéines prions, et l'agrégation de protéines amyloïdes sont pratiquement évitées par la combinaison de bêta-caséine et de ligands anti-amyloïdes de bas poids moléculaire. Cependant, les interactions avec d'autres protéines étaient si graves que la coagrégation était presque instantanée et même la présence de vraies chaperonines n'a pas empêché ce processus. De plus, la bêta-caséine glyquée a montré sa capacité à s'agréger seule, à devenir partiellement ordonnée, mais encore plus, l'a forcé à accélérer l'agrégation des autres protéines. En outre, la bêta-caséine glyquée a montré sa capacité à s'agréger par elle-même et à former des agrégats

spiralement enroulé ordonnés fluorescent spécifiques avec un colorant et un ligand anti-amyloïde thioflavine T. La présence de protéine prion a grandement facilité la formation de ces agrégats, leur nombre augmente, les agrégats eux-mêmes sont deux fois plus longs et ils acquièrent également une structure ramifiée émanant d'un centre commun, ce qui les rend visuellement étoilés. Dans ces conditions, nous avons montré le processus de formation des agrégats en spirale, en partant de simples structures en étoile, dont les filaments divergent d'un noyau d'agrégation. Au fil du temps, ces structures se tordent autour d'un ou de plusieurs axes, formant des agrégats en spirale. Au contraire, la présence de ligands anti-amyloïdes a supprimé la formation d'agrégats en spirale. La curcumine a provoqué une déformation et un fort raccourcissement des spirales, l'acide 3,4-diméthoxycinnamique empêchant presque complètement leur formation. Ainsi, nous doutons de l'exactitude de l'utilisation de la thioflavine T en tant que détecteur sélectif des structures amyloïdes, car, apparemment, il peut également interagir avec les produits de glycation, ce qui entraîne une augmentation de son intensité de fluorescence.

L'interaction de la protéine prion glyquée et de la bêta-caséine est spécifique aussi. Au stade de l'interaction initiale, les protéines forment des agrégats sphériques avec une structure désordonnée et une légère fluorescence de la thioflavine T, apparemment causée par son interaction avec les produits de glycation. Cependant, au cours du traitement thermique, ils sont enrichis en structures amyloïdes détectées par la thioflavine, qui interagissait avec eux et avec les produits de glycation simultanément. Apparemment, dans ce cas, l'effet antiamyloïde de la curcumine et de l'acide 3,4-diméthoxycinamique disparaît presque complètement, ce qui est probablement dû à une agrégation non spécifique des protéines qui empêche la diffusion des ligands antiamyloïdes à l'intérieur des agrégats sans interférer avec les structures internes conversion.

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Introduction

The central dogma of structural biology is that proper folding of proteins is necessary to perform their biological function. Accordingly, the lack of a stable conformation is a negative factor, since unstructured proteins within the dogma were considered as unstable components lacking physiological activity and prone to aggregation. Over time, proteins were found that function normally in an unfolded conformation, but the problem of improper folding of proteins became more and more obvious with time. Pathological aggregation of proteins is a significant problem of modern biochemistry (Leighton & Allison, 2016; Mossuto, 2013; Mulligan & Chakrabartty, 2013). Many diseases are considered to be due to improper folding of proteins and their subsequent aggregation (Geschwind, 2015; Giráldez-Pérez, Antolín-Vallespín, Muñoz, & Sánchez-Capelo, 2014; Oczkowska, Kozubski, & Dorszewska, 2014; Thal & Fändrich, 2015). At the moment, these diseases remain incurable, and any therapy against them is palliative.

Transmissible spongiform neuropathies (TSEs), also known as prion diseases, are neurodegenerative disorders deadly for both humans and animals. Prion protein (PrP) is known for its ability to aggregate into soluble oligomers that in turn associate into amyloid fibrils. One of the viable approaches towards prophylaxis and treatment of prion diseases (as well as other conformational pathologies) is based on the compounds capable of binding to the prion protein (or its aggregates), thus inhibiting its conversion into the pathologic form and ultimately slowing down further aggregation. Neurodegenerative diseases are associated with accumulation of amyloid-type protein misfolding products. Preventing the formation of these infective and neurotoxic entities represents a viable strategy to control prion diseases. Numerous attempts to find dietary compounds with anti-prion properties have been made, however the most promising agent found so far was curcumin, which is poorly soluble and merely bioavailable.

Protein glycation by various sugars and reactive dicarbonyl compounds is considered to be one of the most important posttranslational modifications, which cause numerous consequences (Iannuzzi, Irace, & Sirangelo, 2014; Oliveira et al., 2013). These include, in the first place, changes in the functioning of glycated proteins, as well as the formation of protein aggregates stabilized by covalent bonds between the final glycation products and amino groups of proteins. Identification of glycated proteins is widely used to diagnose diseases associated with hyperglycemia, neurodegeneration (J. Li, Liu, Sun, Lu, & Zhang, 2012; Vistoli et al., 2013) and aging (Baynes, 2001; Ulrich, 2001). The glycation of proteins plays an important role in the formation of pathological amyloid structures since it affects both amyloid transformation and degradation of the modified proteins with proteasome systems involving ubiquitylation (Adrover et al., 2014).

The research on food proteins, the glycation of which intensively occurs in the process of cooking at high temperatures in the presence of endogenous or externally added sugars, stands apart (Roldan et al., 2015). On the one hand, the study of glycation of food proteins is necessary to determine the effect of such a modification on the digestion of proteins in the gastrointestinal tract (Hellwig, Matthes, Peto, Löbner, & Henle, 2014b; Zhao, Le, et al., 2017; Zhao, Li, et al., 2017), on the allergenicity of proteins (Iwan et al., 2011; Lehmann et al., 2006; Suhr, Wicklein, Lepp, & Becker, 2004) and on their effect on the microbiota (Dominika et al., 2011; Qu et al., 2017). On the other hand, it is convenient to use food proteins available in large quantities as model objects in the study of the role of glycation in the induction of aggregation and amyloid transformation of proteins in order to understand the processes occurring during pathological disturbances in the development of amyloidosis.

In a number of works, it was shown that glycation of milk caseins by sugars and aldehydes causes changes in their structure, revealed by traditional methods (circular

dichroism, ThT fluorescence, etc.) and stimulates the aggregation of proteins. Casein glycation, as a rule, worsened their absorption in the gastrointestinal tract (Hellwig, Matthes, Peto, Löbner, & Henle, 2014a) and reduced allergenicity. Along with this, glycation of kappa-casein stimulated the amyloid transformation of this protein (Jindal & Naeem, 2013a). A similar effect was exerted by glycation on the aggregation of serum albumins, which under certain conditions underwent amyloid transformation (Bouma et al., 2003a; Girish & Prasada Rao, 2016). The most detailed effect of glycation on the properties of beta casein was investigated in the work of Reza Yousefi (Yousefi et al., 2017). A study was made of the effect of glycation of beta-casein with D-glucose on the structural properties and aggregation of the protein. After glycation, the protein was thermoaggregated and then, using the circular dichroism method, as well as measurements of the intrinsic fluorescence and fluorescence intensity of ThT and ANS, structural changes in the protein were studied. Structural changes in glycated casein have been identified, but no data have been obtained about the stimulation of amyloid conversion of the glycated protein. It has also been shown that the chaperone-like activity of glycated beta casein is enhanced, and the allergenicity remains practically unchanged.

Unfortunately, information about the stimulation by glycation of an amyloid transformation of proteins, including beta-casein, is not well defined. On the one hand, it is possible to detect signs characteristic of amyloid structures (primarily changes in the fluorescence of ThT (Jindal & Naeem, 2013a)) by various indirect methods, and on the other hand, using direct methods, primarily electron microscopy, it is not possible to reveal characteristic beta-casein amyloid fibrils (Yulia Y Stroylova et al., 2011). In the present work, we investigated the thermal aggregation of glycated beta-casein, paying particular attention to the effect of ThT on the formation of various aggregates, including unusual spiral structures, first discovered in this work. The

observations made appear to be important in analyzing the results obtained using the popular fluorescent labeling by ThT, which probably interacts with the final glycation products.

Abbreviations

β-CN – bovine milk beta-casein, AGE – advanced glycation end-product, BCA – bicinchoninic acid, BLG - ß-lactoglobulin; CM-lysine - carboxymethyl-lysine; DLS – dynamic light scattering, 3,4-DMCA - 3,4-dimethoxycinnamic acid; DSC - differential scanning calorimetry, DTT – dithiotreitol, EGTA – ethylene-glycol tetraacetic acid, GAPDH - glyceraldehyde-3-phosphate dehydrogenase; HPLC — high performance liquid chromatography; IPTG - isopropyl-thiogalactopyranoside; ITC - isothermal titration calorimetry, MES – 2-(N-morpholino)ethanesulfonic acid, NBT – nitro blue tetrazolium, PAAG - polyacrylamide gel; PAGE - polyacrylamide gel electrophoresis; PrP - prion protein; SDS - sodium dodecyl sulfate; TEM – transmission electronic microscopy, TFA – trifluoroacetic acid, ThT – thioflavin T.

The aim and objectives of the work

The aim of the work was to identify new amyloidogenic and amyloidoprotective factors that affect the ability of proteins to pass, provoke and suppress pathological aggregation.

Tasks:

- isolate and purify proteins possessing either amyloidogenic properties or the ability to suppress the pathological aggregation of other proteins;
- to demonstrate the ability of amyloidogenic and amyloidoprotective proteins to interact with each other and act on other polypeptides;
- identify new anti-amyloid ligands and study their effect on different types of protein aggregation;

- choose the conditions for glycation of proteins of the studied proteins and study the change in their properties after glycation;
- assess the ability of anti-amyloid ligands to inhibit amyloid conversion and aggregation of glycated proteins.

Scientific innovations of the work

The work was first shown that 3,4-dimethoxycinnamic acid effectively but not completely suppresses amylodization and aggregation of the prion protein (PrP). The effect of curcumin and its analogues on amyloid conversion and protein aggregation has been studied in detail.

β-casein, which has chaperonin-like activity, was found to significantly suppresses any aggregation of the prion protein, and together with low-molecular-weight anti-amyloid ligands, it is able to almost completely block amyloidogenesis.

Glycation of β-casein was been proven to not only deprive it of the ability to prevent the improper folding of proteins, but vice versa, causes its own aggregation, as well as stimulates its ability to cause aggregation of other proteins. Thus, non-enzymatic glycation of milk proteins was shown to impair their function and may lead to their aggregation. In addition, glycated β-casein showed the ability to form specific ordered helical aggregates after interaction with the dye thioflavin T. The presence of prion protein greatly facilitated the formation of these aggregates, while the addition of anti-amyloid ligands, on the contrary, suppressed it.

Glycation of prion protein leads to a decrease its amyloidogenicity, which confirms the results obtained previously by other researchers. At the same time, it was found that this modification somewhat facilitates non-specific aggregation of prion protein. Apparently, for the same reason, the antiamyloid effect of curcumin and 3,4-DMCA on the aggregation of the test protein was weakened, since after modification the

aggregation proceeded more in a non-specific way, which was less inhibited by 3,4-DMCA and especially curcumin.

The interaction of glycated prion protein and β-casein is also special. The proteins firstly form spherical aggregates, at initial stage with disordered structure, but subsequently they became enriched with amyloid structures. Apparently, in this case, the interaction of amino acids inherent in non-specific protein aggregation prevents the penetration of anti-amyloid ligands inside the aggregates, without interfering with their internal structures to undergo amyloid conversion, which leads to the almost complete disappearance of curcumin and 3,4-DMCA effect. Thus, changes in the property of amyloidogenic prion protein as a result of glycation were studied.

The practical significance of the research

The effectivity of 3,4-dimethoxycinnamic acid for significantly suppression of the prion protein amyloidization has been confirmed.

An effective *in vitro* method for the complete inhibition of the prion protein amyloidogenesis using antiamyloid ligands and a protein with chaperone-like activity was found.

Usage of thioflavin T fluorescence method for detection of amyloids in glycated proteins is incorrect because of interactions between glycation products and ThT and demands additional confirmations.

The effect of the widespread in nature protein modification glycation on the properties of β-casein is shown, the effects of the modification can be both dangerous and applicable for the rapid removal of proteins from solution. The effect of glycation on the amyloidogenic properties of the prion protein was also studied.

Propositions for defence

1. Curcumin has a more effective anti-amyloid agent with a wider specificity compared to 3,4-dimethoxycinnamic acid. 3,4-DMCA, on other hand, more effectively suppresses protein aggregation.

2. D-glucose β-casein glycation deprives excludes its chaperone-like activity, activates its own aggregation and the involvement of other proteins in the aggregation.

3. Glycated β-casein instead of a naturally unstructured form begins to form an ordered secondary structure.

4. Glycated β-casein is able to specifically interact with thioflavin T to form helical structures.

5. Glycation of prion protein reduces its susceptibility to anti-amyloid ligands.

6. The combined effect of anti-amyloid ligands and the chaperone-like activity of β-casein makes it possible to almost completely suppress amyloid conversion and aggregation of prion protein.

7. Glycation of prion protein lowers its amyloidogenicity, but enhances its ability to non-specific aggregation.

Approbation of the results

The results of the work were presented at scientific seminars of the Department of Animal Cell Biochemistry, A.N. Belozersky Research Institute of physico-chemical biology (September 2017, August 2018). The results of the work were also reported at 3 seminars held at the National Institute for Agronomic Research, Nantes, France (L'Institut National de la Recherche Agronomique, Nantes, France). Some results were presented at the poster session of the conference "Biomembranes 2018" (Dolgoprudny, Russia).

The results of the work were used in publications:

1. Kudryavtseva SS, Stroylova YY, Zanyatkin IA, Haertle T, Muronetz VI. (2016). Inhibition of Chaperonin GroEL by a Monomer of Ovine Prion Protein and Its Oligomeric Forms. *Biochemistry (Mosc).*, *81*(10):1213-1220.
2. Tishina, S. A., Stroylov, V. S., Zanyatkin, I. A., Melnikova, A. K., Muronetz, V. I., & Stroylova, Y. Y. (2017). Cinnamic acid derivatives as the potential modulators of prion aggregation. *Mendeleev Communications*, *27*(5), 493-494.
3. Zanyatkin, I., Stroylova, Y., Tishina, S., Stroylov, V., Melnikova, A., Haertle, T., & Muronetz, V. (2017). Inhibition of Prion Propagation by 3,4-Dimethoxycinnamic Acid. *Phytotherapy Research*, *31*(7), 1046-1055.

Other scientific works published on the thesis topic:

1. I.A. Zanyatkin, V.I. Muronetz. Activation of mammal prion protein specific aggregation by glycated β-casein. "Biomembranes-2018 International conference, Book of abstracts", Dolgoprudny, 2018, p. 382.

Personal contribution of the author

The main results were obtained personally by the author. Personal contribution is the analysis of literature data, planning and conducting experiments, as well as in the processing and analysis of the data, the preparation of publications.

Molecular docking procedures were carried out by Ph.D. V.S. Stroilov (N.D. Zelinsky Institute of Organic Chemistry, RAS), experiments on circular dichroism spectroscopy were conducted with the participation of Ph.D. A.M. Arutyunyan (A.N. Belozersky Research Institute of Physico-Chemical Biology), samples for electron microscopy were analyzed with the participation of Ph.D. E.V. Sheval and A.V. Lazarev (A.N. Belozersky Research Institute of physico-chemical biology), experiments with coaggregation of prion protein and chaperonins were carried out with S.S. Kudryavtseva, setting up techniques in the laboratory in Nantes was carried out with the help of the staff Yvan Choiset and Hanitra Rabesona (INRA).

Literature review

Food and milk proteins

Milk is one of the essential components of children's nutrition and a drink that is often consumed by adults. Milk is a very useful product due to the high content of easily digestible proteins, peptides, vitamins and other important substances for mammals. The composition of milk depends on the source. For example, in cow's milk, protein concentration is 3.3% (according to the USDA National Nutrient Database), protein is higher in goat milk (concentration is 3.6%), and in human milk, by contrast, more than 2 times less and is 1.5%. The main milk proteins are caseins, α-lactalbumin, β-lactoglobulin. Their ratio also depends on the source of milk. In cow's milk, caseins are the dominant proteins, the most common among them is αS-casein, in goat milk the concentration of αS-caseins is lower, and in human milk, on the contrary, α-lactalbumin, β-lactoglobulin prevail.

In our work, we planned to study firstly caseins, whose properties are described below.

Casein (from lat. Caseus - "cheese") is the name for a family of phosphoproteins (αS1, αS2, β, κ). They are usually found in milk, where they make up to 80% of cow's milk proteins and from 20% to 45% of human milk proteins (Kunz & Lönnerdal, 1990). Caseins have a wide range of applications in the food industry, from the main component of cheese to food additives. As a component of an important food resource - milk - casein is a source of amino acids, can carry carbohydrates, and also bind calcium and phosphorus. (“Casein”, 2011)

Casein contains a significant number of proline residues that do not interact with each other and do not allow its polypeptide chain to form extended ordered structures. Normally, it lacks such a stabilization factor as disulfide bridges: for example, β-casein does not contain cysteines at all. As a result, the percentage of peptide chain involved in the tertiary structure is low. Caseins are relatively hydrophobic proteins, due to

which they are not easily dissolved in water in the form of monomers. Therefore, in milk casein is usually represented in the form of aggregates, the so-called "casein micelles".

Despite the similarity at first sight with the micelles formed by surfactants, the internal region of casein micelles is hydrophilic. Casein molecules inside micelles are retained due to calcium ions and hydrophobic interactions. There are several models explaining the formation and internal structure of casein micelles (Dalgleish et al., 1998). One of them says that inside the micelle consists of several subvesicles held outside by κ-casein (Lucey, 2002; Walstra, 1979). According to another model, the core of the micelle is composed of casein fibrils (C Holt, 1992). One of the new models suggests the formation of cross-links between casein molecules (Horne, 1998). All models are united by the fact that k-casein is a stabilizing factor in them.

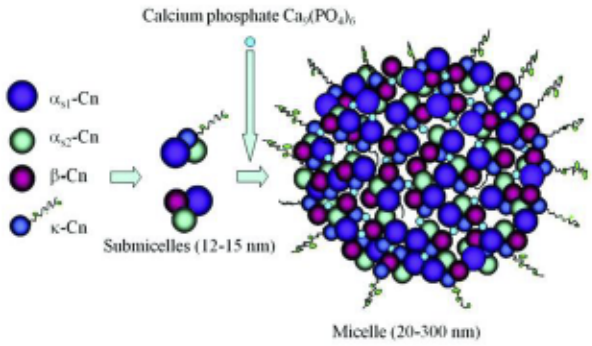


Fig. 1. The structure of casein micelles according to subvesicles model (Lucey, 2002; Walstra, 1979).

The isoelectric point of caseins is 4.6. Since the pH of milk is usually 6.6, casein molecules normally have a negative charge.

The most obvious function of casein is a food source that is easily digestible by amino acids, which is why it is so important for young mammals (Thorn, Ecroyd, Carver, & Holt, 2015).

Another obvious function of caseins is the ability to bind phosphates and calcium ions. Moreover, the formation of casein micelles is considered to be a necessary mechanism

to prevent mammary glands calcification. A typical casein micelle includes 104 protein molecules in ~ 800 CCP nanoclusters distributed in a hydrated protein matrix (Carl Holt, 2013). Based on the ability to bind calcium, caseins are divided into calcium-sensitive (αs1-, αs2-, and β-casein) and calcium-insensitive (κ-casein). Being calcium-insensitive, K-casein itself in solutions of calcium salts is readily soluble, but it also has the ability to interact with other types of calcined caseins, stabilizing them in the form of a suspension (Qi, 2007).

Finally, an important casein ability is to influence the proper assembly of proteins, like the chaperones (Carl Holt, 2013). Moreover, caseins can work as "samoshaperons": forming micelles, β-casein prevents spontaneous self-aggregation typical of αS2 and κ-caseins (Carl Holt, 2013; Thorn, Ecroyd, Sunde, Poon, & Carver, 2008).

Unorganized and amyloid protein aggregation

Increasing of protein molecule complexity and its amino acid sequence length, brings to more difficulties for the protein to maintain its secondary and tertiary structures under adverse conditions. The phenomenon of denaturation is precisely the unfolding of a protein molecule secondary and tertiary structures with the formation of an irregular statistical ball. Some proteins and even enzymes have the ability to spontaneously restore structure and even enzymatic activity, at least partially, but many proteins without special cellular machines, that restore the spatial structure of proteins, are incapable of renaturation. On the contrary, after the molecule unfolding into a statistical ball and subsequent disappearance of the factor that led to denaturation, many regions of the protein molecule open to interaction, which leads to the formation of non-covalent bonds between protein molecules, which in turn leads to their adhesion into large conglomerates and precipitation. Naturally, the protein in this state can no longer perform its function.

Most milk proteins are able to undergo precisely non-specific aggregation. Actually, that exactly happens, if, for some reason, milk as an emulsion loses its stability and stratifies. The tendency of caseins to nonspecific aggregation is well known (Larissa M. Mikheeva, Natalia V. Grinberg, Valerij Ya. Grinberg, Alexei R. Khokhlov, & Cornelius G. de Kruif §, 2003; Portnaya et al., 2006). However, many proteins, including food ones, are able to undergo ordered aggregation. An example of such aggregation is amyloidosis.

For the first time this term was used by Rudolf Virkhov in relation to protein fibrous structures found in the liver, which, like starch, were stained with iodine. The unambiguous reason of amyloidosis development has not yet been named, but at the same time it is known that in some diseases amyloidosis develops as a complication, for example, in tuberculosis, rheumatoid arthritis, chronic osteomyelitis, lymphogranulomatosis, plasmacytoma. Amyloid aggregates are formed from various proteins; currently, only for human 20 proteins that are prone to this type of aggregation are known (Selkoe, 2003). Different amyloidogenic proteins are structurally and functionally unrelated, but they form are very similar amyloid fibrils. Initially, the structure of amyloid fibrils was represented as flat anti-parallel β-layers located perpendicular to the axis of the fibrils. Hydrogen bonds connecting polypeptide chains are located along this axis (Sunde & Blake, 1997). Later, more diverse theories began to appear, based on the interaction between the β-structures of protein molecules.

Amyloid aggregation is a potentially dangerous process. A common property for this type of aggregation is a change in the secondary structure of a protein, its enrichment with β-layers, with which protein molecules interact with each other, forming very stable aggregates. The formation of amyloid structures is one of the histological signs of Alzheimer's disease (Ballard et al., 2011; Tiraboschi, Hansen, Thal, & Corey-

Bloom, 2004), moreover, at least one infectious agent is known, the consequences of infection of which are amyloid aggregates - prion protein (Prusiner, 1982). Other proteins are able to form similar aggregates (K. Pan & Zhong, 2015), but their toxic effect has not yet been proven. There is evidence of the ability of some of them (for example, cow κ-casein) to form amyloid fibrils (Farrell, Cooke, Wickham, Piotrowski, & Hoagland, 2003), especially after modifications (Koudelka, Dehle, Musgrave, Hoffmann, & Carver, 2012). Amyloidization of β-lactoglobulin (Elofsson, Dejmek, & Paulsson, 1996; Raynes, Day, Crepin, Horrocks, & Carver, 2017) is being actively studied too, similar properties are known for alpha lactalbumin (Ebrahim-Habibi, Amininasab, Ebrahim-Habibi, Sabbaghian, & Nemat-Gorgani, 2010; Goers, Permyakov, Permyakov, Uversky, & Fink, 2002). Also, although the conditions for this process have not yet been found, it is believed that αS1-casein is also capable of amyloidization (Carl Holt, 2013). However, the fact that a protein is formed in amyloid structures is itself considered potentially dangerous.

Protein glycation in nature

Protein glycation by various sugars and reactive dicarbonyl compounds is considered to be one of the most important post-translational modifications causing numerous consequences (Iannuzzi et al., 2014; Oliveira et al., 2013). These primarily include changes in the functioning of glycated proteins, as well as the formation of protein aggregates stabilized by covalent bonds formed between the end products of glycation and the amino groups of proteins that are free in the native state. Protein glycation plays an important role in the formation of pathological amyloid structures, since it affects both amyloidization itself and the degradation of modified proteins by proteosomal systems involving ubiquitinylation (Adrover et al., 2014).

The main pathway for the appearance of glycated proteins in the body is a spontaneous non-enzymatic reaction. This process is observed at high concentrations of free

carbohydrates in the medium, as a result, often takes place in patients with diabetes. This reaction involves reducing sugars (glucose, fructose, galactose, etc.) and free NH-groups in the protein, mainly lysine, less often arginine. In the first stage of the reaction, a Schiff base is formed, which can serve as the starting material for many variations. One of these is methylglyoxal formed from the intermediate 3-deoxyglucosone; the other ones are Amadori products or fructosamine. Schiff bases and Amadori products are classified as early glycation products. The amount of the body's proteins modified by this way (for example, blood hemoglobin) is proportional to the concentration of glucose or other reducing sugars in their environment: the amount of modified proteins increases with increasing glucose levels in the blood and decreases with its decrease.

Amadori products can further undergo a series of slow irreversible rearrangements with the formation of the so-called late, or end, glycation products (AGE). The amount of proteins modified by AGE does not decrease with a decrease in glucose level and only grows with time. Moreover, some of the AGEs can form covalent bonds (crosslinks) with the amino groups of other protein molecules. One of the most common representatives of AGE is Nε-carboxymethyl lysine (here in after KM-lysine), which is capable with hydrogen peroxide releasing (i.e., active oxygen forms), or with dehydration to form Endion Amadori, which in turn can form crosslinks between protein side chains (Younus & Anwar, 2016). In addition, during the "maturation" of glycation products, relatively low molecular weight nonprotein products can be formed, such as methyl glyoxal derivatives, which have a high reactivity to modify other proteins.

Proteins can be glycated before eating, after which the glycation products of these proteins can be ingested. For example, glycation is often used to add protein food a brighter taste. However, the digestibility of these modified amino acids may vary

significantly(Hellwig et al., 2014a; Zhao, Le, et al., 2017; Zhao, Li, et al., 2017). In this regard, separate studies have been carried out on food proteins, the glycation of which occurs intensively during cooking at high temperatures in the presence of endogenous or externally added sugars (Roldan et al., 2015). It has been shown that lysinoalanine and casein-bound N-ε-(γ-glutamyl)-L-lysine are absorbed to a very small extent. In the course of new research, the possible formation of bis-carboxymethyl lysine during digestion was shown. In general, the digestibility of glycated casein decreased in comparison with the native protein from 78.3% to: in the case of undirected glycation - 61.4%, protein enriched with CM-lysine - 57.6%, enriched with lysyl-alanine - 56%. However, the effect of glycation on the splitting was relatively small. The distribution of glycation products in the resulting peptide fractions, separated by chain length, vary for different products: lysine-alanine remains mainly in long peptide chains (with M = 4–20 kDa) and therefore is poorly absorbed, fructose lysine is contained in shorter chains (M = 1-4 kDa) and therefore passes into the body more easily, CM-lysine is localized in the shortest peptides (M = 0.5-1 kDa) and therefore absorbed at the level of unmodified amino acids.

In summary, the reduced digestibility of the modified protein and the different absorbability of glycation products of amino acids leads to a large difference in the bioavailability of the modifying agents: CM-lysine - about 50%, fructozozolysin - 40%, lysinoalanine - not more than 10% of the possible. Thus, CM-lysine is the most bioavailable form of protein glycation products (Hellwig et al., 2014b), which suggests that it is possible for glycation products to enter the body.

Consequences of non-enzymatic protein glycation

Numerous studies have shown that protein glycation often leads to disfunction of proteins, even as common modification *in vivo*. Protein glycation plays an important role in the formation of pathological amyloid structures, since it affects both

amyloidization itself and the degradation of modified proteins by proteasomal systems (Adrover et al., 2014). The identification of glycated proteins is widely used to diagnose diseases associated with hyperglycemia, neurodegeneration (Vistoli et al., 2013) and aging (Ulrich, 2001).

In the majority of works (Bouma et al., 2003b; Girish & Prasada Rao, 2016), experiments on the glycation of food proteins were carried out on serum albumin, most often bovine. In all these works, it was shown that protein glycation facilitates amyloidization. Studies of the effect of glycation of kappa-casein on its ability to aggregate were also carried out (Jindal & Naeem, 2013b), and similar results were obtained: it was confirmed that protein amyloidization was facilitated after glycation by thioflavin T fluorescence, circular dichroism and infrared spectroscopy with Fourier transform.

Most of researches related to the glycation of amyloidogenic proteins has shown that this modification accelerates their conversion. A similar tendency was found for β-amyloid (Jana, Batkulwar, Kulkarni, & Sengupta, 2016), it is also known about the similar effect of glycation on the behavior of apomyoglobin, the glycation of which promotes the formation of highly toxic oligomers (Iannuzzi et al., 2015). There is evidence that high blood sugar levels, which increase the risk of non-enzymatic protein glycation, can be a risk factor for the development of not only type I diabetes, but diseases associated with pathological aggregation, such as Alzheimer's or Parkinson's disease (Guerrero, Vasudevaraju, Hegde, Britton, & Rao, 2013; Vicente Miranda, El-Agnaf, & Outeiro, 2016). Moreover, it was confirmed that, for example, in the case of Parkinson's disease, the glycation of α-synuclein and the accumulation of late AGE glycation products precede the formation of Levi bodies and the development of parkinsonian symptoms and does not disappear during protein aggregation. At the same time, the effect of glycation on amyloidogenic proteins may be different. Thus,

several researchers have shown that glycation of the prion protein led to the suppression of the formation of amyloid fibrils (Panza, Dumpitak, & Birkmann, 2010) and a sharp decrease in infectivity (Suyama et al., 2007), however, this phenomenon has not yet been studied in sufficient detail.

The effect of glycation on caseins

Casein glycation studies were carried out mainly *in vitro*. This was the way (Moeckel, Duerasch, Weiz, Ruck, & Henle, 2016) to study the effect of glycation on micellar and monomeric forms of casein (the researchers used total casein without separation of isoforms). Laboratory sodium caseinate was used as a source of monomeric forms, and natural milk casein as a source of micellar form. The casein from milk was firstly purified by filtration from the fat fraction and subjected to precipitation at the isoelectric point. The modification was carried out by boiling purified casein (imitation of milk boiling). The resulting products were subjected to acidic and enzymatic hydrolysis. The obtained preparations were analyzed for the content of: Amadori products (N-fructosyl-lysine -> furosin as a result of acid hydrolysis), lysine dimers of glyoxal derivatives and pentosidine (after acid hydrolysis), pyrroline (after enzymatic hydrolysis), CM-lysine; analyzed the amino acid composition of the hydrolysis products (search for lysine and crosslinks between the amino acid residues of lysine alanine), determined the degree of polymerization by gel chromatography (calibration was carried out using polystyrene sulfonate), looked for changes in size (DLS) and surface structure (scanning electron microscopy) micelles.

As a result, the formation of Amadori products for micelles was shown to be slightly more active: there was an increase in pyrroline in the micellar form in comparison with the monomeric, the amounts of KM-lysine were approximately the same. During glycation, the di- and trimers remained minor fractions, and their concentration did not exceed 10% of the total protein (while remaining approximately the same throughout

the boiling time). Either monomers or vice versa prevailed in solutions, oligomers with more than 4 protein molecules. For casein from micelles, the process of formation of oligomers was faster and after 4 hours of boiling captured 60% of the protein, while monomeric casein was included in oligomers only ~ 17%.

In the absence of glucose, glycated caseins (especially the micellar form) formed aggregates due to lysyl-alanine crosslinks. Late glycation products, like pentozidine, were not found. Glycated protein aggregated much more actively than it could due to only lysyl-alanine cross-links, i.e. new unexplored crosslinks caused by glycation were formed (for example, glucosepan - lysine-arginine crosslinking).

The particle size almost did not change from glycation level. Micelles without glycation after long-term heating began to form the monomer fraction. Glycated micelles formed monomers less actively. According to microscopy, glycated micelles were more stable (unglycated ones started to form small aggregates), but their surface was less structured (Moeckel et al., 2016).

Another group of researchers (Jindal & Naeem, 2013b) conducted a study of glycation effect on k-casein. To determine the properties of the modified protein, they used ANS-fluorescence, ThT fluorescence (theis way allows to detect amyloidization), self-fluorescence of glycation products, CD, and IR spectroscopy with Fourier transform. The fluorescence of ANS showed firstly an increase, and then a decrease in intensity, which the authors associated with the melting of the protein structure at initial stages, and later formation of a new structure. Moreover, this new structure enhanced the ThT fluorescence and led to a shift of the CD peak in the far UV region to a longer wavelength region. In the near-UV region, incubated with glucose, κ-casein showed only a decrease in the value of the total CD peak, and in the presence of fructose, the protein showed a decrease in the value and shift of the total CD peak in the long-wavelength region. According to IR spectroscopy, over time, the protein has a peak of

absorption characteristic of enriched β-structures. However, with all this, researchers have repeatedly come across data that violate trends, and explained that by formation of "molten globule" as intermediate product (Jindal & Naeem, 2013b).

According to the conditions of glycation, the study of the Yousefi team (Yousefi et al., 2017) can be considered the closest to our work. They investigated the effect of β-casein glycation with D-glucose on amyloid conversion and protein aggregation. The protein modification conditions very similar to ours were used: a slightly lower concentration of sugar was compensated by a long incubation time. Also an additional thermoaggregation of protein was conducted. Experiments were performed to evaluate the chaperonin-like activity of β-casein glycated in this way, which showed an increase in the ability of the protein to suppress the aggregation of other proteins, with the exception of human hemoglobin.

Unfortunately, the information on the glycation stimulation of proteins amyloid transformation, including β-casein, is not well defined. On the one hand, using various indirect methods, it is possible to detect signs characteristic of amyloid structures (first of all, changes in thioflavin T fluorescence (Jindal & Naeem, 2013b)), and on the other hand, using direct methods, for example electron microscopy, detection of characteristic amyloid β-casein fibrils manages wasn't successful (Yulia Y Stroylova et al., 2011).

Prion proteins as known sources of amyloid structures

Prions (PrP) - a group of proteins found in mammals and yeast. A distinctive feature of prion proteins is their ability to form 2 stable conformations: native protein (PrP^{C}) and infectious prion (PrP^{Sc}). The infectious form, by interaction with a protein in the native conformation, causes changes in the tertiary structure in it like its own, i.e. works a carrier of information about the structure of the protein molecule (Prusiner & Kingsbury, 1985).

Yeast prion protein is not potentially dangerous for its carriers, unlike mammalian protein. The mammalian prion protein (PrP) is a protein localized on the outer side of the cytoplasmic membrane and, to a lesser extent, in the extracellular medium. The protein can be inserted into the cell membrane in any orientation, both the N- and C-terminal domains outside, while the transmembrane domain remains in the region of residues 111-134. In addition, it can be fixed on the outside of the cell membrane by means of a glycophosphatidyl inositol "anchor" (Prusiner & Kingsbury, 1985). Inside the cells, this protein is stored either in EPR, from where it is transported to the cell membrane after synthesis, or in endosomes that do not contain lysosomal markers, i.e. not intended for splitting content. This protein pool can be enriched from the cell membrane by endocytosis, after which it can either undergo proteolytic cleavage at the site in the region of the N-terminal domain between Thr114 and Met137 (specific amino acids may differ depending on the type of protein host, but in general the region remains highly conserved) or return to the cell membrane (Shyng, Huber, & Harris, 1993). PrP is encoded by the PRNP gene located in chromosome 20 (B.-Y. Choi et al., 2009). The 23 kDa prion protein molecule contains 250-260 amino acids. The protein contains 11 lysine residues, which is glycated first, and 12 arginine residues. PrP is a partially unfolded protein; its structure can be divided into 2 parts based on its structure. The N-terminal unfolded domain includes an address sequence, several repeating octapeptides enriched with histidine and capable of coordinating the binding of divalent metal ions, charged and hydrophobic clusters. The C-terminal structured part of the molecule in the native conformation forms 3 α-helices (H1, H2 and H3) and 2 loops between them. The spirals H2 and H3 are linked by a disulfide bridge. The structured part of the molecule, when it enters the form enriched with β-layers, is resistant to the action of proteinase K. At the C-terminus there is a glycophosphatidylinositol binding site through which the protein can be anchored in the membrane.

Information about prion protein functions of mammals is poor. Presumably, they are involved in signaling cascades of apoptosis; it is possible that they are designed to concentrate bivalent metal ions (Colby & Prusiner, 2011) and signaling molecules that control cell adhesion (Petit, Besnier, Morel, Rousset, & Thenet, 2013) at the cell membrane; there is information about the participation of prions in embryogenesis and the maintenance of the pluripotency of tissue-specific stem cells (Aguzzi & Polymenidou, 2004), and the management of circadian rhythms (Lawson, Collins, Masters, & Hill, 2005). In addition, it was shown that the expression of prion protein was greatly reduced in animals with a type 2 diabetes model (Pham, Dhar, Khalaj, Desai, & Taghibiglou, 2014).

Amyloid conversion of prion protein

It is not known exactly which amino acids and parts of the PrP protein chain are involved in amyloid conversion. One of the traditional theories says that in the infectious conformation of the protein, the α-helix H1, together with a part of the α-helix H2 and a hydrophobic cluster from the unstructured domain, form large "β-sheets" (K. M. Pan et al., 1993). With these "sheets", prion molecules stick together, first forming oligomers, and then a rigid fibril. Modern studies also indicate the importance of the C-terminal domain in the process of amyloid conversion, even amino acids have been identified, the replacement of which prevents the transition of PrPC into the infectious form, for example, Tyr-169 (Kurt, Jiang, Bett, Eisenberg, & Sigurdson, 2014). In addition, the currently known alleles of sheep prion protein also differ in point amino acid substitutions in the C-terminal domain (for example, in the ARR - VRQ allele series, the tendency to form amyloid aggregates increases, these proteins differ in amino acids at positions 136, 154 and 171). It was also shown that PrP, which is located in the cytoplasmic membrane or EPR membrane with the C-terminal domain in the EPR cavity or outside the cell, is primarily involved in the process of amyloidization in vivo. At the same time, there are data showing that the N-

terminal unstructured domain is quite capable of forming amyloid structures, but by a different mechanism than the C-terminal domain. As a result, spherical aggregates, not elongated, are formed (Shiraishi, Utsunomiya, & Nishikimi, 2006).

The mechanism of amyloidosis is currently unknown. Studies (Rezaei et al., 2005) have shown that, regardless of the aggregation model, prion amyloidization passes through a stage of intermediate oligomers. A feature of these aggregates is that they consist of a certain number of monomers, for example, sheep PrP^{Sc} forms conglomerates of 12 or 36 molecules. In addition, it was shown (Rezaei et al., 2005) that oligomers are the most dangerous infectious agent, as they are more resistant to proteolysis than monomers and at the same time not as bulky as fibrils.

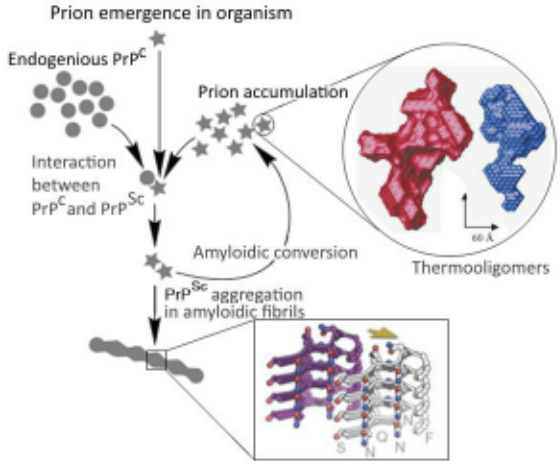


Fig. 2. The principle of prion protein amyloid conversion.

This idea is also confirmed by the fact that diseases associated with prion alleles and more prone to the formation of oligomers and slower forming fibrils, are much more transient (B.-Y. Choi et al., 2009).

Regarding the complete mechanism of amyloidization and aggregation of prion protein, several theories have been proposed (see Fig. 3):

A) The matrix-assembly model assumes the need for a fibril embryo, when interacting with it, PrP^C changes its conformation to an infectious and then sticks to it (Lopes & Santos, 2012).

B) According to the heterodimer model, protein conversion occurs through the interaction of the PrP^C monomers and PrP^{Sc} (Jarrett & Lansbury, 1993).

C) The nucleation model is based on the assumption that the prion protein in the body forms both native and infectious forms, which are linked by a reversible reaction. The formation of aggregates shifts this equilibrium towards the converted form (Prusiner, 1991).

D) According to the conformational conversion model, PrPC can change the conformation only as part of an oligomer composed of native protein molecules (Serio et al., 2000).

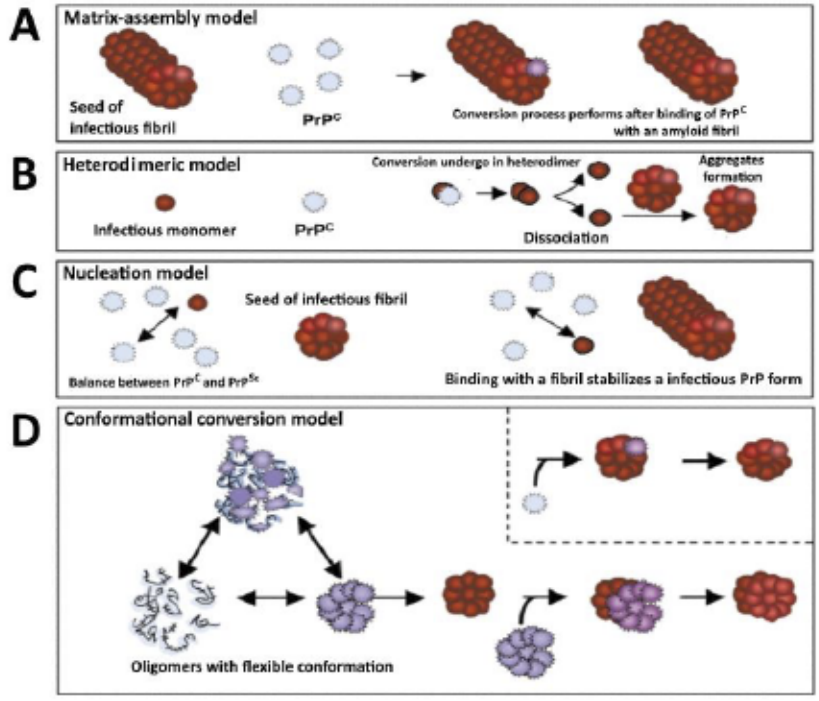


Fig. 3. Models of prion conversion.

The mechanism of amyloid fibrils cytotoxic effect is poorly studied (Poggiolini, Saverioni, & Parchi, 2013; Rezaei et al., 2005). There is evidence that prion amyloidization leads to oxidative stress of cells, activation of the complement system, disruption of EPR, which leads to autophagy and apoptosis of cells. Perhaps prion aggregates can interact with cellular membranes and destroy them (Rezaei et al., 2005). In mammals, the cytotoxic effect of prion is manifested mainly in nerve tissues: on histological sections, the brain of an organism infected with PrPSc becomes similar to a sponge due to a strong increase in endocytosis vesicles in neurons (Kumar, Vinay; Abbas, Abul; Aster, 2015). Subsequently, the neurons begin to die en masse, which leads to the accumulation of astrocytes in the lesions, while no inflammation occurs (Belay, 1999). Due to the morphology of the affected nerve tissue, all diseases of the prion etiology are called spongiform encephalopathies (scrapie of sheep, "mad cow disesase" for cattle; kuru for sheep, Creutzfeld-Jacobs syndrome for humans etc.) (Prusiner & Kingsbury, 1985). Some of them have been known for a long time (for example, sheep scrapie, also known as "scratcher").

Human prion diseases were discovered in the 50s: among the Aborigines of New Guinea, in whom ritual eating of deceased tribesman brain was common, scientists found a disease known as kuru. However, it was only in the 80s of the 20th century that the cause of this disease was shown to be a prion protein (Prusiner & Kingsbury, 1985). Some of the spongiform encephalopathies are hereditary, some are capable of horizontal transference.

Table 1. Diseases of prion etiology and organisms susceptible to them.

Affected animal(s)	**Disease**
Sheep, goat	Scrapie
Cattle	Bovine spongiform encephalopathy (BSE), mad cow disease

Mink	Transmissible mink encephalopathy (TME)
Elk, deer	Chronic wasting disease (CWD)
Cat	Feline spongiform encephalopathy (FSE)
Ostrich	Spongiform encephalopathy (Not been shown to be transmissible.)
Human	Creutzfeldt–Jakob disease (CJD)
	iatrogenic Creutzfeldt-Jakob disease (iCJD)
	variant Creutzfeldt-Jakob disease (vCJD)
	familial Creutzfeldt-Jakob disease (fCJD)
	sporadic Creutzfeldt-Jakob disease (sCJD)
	Gerstmann–Sträussler–Scheinker syndrome (GSS)
	Fatal familial insomnia (FFI)
	Kuru

There are currently several known routes for PrP^{Sc} to enter the body. This is mainly the use of food containing nerve tissue (an example of that is kuru). There is an assumption that prions can be transmitted through poorly cleaned medical, for example, dental, instruments (PrP^{Sc} is very resistant to traditional methods of disinfection, such as UV irradiation (McDonnell, 2008; Tamgüney et al., 2009)). Prions cannot pass through the epithelium, therefore they enter the bloodstream only through microtraumas. Further, infectious proteins through the cells of the immune system get to lymphatic system, later they are found in the nerve tissues, where they have the most destructive effect (Kovacs & Budka, 2008).

The increase in the intensity of prion diseases study occurred in the 90s, when in the UK, due to the violation of animal feed production technology, there was an outbreak of scrapie among the sheep and the bovine spongiform encephalopathy among cows (P. G. Smith & Bradley, 2003). Until then, it was believed that the spread of prion diseases was strictly limited to interspecific barriers, but by 1997 there were reports from Great Britain and France about human infection after eating bull meat with "mad cow disease" (Bateman et al., 1995; Cousens, Vynnycky, Zeidler, Will, & Smith, 1997). At the moment, despite the insignificant number of obvious cases of prion diseases in humans, many experts believe that there is a high degree of risk of manifestations of "slow" infections.

For today methods for prion diseases treatment and, in general, amyloidosis has not been developed. Their search is conducted in the following directions (Mason, Kokkoni, Stott, & Doig, 2003):

1. Inhibition of amyloidogenic protein expression or its stabilization in the native form using low molecular weight organic ligands;

2. Inhibition of infectious protein release from host cell through the activation of intracellular proteases;

3. Direct inhibition amyloidogenic protein aggregation of using organic ligands or vaccines;

4. Inhibition of amyloidosis effects, possibly not directly related to it (for example, oxidative stress) and alleviating the symptoms of the disease without directly suppressing the pathogenic process.

The first strategy is most effective, but it leads to synthesis of both infectious and native protein suppression, which can perform a vital function in the body (Ferreira, Saraiva, & Almeida, 2012). For example, it was shown that mice with a knockout PRNP gene

are characterized by an increased tendency to outbursts of unmotivated aggression (Maglio, Perez, Martins, Brentani, & Ramirez, 2004). Activation of proteases can also lead to a negative systemic effect, for example, to the concomitant proteolysis of the necessary native polypeptides. The use of the latter strategy is difficult due to the poor knowledge of the cytotoxic effect of amyloid aggregates (McDonnell, 2008). Perhaps the direction of activation of the anti-inflammatory system through the suppression of epoxide hydrolase and an increase in the concentration of epoxygenated fatty acids will be promising. However, this pathway increases cell survival, but does not eliminate the root causes of amyloid aggregates (Poli et al., 2013). Therefore, the main efforts of scientists are aimed at finding compounds that can directly inhibit amyloidogenesis. Quite a few such substances are already known: for example, it has been shown that β-amyloid peptide aggregation can suppress both low molecular weight ligands, such as rifampicin, hemin, and nearby porphyrins, as well as larger molecules, such as β-cyclodextrin (Ferreira et al., 2012). Prion itself also binds cationic porphyrins, which suppresses its aggregation, and it is known that this process is pH-dependent (Xiao et al., 2013). The ability of protein molecules to interact with PrP Sc has also been shown (Shiraishi, Inai, Hirano, & Ihara, 2011). Amyloidogenesis of another transthyretin protein can inhibit both small ligands (already known 18 (Ferreira et al., 2012)) and, for example, polyphenols (Maglio et al., 2004). Substances capable of in vitro binding to PrP molecules (Perez-Pineiro et al., 2011) are already known, and some, presumably, even suppress (Hafner-Bratkovič, Gašperšič, Šmid, Bresjanac, & Jerala, 2008) the formation by prion of intermediate oligomers — the main infectious agents (Rezaei et al., 2005) —and fibrils, which are attributed to most of the damage caused to cells (McDonnell, 2008). Moreover, to suppress aggregation, the ligand can bind, not only with the C-terminal, but also with the N-terminal unstructured domain (Shiraishi et al., 2011).

Glycation of prion protein

Prion protein undergoes some post-translational modifications in vivo, including glycosylation. It was shown that up to 2 glycophosphatidyl-inositol (GPI) oligosaccharides are attached in the native form to the C-terminal domain of the prion protein. In this case, the modification occurs on the asparagine residue (Haraguchi et al., 1989) in the region of amino acids from 231 to 254, while the attached oligosaccharides have a wide variation, which gives rise to 400 different isoforms of the prion protein and its converted form (Endo, Groth, Prusiner, & Kobata, 1989). Further studies have shown that protein molecules glycosylated by oligosaccharides containing 3 or 4 branches predominate in the amyloidized form (Rudd et al., 1999). C-terminal glycosylation can also affect the degree of glycation of the N-terminal residues of the prion protein, but not directly, but depending on the sequence of amino acids contained in the glycosylation region: molecules that do not contain sites for glycophosphatidyl inositol "tails" are also not glycated and N-terminal domain (A R Walmsley, Zeng, & Hooper, 2001), but at the same time N-terminal glycation was carried out again, even if the C-terminal signal sequence was cleaved prior to its glycosylation or was itself present, but did not contain asparagins (Adrian R Walmsley & Hooper, 2003).

The question of glycation pathological influence on PrP remains open. It is known that in organisms affected by various forms of spongiform encephalopathy, the converted prion binds late glycation products at Lys23, Lys24, and Lys27 sites. Similar information is available on the Arg37 site (Y.-G. Choi et al., 2004). It is unclear whether these modifications are the cause or consequence of amyloid conversion, however, it has been shown that modifications occur in parallel with amyloidization and that this glycation pathway leads to the formation of cross-links between PrP^{Sc} molecules, which contributes to an increase in resistance of protein aggregates to proteolysis (Salahuddin, Rabbani, & Khan, 2014). It was found that prion, as a carrier

of information, stores in itself information about not only the secondary protein structure, but also about level of its modification - so after infecting animals with prion, the level of PrP glycation in their bodies aspired to that was given by the infectious agent (Collinge, Sidle, Meads, Ironside, & Hill, 1996). There is also information about the glycation of prion in the composition of amyloid structures and in the unstructured region that undergoes amyloid conversion and is involved in the formation of β-folds that are resistant to the degradation by proteinase K (Y.-G. Choi et al., 2016). In addition, it is known that a prion protein, unglycated at the Thr180 and Thr196 sites, is usually localized inside the cells, whereas the protein monoglycated at any of these sites is outside the cells. In addition, the protein, mutated in close to these positions in order to prevent modification by sugars, changed its tendency to amyloidization. The replacement of threonine with alanine at a position closer to the N-terminus, significantly increased the resistance of the protein to infectious prions, and also excluded its transport between cells (Gorodinsky & Harris, 1995), which, accordingly, greatly complicated the spread of infection through the nervous system. A similar replacement of threonine in the position closer to the C-end did not exclude amyloid conversion of the protein completely, but significantly slowed it down (DeArmond et al., 1997). C-terminal glycosylation was also partially correlated with neurodegenerative diseases, although it did not rule out their development. Studies of protein samples from organisms showed that monoglycosylated PrP^{Sc} usually prevailed in sporadically arising prion diseases, and diglycosylated in hereditary forms of the same diseases (Biacabe, Laplanche, Ryder, & Baron, 2004; Casalone et al., 2004).

At the same time, experiments with recombinant PrP in vitro have shown that uncontrolled non-enzymatic glycation of the prion protein suppresses its amyloid aggregation (Kudryavtseva, Melnikova, Muronetz, & Stroylova, 2018; Panza et al.,

2010). The glycated PrP underwent amyloid conversion much slower, regardless of which agent it was modified by.

Chaperones and their interaction with misfolded proteins

Molecular chaperones ("chaperon" (Fr.) - an elderly lady accompanying a young girl to the balls, or a mentor accompanying a group of young people) are a class of proteins whose main function is to restore the correct native tertiary or quaternary structure of proteins, as well as formation and dissociation of protein complexes.

The term "molecular chaperone" was first used (Laskey et al., 1993) in the description of the nuclear protein of nucleoplasmin, which is able to prevent the aggregation of histone proteins with DNA during the formation of nucleosomes. Many chaperones belong to the group of "heat shock proteins" (heat-shock proteins), that is, their expression in the cell increases dramatically in response to elevated temperatures or other causes of cellular stress (Ellis & van der Vies, 1991). The reason for this behavior is that the folding of the protein and the stability of its secondary and tertiary structures are strongly influenced by heat, and therefore some chaperones act to prevent or correct the damage caused by thermal denaturation and incorrect folding. Other chaperones are involved in the folding of complex proteins, linking them directly in the process of the synthesis of the folding protein on the ribosome. Some of the proteins synthesized in the cell are usually the simplest structurally, can spontaneously fold in the absence of chaperones, but most complex enzymes, especially with a non-protein active center, can adopt the correct conformation only with the assistance of folding factors. Particularly often, chaperones are needed in the synthesis of membrane proteins or proteins directed to the endoplasmic reticulum (EPR) for further post-translational modifications and transport. This is due either to the fact that modifications must be made before complete folding of the protein, or the need to avoid inappropriate

interaction of the hydrophobic regions of the protein, intended either to form an active center or to anchor in the membrane.

Chaperones have been found in almost all organisms. A very accessible object for research is the GroEL/GroES bacterial chaperonin complex. Its closest homologue is the eukaryotic "heat shock protein" Hsp60. This protein is found in mitochondria. In them, it, firstly, facilitates the transport of proteins through the membrane into the mitochondria, and secondly, ensures the correct folding of 15 to 30% of all proteins of the cell (Ranford, Coates, & Henderson, 2000). Eukaryotic chaperones are divided into basic, lectin and non-classical. One of the famous eukaryotic chaperonins is TriC chaperonin. It is located in cytoplasm and is responsible for the correct folding of about 10% of all eukaryotic proteins. Structurally, it is similar to the bacterial GroEL/GroES complex, but does not require cochaperone for its functioning (in the GroEL/GroES complex, the GroES protein is cochaperone). It is also noteworthy that eukaryotic cytoplasmic chaperones are homologous to the archaea chaperones (Gupta, 1990).

Molecular chaperones are divided into two groups. Classical chaperones bind to the synthesized polypeptide chain, fixing it on their surface and thus blocking the folding of the "patronized" protein (including the wrong one) until it gets into conditions that promote proper folding. They also participate in the transmembrane transport of proteins, keeping the polypeptide chain of their "protege" in the unfolded state, because it is in this form that the protein chains are "pulled" through the membrane. True chaperonins exist just to create favorable conditions for protein folding, and the mechanism of their action — non-covalent attachment to proteins and their "unwinding" using the energy of ATP hydrolysis — is conservative. In contrast to fairly simple built chaperones (consisting of one or two polypeptide chains), chaperonins are often complex oligomeric structures that together form an isolated cavity from the external environment, also called the Anfinsen cell. The protein is

"loaded" into the chaperonin (usually detaching from the chaperone molecule) in the state of "molten globule" and binds the hydrophobic regions of the "walls" of the central channel of the chaperonin molecule. This interaction stimulates the addition of ATP, which changes the structure of the chaperonin (the cavity is isolated from the external environment by either closing the channel with the chaperonin itself (eukaryotic TriC), or by binding to a special co-chaperonin that blocks the passage (GroEL/GroES complex) are shielded), and the protein is partially released, moving into the central channel. Spontaneous folding will continue until ATP hydrolysis occurs and the chaperonin is transferred to a state capable of binding a partially unfolded protein. The closer the structure is to the native one, the smaller are the areas "recognized" by chaperonin. Finally, the foldable protein reaches its native conformation and leaves the cavity.

In most cases, chaperones are inhibitors of protein aggregation and contribute to their refolding (Jeng, Lee, Sung, Lee, & Tsai, 2015). In the body, chaperonins can be applied by activating the capacity of the organism itself, to a lesser extent by chaperonins addition from the outside (Hartl, Bracher, & Hayer-Hartl, 2011; Jeng et al., 2015; Papsdorf & Richter, 2014). Moreover, this may not even require any complex transfection or other non-native routes of protein introduction into the body. For example, another protein of the casein family, β-casein, is known for its chaperone-like activity (Yousefi et al., 2009; Zakharchenko et al., n.d.). At the same time, molecular chaperones, apparently, do not create a new pathway for protein folding, but only accelerate the folding along the proper protein path and protect it from accidental folding. But if the misfolding is not stochastic, but ordered, the chaperonins may not be effective. Thus, GroEL/GroES and TriC chaperonins are apparently unable to prevent amyloid conversion of the prion protein and even, on the contrary, provoke it to accelerated amyloidization (Kiselev et al., 2011; Y. Y. Stroylova, Kiselev,

Schmalhausen, & Muronetz, 2014), which, however, requires additional testing. In addition, there is already information about usage of chaperone activity inhibition for medical purposes. For example, the suppression of mitochondrial protein complexes Hsp90 activity is considered as a potentially useful method for the elimination of oncological formations (Schmid, Haslbeck, Buchner, & Somoza, 2008). In addition, there are proposals for selective inhibition of viral chaperones, which ensure the assembly of the capsid of the human immunodeficiency virus, which, accordingly, should reduce its virulence (Avilov, Boudier, Gottikh, Darlix, & Mély, 2012).

Subjects of research

Amyloidogenic proteins

Sheep prion protein PrP (23 kDa, 256 a / c, GenBank: AAC78726.1), used in the work, belonged to an aggregation prone allele (amino acids Val-136, Arg-171, Gln-154 are characteristic of the allele, therefore designated VRQ).

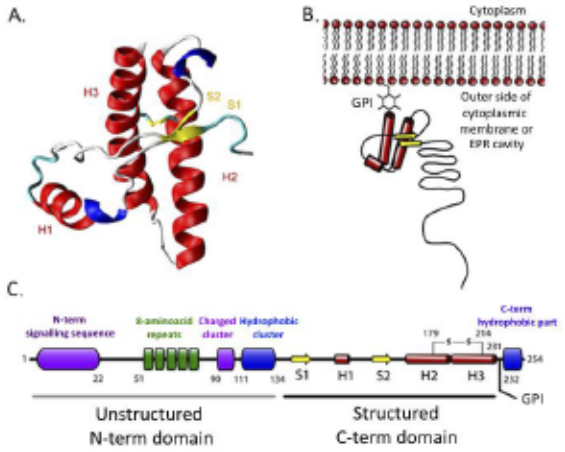


Fig. 4. A) Model of sheep prion protein structure, B) The way of PrP anchoring in the membrane, C) Domains in prion protein amino acid chain.

The prion protein molecule is divided into 2 domains. The unstructured N-terminal domain includes octapeptide repeats, enriched with histidine, charged and hydrophobic clusters, which are necessary for anchoring in the membrane. The C-terminal domain includes 3 long α-helices and 2 short β-sheets. After amyloid conversion, the α-helix

H1 undergoes disassembly and with a portion of the unstructured domain is involved in the formation of extensive β-layers, with which the protein molecules interact with each other.

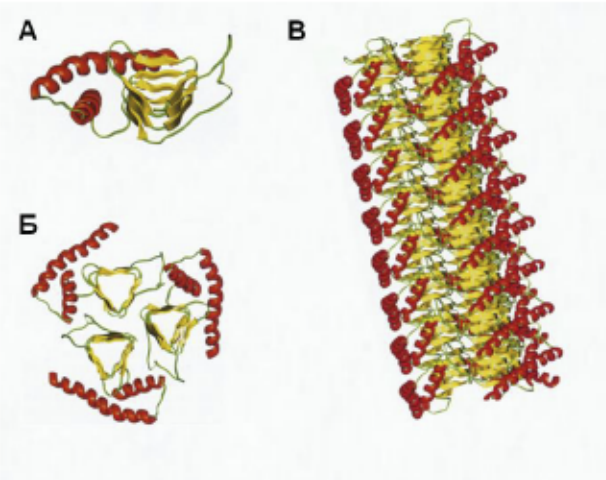


Fig. 5. The proposed structure of PrP^{Sc} (A), the trimer formed by this protein (B) and protofibril (C).

The amino acid sequence of sheep prion protein:

```
  1 mvkshiqswi lvlfvamwsd vqlckkrpkp gggwntggsr ypgqgspggn ryppqggggw
 61 gqphgggwgq phgggwgqph gggwgqphgg ggwgqgggshs qwnkpskpkt nmkhvagaaa
121 agavvgglgg ymlgsamsrp lihfgndyed ryyrenmyry pnqvyyrpvd rysnqnnfvh
181 dcvnitvkqh tvttttkgen ftetdikime rvveqmcitq yqresqayyq rgasvilfss
241 ppvillisfl iflivg
```

Lysine residues, which undergoes glycation in the first place (total 11), marked red, arginine - blue (total 12). Plus 1 alpha-amino group - a total of 24 free amino groups per one molecule of unmodified protein. The N-terminal signal sequence, which is removed during processing of protein and absent in recombinant one, is underlined. Amino acids 1-131 are part of the N-terminal unstructured domain; 131-256 - C-terminal structured.

In our experiments, we used recombinant sheep ovarian recOvPrP protein obtained by expression in a special strain of E. coli bacteria transformed with a plasmid with the gene of the target protein under the lactose operon.

κ-casein - 190 aa, (23 kDa, 256 a / c, GenBank: CAA33034.1) - is a minor component of milk proteins. only this casein is unable to bind calcium. The localisation of κ-casein on the surface of casein micelles stabilizes the boundary between the hydrophobic zones of the micelle and the hydrophilic environment.

One of the main properties of caseins is their natively unfolded structure (Naqvi, Irani, Katanishooshtari, & Rousseau, 2016), which makes it difficult to compose the 3D structures of these proteins. So, for κ-casein, models of its structure are mainly presented.

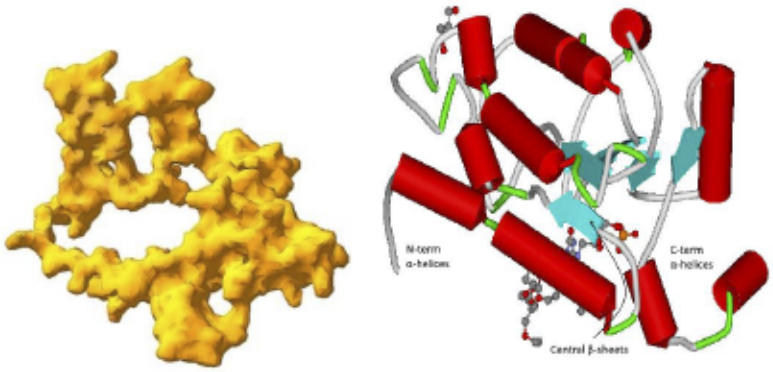
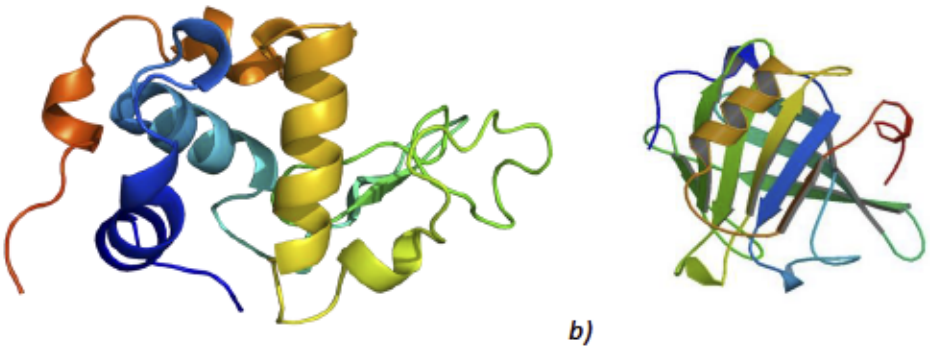


Fig. 6. Model of the surface of the κ-casein molecule and a computer model of a 3D structure.

α-lactalbumin - (14.1 kDa, 123 a/a, GenBank: CAA29664.1) - another milk protein. α-lactalbumin is known to be as a component of lactose synthetase (Hill & Brew, 1975). But according to modern data, α-lactalbumin may have other more intriguing functions. According to these studies (Håkansson, Zhivotovsky, Orrenius, Sabharwal, & Svanborg, 1995; Svensson et al., 1999), the multimeric α-lactalbumin complex can bind on the cell surface, enter the cytoplasm, accumulate in the cell nucleus and brings to apoptosis.



a) *b)*

Fig. 7. Tertiary structure of a) α-lactalbumin; b) β-lactoglobulin.

ß-lactoglobulin - (18.4 kDa, 162 aa, GenBank: CAA32835.1) is the second with α-lactalbumin, component of the whey protein milk fraction. The main function of a protein is a source of amino acids. In addition, BLG can bind many hydrophobic molecules, playing a role in their transport. It was also shown that β-lactoglobulin is able to bind iron using siderophores (Roth-Walter et al., 2014), which may play a role in the fight against pathogens.

Chaperonins and proteins with a chaperone-like activity

Chaperonins GroEL (also known as Hsp60) and GroES (also known as Hsp10) are heat shock bacterial proteins, one of the most well-known examples of proteins that promote the correct folding of other peptides. The name "heat shock proteins" was given them for the sharp increase in their expression in the case of heat stress E.coli (Schlesinger, 1990). GroEL protein, which forms a complex of 14 subunits in the form of two hollow barrels connected by the ends, has true chaperone activity. Protein GroES is its essential partner, forming a “lid” for each of these barrels, consisting of 7 subunits.

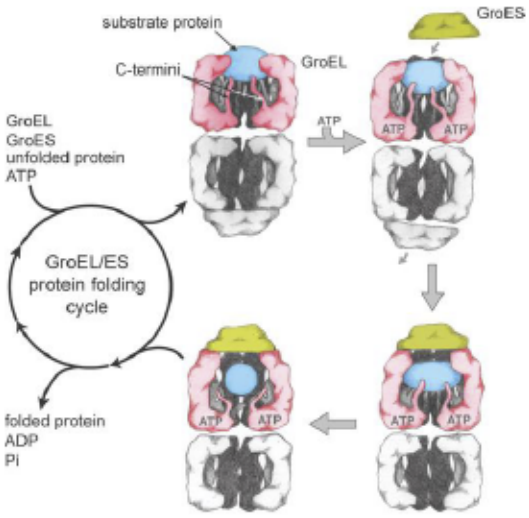


Fig. 8. Diagram of the chaperonin complex GroEL-GroES structure and the main idea of its work.

GroEL has broad specificity: it is able to bind about 1000 proteins out of 4300 encoded in the genome of E. coli. This fact allows it to use it in the study of interaction with many proteins, even those that are not typical for bacteria, especially since even human Hsp60 was found to have very similar structure and mechanism of action. However, the functioning of the complex requires the presence of ATP: the binding of the complex to an incorrectly folded protein is easy, but energy is required to release the folded peptide (Grantcharova, Alm, Baker, & Horwich, 2001).

Heat shock proteins, and the GroEL/GroES complex in particular, are very important for maintaining cell activity — mutations in their genes lead to cell death or serious diseases. That's why the study of cases of suppression of chaperone activity is improtant. For example, it is known that the oxidized form of glyceraldehyde-3-phosphate dehydrogenase can irreversibly bind to the GroEL/GroES complex and block the folding of the non-oxidized form (Naletova, Muronetz, & Schmalhausen, 2006). The effect of similar binding on the refolding of other proteins is not well understood, in addition, it is possible that other modifications may block the chaperonins functioning, that's why we chose this complex as an object for research.

β-casein - 224 aa, (GenBank: AAA30431.1) - in addition to noted earlier functions (the source of amino acids in food and the binding of calcium ions), β-casein and its fragments are involved in many other biological functions. Thus, the peptide cazoparan was shown to be involved in the activation of phagocytosis by macrophages and peroxidase release. Another peptide, casohypotensin, is known to be a potent inhibitor of endooligopeptidase A, a thiol-activated protease capable of destroying bradykinin and neurotensin and hydrolyzing enkephalin-containing peptides with enkephaline products. β-caseins are also a source of casomorphins, peptides capable of activating opioid receptors (Miyamoto et al., 1987). We were most interested in its ability to suppress non-specific aggregation of other proteins.

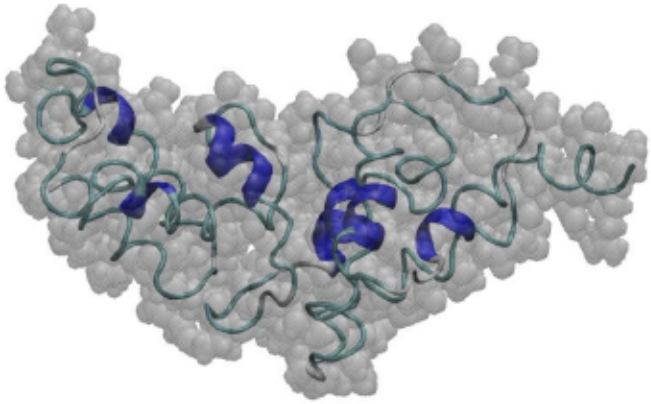


Fig. 9. Tertiary structure of β-casein (Pérez-Fuentes, Drummond, Faraudo, & Bastos-González, 2017)***, obtained by molecular modeling of the molecular dynamics*** (Humphrey, Dalke, & Schulten, 1996).

The amino acid sequence of β-casein:

```
  1 mkvlilaclv alalarelee lnvpgeives lssseesitr inkkiekfqs eeqqqtedel
 61 qdkihpfaqt qslvypfpgp ihnslpqnip pltqtpvvvp pflqpevmgv skvkeamapk
121 hkempfpkyp vepftesqsl tltdvenlhl plpllqswmh qphqplpptv mfppqsvlsl
181 sqskvlpvpq kavpypqrdm piqafllyqe pvlgpvrgpf piiv
```

Lysine residues, which undergoes glycation in the first place (totally 12), marked red, arginine - blue (totally 4). Plus 1 alpha-amino group - a total of 17 free amino groups per one molecule of unmodified protein.

Low molecular weight inhibitors of amyloid conversion and aggregation

A few researchers previously have shown the ability of the plant alkaloid curcumin to bind a prion protein molecule and suppress its amyloid conversion and aggregation (Hafner-Bratkovič et al., 2008). This plant polyphenol, extracted from the spice turmeric, which is widespread in India, has shown its effectiveness against the amyloiodic conversion of proteins both in vitro and in vivo (C.-F. Lin, Yu, Jheng, Chung, & Lee, 2013). Various neurodegenerative diseases, including Alzheimer's, Parkinson's, Huntington's, and prion's diseases (Maiti & Dunbar, 2018).

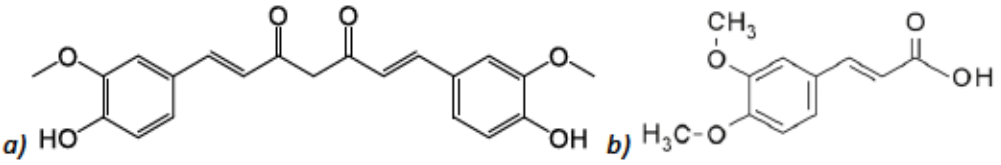


Fig. 10. Structures of: a) curcumin b) 3,4-dimethoxycynamic acid

At the same time, its binding sites on the prion protein molecule were predicted by the molecular docking method, however, they remain unconfirmed by direct experiments. Using the Lead Finder software, the possible binding sites for curcumin on PrP molecule were predicted (the results of docking and connection-analogues of curcumin were kindly provided by Dr. V. Stroilov). This docking was calculated to the native conformation of PrP molecule, but not to the infectious form of the PrP^{Sc} protein. The putative curcumin binding region is located in a shallow pocket on the surface of sheep prion protein, starting from the contact area of the β-leaf site with the H2 helix and continuing towards the H1 helix.

Materials from this chapter were published in articles (Tishina, S. A., Stroylov, V. S., **Zanyatkin, I. A.** et al., 2017; **Zanyatkin, I.**, Stroylova, Y., Tishina, S, 2017).

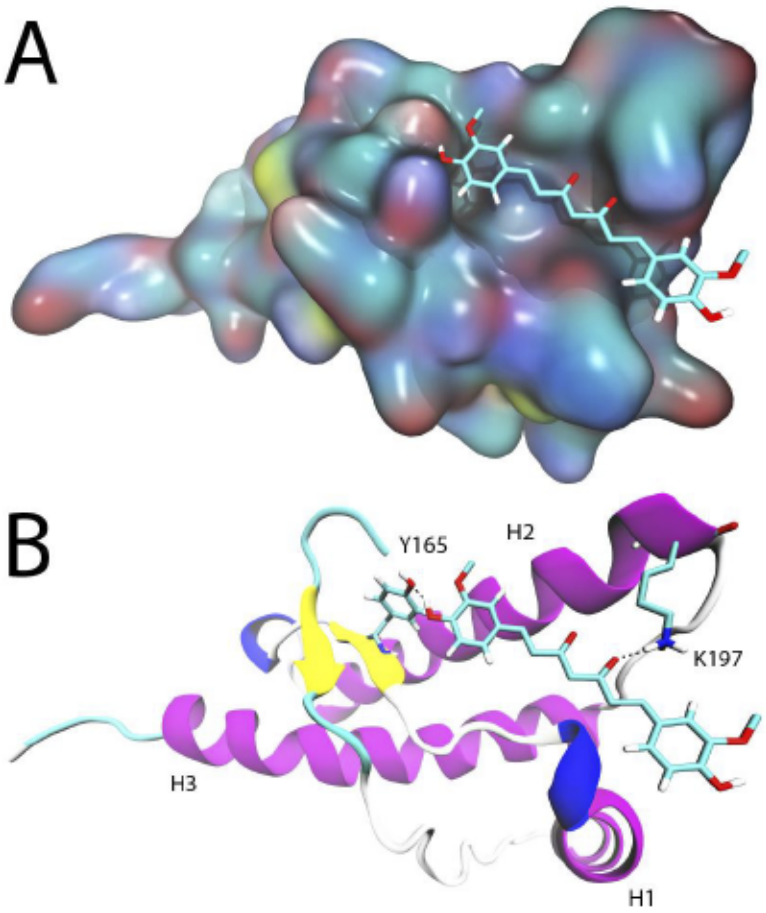


Fig. 11. Results of molecular docking of the prion protein (PDB ID 1uw3) with curcumin. A: Image of the surface of a prion protein molecule with bound curcumin. Negatively charged areas are shown in red, positively charged in blue, polar in yellow, and hydrophobic in white. B: The elements of the structure that provide curcumin binding. Amino acid residues directly involved in curcumin binding are noted: Y165 and K197. Alpha-helixes are colored purple, β-sheets are yellow, loops are white, an unstructured domain originates from alpha-helix H3, not shown in the figure.

By the same method of molecular docking, we predicted that binding site for curcumin is available for 3,4-dimethoxycinnamic acid, which is structurally very similar to half the curcumin molecule. Also for dimethoxycinnamic acid, a search for alternative binding sites with the prion protein molecule was conducted, during which an energetically more favorable site was found (Fig. 11). This site is located in the contact area of the H2 and H3 helices of a structured domain, the binding of 3,4-dimethoxycinnamic acid in it is provided by hydrogen bonds and electrostatic interactions with residues R139 and N162. 3,4-dimethoxycinnamic acid was shown to

have a higher affinity to the prion protein than curcumin — the calculated ΔG binding is -4.5 kcal/mol, in contrast to -3 kcal/mol to the curcumin binding site. At the same time, due to lower molecular weight and more hydrophilic groups, 3,4-DMCA quite expectedly has better solubility in the aqueous phase than curcumin: the apparent solubility of 3,4- DMCA determined by us exceeds the solubility of curcumin by an average of 4-5 times in the form acid, and 10 times if cinnamic acid is present in the form of sodium salt. The binding site found is a smaller pocket than for curcumin; therefore, it is not suitable for its binding and is specific only for 3,4- DMCA.

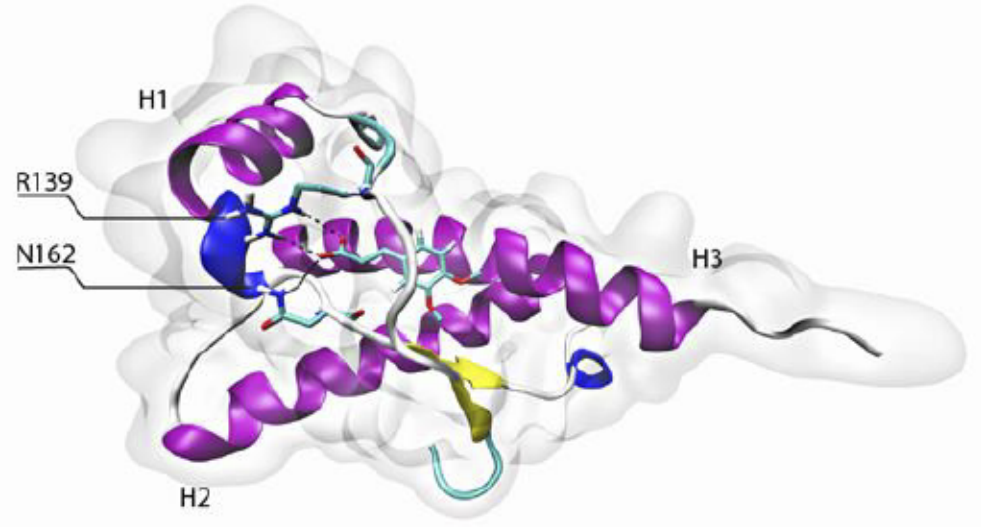


Fig. 12. The proposed binding site on prion protein molecule (PDB ID 1uw3) for3,4-dimethoxycinnamic acid. Alpha-helixes are colored purple, β-sheets are yellow, loops are white, an unstructured domain originates from alpha-helix H3, not shown in the figure. Amino acid residues directly involved in curcumin binding are noted: R139 and N162. The surface of the molecule is shown in gray.

In order to experimentally determine the binding affinity of 3,4-DMCA with a prion protein, we used the method of isothermal titration calorimetry (ITC). This method allows to estimate the thermal effect and the binding constant of ligands; in case of the prion protein, it was used a lot of times to evaluate its binding to metal ions such as zinc, copper, cobalt (Davies, Marken, Salter, & Brown, 2009; Nadal, Davies, Brown, & Viles, 2009). Differential scanning calorimetry (DSC) was used to detect changes in the properties of the prion protein after binding to the ligand.

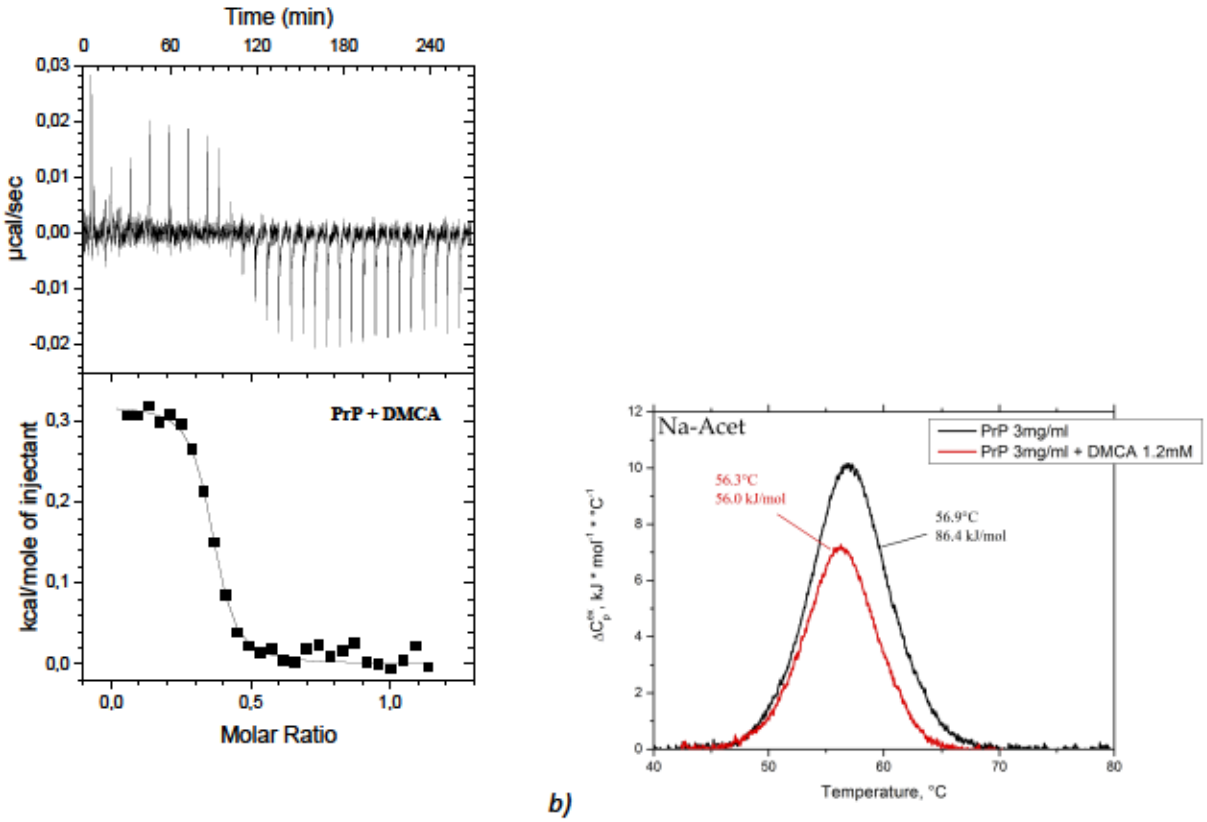


Fig. 13. A - curves of enthalpy changes depending on the concentration of 3,4-DMCA. ITC analysis. B - change in thermal stability of the prion protein in the presence of 3,4-DMCA. DSC analysis.

In acidic conditions (pH 4.0) the binding of 3,4-DMCA with a prion protein is characterized by the following binding parameters: protein: ligand ratio = 1: 0.35, K = binding constant = 2.47 * 106 M-1 ± 4.59 * 105 (dissociation constant = 405 nM). The binding constant has an order of 10^6, which means good binding. At the same time, experiments showed that the stoichiometry of the protein: ligand complex is 3:1. This fact may indicate that the binding of one DMCA molecule hinders the binding of the others, i.e. on the negative cooperativity of binding, or on the initial inaccessibility of the binding sites of 3,4-DMCA on part of the prion protein molecules. Perhaps, in this case, the situation is similar to that demonstrated by the known effective anti-amyloid agent curcumin, which causes conformational changes in the tertiary structure of amyloidogenic proteins (P. K. Singh et al., 2013). It can be assumed that DMCA causes

in the prion protein conformational rearrangements that prevent the binding of prion monomers into large aggregates.

According to previous experiments, in experiments with cell culture of neuroblastoma, it was shown that 3,4-DMCA does not have a significant level of toxicity, while it better protects cells from the harmful effects of PrP^{Sc} than curcumin (Tishina et al., 2017).

Alternative potential ligands

Additionally, we decided to test another 2 ligands, about which there are no comprehensive information on anti-amyloid properties, but they demonstrated a cytoprotective effect on cell cultures, and also showed the presence of antioxidant properties, like curcumin.

Pentamidine isethionate. In our work it was proposed to include it in view of the structural similarity with curcumin.

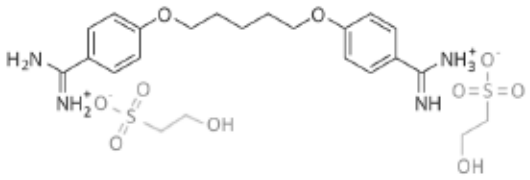


Fig. 14. The structure of pentamidine in the form of an isethionate salt.

Pentamidine uses as an antimicrobial agent in the treatment of trypanosomiasis, leishmaniasis and for the prevention of pneumonia in people with weak immunity (Kruizinga, Bresters, Smiers, Lankester, & Bredius, 2017). It can be injected in organism by intravenous and intramuscular ways, or even by inhalation. However, it has a list of side effects of irritation: cough, runny nose, sneezing, emetic urge, from systemic problems a decrease of blood pressure and temporary renal failure were noted.

The mechanism of the antimicrobial action of pentamidine is not known in detail. It was confirmed to affects the processes of transcription and translation in prokaryotic organisms. Presumably, ligand is able to bind to adenine residues in the composition of nucleic acids (Cory, Tidwell, & Fairley, 1992), form cross-links between molecules and block the reading of information from the polynucleotide chain. It was also shown that it inhibits the activity of topoisomerase enzymes in the mitochondria of *Pneumocystis jirovecii* and the parasites of the *Trypanosoma* family, which, accordingly, inhibits the growth of pathogens.

In addition, there is information about the applicability of pentamidine as an anticancer drug (Zerbini et al., 2014). Presumably, this effect is associated with the ability of pentamidine to inhibit the interaction of the Ca^{2+}-binding protein S100B and the anti-oncological transcription factor p53, which increases the efficiency of the latter and inhibits cancer growth (J. Lin et al., 2004). These assumptions were confirmed in *ex vivo* experiments (J. Smith et al., 2010).

Resveratrol

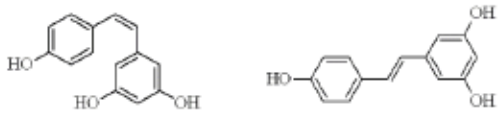


Fig. 15. Resveratrol cis- and trans-isomers.

This ligand contains in red wine. Known antioxidant (Kulkarni & Cantó, 2015). Plants often use as phytoalexin for inhibition of bacteria and fungi growth.

The positive effect of resveratrol on the prevention of cardiac muscle fibrillation and heart attacks has been shown (Chong et al., 2015). that this effect was assumed to be associated with the regulation of resveratrol by the expression of phosphoinositol-3-kinase (PI3K)/AKT/endothelial nitroxide synthase proteins, which in its turn led to an increase in the operation of this signaling pathway. In addition, the antioxidant

properties of resveratrol reduce the probability of the components of low-density lipoproteins oxidation, from which atherosclerotic plaques can form (Kovanen & Pentikäinen, 2003; MacCarrone, Lorenzon, Guerrieri, & Agrò, 1999).

Resveratrol, as a curcumin, is proven to regulate post-translational modifications of the anti-oncogenous transcription factor p53 (Xu et al., 2018).

Studies have shown that resveratrol was able to have a positive effect on the body in neurodegenerative diseases. Polyphenol facilitated the non-amyloidogenic cleavage of the β-amyloid precursor protein, facilitated the elimination of amyloid β-amyloid peptides, and reduced neuronal damage. At the same time, the mechanism of its effect is not yet known (F. Li, Gong, Dong, & Shi, 2012). Probably, the most effective way to influence resveratrol on neurodegenerative diseases is not a direct inhibition of aggregation and destruction of amyloid aggregates, but an effect on intracellular mechanisms [4], but this assumption requires verification. In addition, there is evidence that resveratrol is able to suppress the aging of organisms through the suppression of cAMP phosphodiesterase (Park et al., 2012).

Materials and methods

The following reagents were used: Polyclonal rabbit antibodies against bovine milk β-casein (Barinova, Khomyakova, Semenyuk, Schmalhausen, & Muronetz, 2018); goat anti-rabbit IgG Fc fragment conjugated with AlexaFluor 555 fluorescent label (Thermoficher scientific). Yeast extract (Helicon), tryptone (Helicon), dry components for LB (Amresco), isopropyl-β-D-1-thiogalactopyranoside (Helicon), glycerin (Sigma), Tween-20 (Helicon); antibiotic ampicillin (JSC Synthesis, Russia); EDTA (Fluka), $NiCl_2$ * $6H_2O$ (ultrapure, Sigma), Lysozyme (Sigma), Protease inhibitor kit (Sigma). Ni-GE Sepharose (GE Healthcare), Q Sepharose High Performance (GE Healthcare), $CHCl_3$, EGTA, guanidine hydrochloride (Sigma), imidazole (oscin, Pancreac). CF_3COOH. MOPS (Sigma), MES (Sigma), phosphate-

buffered saline in tablets (Amresco); glycine (Sigma), 2-dimethylaminoethanethiol hydrochloride (Sigma), β-mercaptoethanol (Ferak), DTT (Panreac). D-glucose (Merck); methylglyoxal (Sigma), NAD^+ (Reanal), Coomassie R-250, Coomassie G-250 (Sigma), Nitrosynium terazolium (Sigma), thioflavin T (Sigma), Congo red (Sigma), curcumin (Sigma), 3,4-dimethoxycinnamic acid, pentamidine isethionate (Sigma), resveratrol (Sigma), 3,3'- [3,3'-dimethoxy- (1,1'-biphenyl) -4,4'-diyl] -bis [2-(4-nitrophenyl) -5-phenyl (2H)] tetrazolium (NBT) (Chemapol, Czechoslovakia); bromophenol blue, o-phthalaldehyde (Sigma), bicinchoninic acid (Sigma), 5,5'-dithiobis- (2-nitrobenzoic) acid (Merck). Glyceraldehyde 3-phosphate, CH3COOH (OFS, Himmed), $CH_3COONa * 3H_2O$ (OFS, Reahim), CH_3COONH_4 (OFS, Helicon), acetonitrile (Sigma), CCl_3COOH (Sigma), $NaBH_3CN$ (Sigma), $MnCl_2$, $CaCl_2$ (Sigma); NaCl, KCl (Helicon), $MgCl_2$, KH_2PO_4, SDS (Sigma), $(NH_4)_2SO_4$, (MP Biomedicals); NaH_2PO_4, (Reahim); hydrogen peroxide (Mosreaktiv LLC); ammonia water, acetic acid, hydrochloric acid, ethyl alcohol, sulfuric acid (Himmed); Urea (ultrapure, Amresco), Tris (ultrapure, Amresco), NaCl (ultrapure, Reahim), $CaCl_2$ (ultrapure, Pancreac), $CuSO_4$.

Preparative technics

E. coli competitive strain preparation

This procedure is performed to facilitate the penetration of DNA molecules, in particular plasmids, into the bacterial cell.

Strain BL21 DE3, not yet containing the target plasmid, was grown in LB medium to an optical density of 0.3-0.4, then the cultures were placed in an ice bath. A certain volume of cell suspension (from 5 to 50 ml depending on the amount of biomass) was taken, it was centrifuged for 5 min 4000g, the supernatant was decanted, 1 ml of 0.1 M sterile $CaCl_2$, previously cooled in an ice bath, was added to the sediment. The pellet was resuspended and incubated for 10 min on ice, after which the cells were again

precipitated by centrifugation and the supernatant was discarded. 400 µl of 0.1 M sterile cooled $CaCl_2$ was added to the precipitate and the cells were resuspended. The resulting competent strains were stored at -80°C.

Transformation of competent E.coli strains

This technique allows to obtain new bacterial strains containing plasmids with a nucleotide sequence that encodes the target protein.

Cells of the competent strain were thawed from -80°C in an ice bath. 2-3 µl of plasmid was added to 100 µl of cells and mixed gently. The resulting mixture was incubated for 30 min on ice, then the cells were subjected to heat shock for 42°C for 90 s and incubated again on ice for 10 min. Next, 800 µl of LB nutrient medium was added to the cells and incubated for 1 h at 37°C. The obtained suspensions were frozen at -80°C in LB medium with glycerol.

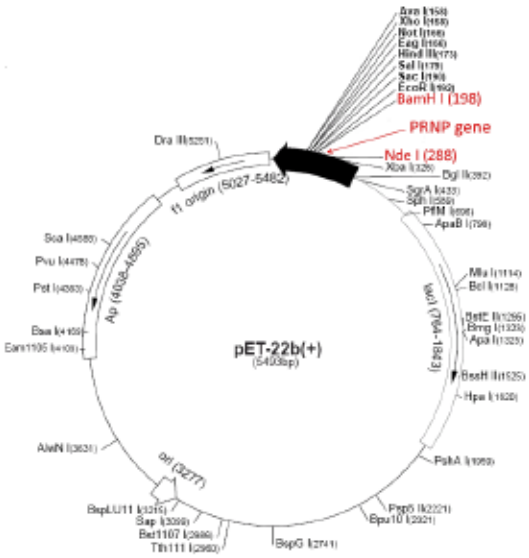


Fig. 16. Diagram of the plasmid pET-22b (+) with the indicated position of the recombinant sheep prion protein gene. Red highlights the restriction sites within which the recombinant sheep prion protein gene has been inserted.

This method was used to obtain the strain E. coli BL21 DE3 containing the plasmid pET 22b+ and capable of expressing the recombinant sheep prion protein.

Production of bacterial lysate with PrP

A sample of frozen culture of *E. coli* BL21 DE3 strain containing the plasmid pET 22b+ with a nucleotide sequence encoding sheep PrP was used. The autoclaved sterilized LB medium was infected with ~ 2 µl of the above culture, incubated overnight at 37°C and stirred. Next, the overnight culture was injected in media containing 100 µg/ml of ampicillin in such amount that the optical density of suspension was 0.1-0.2 (measured at 600 nm versus pure LB medium). The resulting suspensions were incubated at 37°C to an optical density of 0.6–0.8, after which the expression of the target protein in them was provoked by introducing the IPTG solution to a concentration of 1 mM. Cultures were grown overnight.

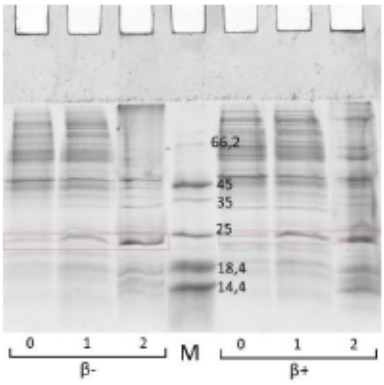


Fig. 17. Electrophoregram of fractions from prion protein separation from bacterial lysate. "B-" - absence of β-mercaptoethanol in samples; "B +" is the presence of β-mercaptoethanol (in case of PrP dimerization). 0 - cell lysate prior to the induction of expression; 1 - cell lysate after expression of the target protein; 2 - inclusion Taurus solution. Red stripes around the prion protein.

The obtained cultures were centrifuged for 10 min at 5000 g and temperature 40°C. The precipitate containing the bacteria was washed with 50 mM Tris-HCl with 0.5 M NaCl pH 7.4. Next, the pellet was resuspended in lysis buffer and incubated for 20 minutes at 37°C and with stirring. Then the bacteria were destroyed by ultrasonic desintegration (Branson Digital Sonifier) 6 pulses of 30 seconds each with an amplitude of 30% of the maximum. The lysate was washed by 50 mM Tris-HCl pH 8.0, then centrifuged at 10,000 g for 15 min and 40°C. The precipitate of Taurus

inclusions containing prion protein was solubilized in 50 mM Tris-HCl buffer with 0.5 M NaCl and 6 M guanidine hydrochloride.

The presence of protein at the stages of expression and isolation was checked by SDS-electrophoresis of samples taken from the medium of *E.coli* prior to the induction of expression, from the final expression culture and from the inclusion body solution in the buffer with guanidine hydrochloride.

Isolation and purification of sheep prion from bacterial lysate

Due to the presence in PrP sequence of octapeptides capable to bind bivalent metal ions, isolation and purification can be carried out in a single step using affinity chromatography on an immobilized Ni^{2+} column (Vrentas, Onstot, & Nicholson, 2012).

Purification steps:

I. Equilibration of column with MAS-Sepharose or Ni-GE Sepharose with immobilized nickel ions by buffer A (5 M urea; 20 mM Tris-HCl pH 7.4; 0.5-2 M sodium chloride; 10 mM imidazole);
II. Injection of inclusion bodies solution in denaturing buffer;
III. Washing the column with buffer A;
IV. Washing the column with buffer B (20 mM Tris-HCl pH 7.4; 0.3 M sodium chloride; 20 mM imidazole);
V. Elution of protein with 1 M solution of imidazole (pH 7.4);
VI. Re-wash the column with buffer A to elute any remaining protein.

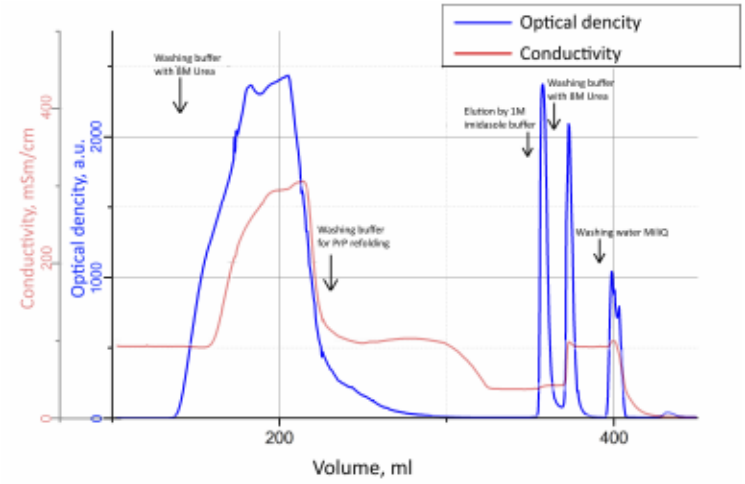


Fig. 18. The elution profile of affinic chromatography of inclusion bodies solution of prion protein.

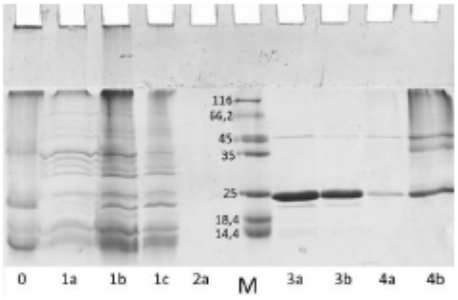


Fig. 19. The composition of the fractions from affinic chromatography of inclusion bodies solution of prion protein.

0 - inclusion bodies solution; 1 - eluate with buffer A with 8M urea. 2 - eluate by buffer B; 3 - eluate by 1M imidazole; 4 - eluate repeated washing with buffer A with 8M urea.

The protein solution obtained by elution with 1M imidazole was dialyzed against 15 mM ammonium acetate buffer pH 4.5 with a sequential decrease of the buffering agent concentration to 5 mM by changing the dialysis solution. Dialysate was freeze-dried. On average, about 1 mg of lyophilized protein was obtained from 1 l of cell culture.

Purification of Native β-Casein

Native β-CN was purified according to the two-step purification procedure (Mercier, Maubois, Poznanski, & Ribadeau-Dumas, 1968) with additional reversed-phase chromatography. Fresh bovine milk was defatted by centrifugation and β-CNs were precipitated at pH 4.6.

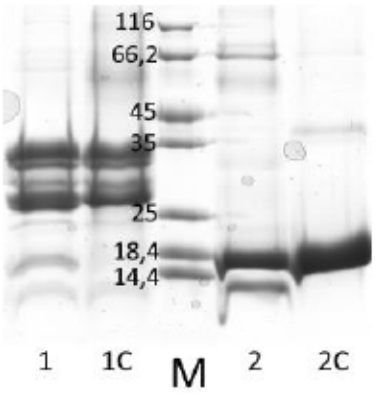


Fig. 20. Analysis of the composition of the separated fractions of milk proteins. SDS electrophoresis. 1 – casein fraction, 1C - control total casein; 2 - isolated fraction of alpha-lactalbumin and β-lactoglobulin, 2C - control β-lactoglobulin.

The pellet enriched with caseins was isolated by centrifugation (5860 g, 60 min, 4 ◦C), washed, lyophilized, and stored at −20 °C until further use. Whole casein fraction in concentration of 100 mg/ml was prepared in 50 mM Bis-Tris pH 6.45, 7 M Urea, 1 mM EGTA, 20 mM DTT, and centrifuged at 10000 g for 10 min. Supernatant was loaded on the column filled with Q Sepharose High Performance and equilibrated by 20 mM Bis-Tris pH 6.45, 5M Urea, 1 mM EGTA, 1 mM DTT. Proteins were eluted by a 0-1M NaCl linear gradient in the same buffer.

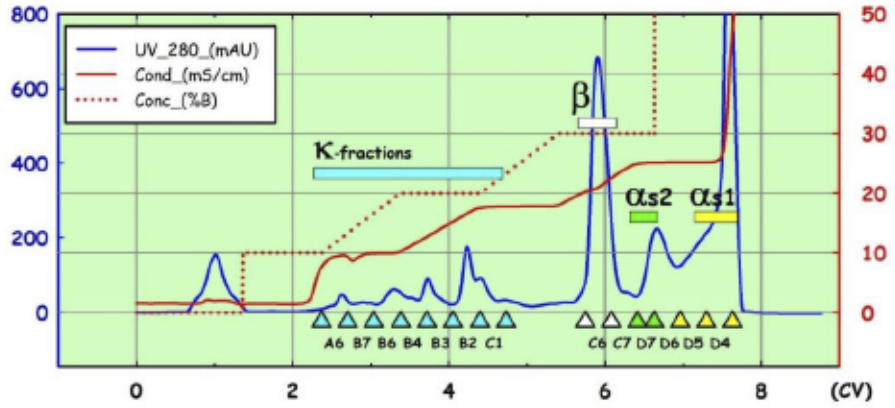


Fig. 21. Elution curve for casein anion-exchange chromatography.
In the process of chromatography, a linear gradient was applied twice: from 0.1 to 0.2 M NaCl and from 0.2 to 0.3 M NaCl.

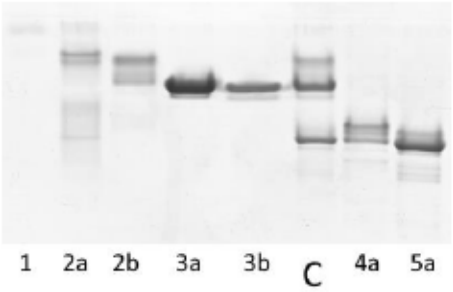


Fig. 22. Results of anion-exchange chromatography of caseins, electrophoresis analysis with urea. Stage 1 - elution of unbound protein; 2a-2b - elution of c-casein (s (NaCl) = 0.1 M); 3a-3b - elution of β-casein (s (NaCl) = 0.2M); 4a — elution of αS1-casein (c (NaCl) = 0.3M); 5 — αS2-casein elution (c (NaCl) = 0.3M). C - control total casein.

Subsequently the protocol was simplified:

1. A linear gradient from 0 to 0.1 M NaCl was removed, since there was no task to separate the κ-casein isoforms. The linear gradient of salt concentration in the range from 0.1 M to 0.2 M NaCl was retained because it was useful for the qualitative elution of β-casein;
2. An extended linear gradient range was introduced in the range of 0.2 M to 0.32 M NaCl in order to provide a clearer separation of the β and αS forms.

During this work, it was also found possible to carry out a separate elution of αS-forms of casein. However, differences in salt concentration for separate elution of these two forms are insignificant, and the process can be monitored only by the buffer conductivity: 24 mS/cm corresponds to elution of the αS2 form, and for αS1-casein the conductivity is 27 mS/cm. So the procedure for their separation was difficult, and the goal was not to divide the forms of αS-casein.

Reversed-phase chromatography

After protein detection by spectrometry and BCA assay, fractions containing pure κ- and β-CN were pooled and purified from low-molecular compounds by reversed-phase chromatography. The column Sep-Pak 20 CC was activated by 75% ethanol and equilibrated by 0.1 % TFA solution on MilliQ. The protein elution was

made by 80% ACN with 0.05% TFA. Protein solution was concentrated, evaporated from acetonitrile using Speed-Vac and was frozen at -20°C.

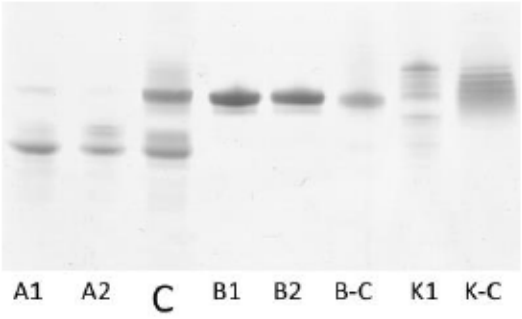


Fig. 23. Analysis of concentrated casein fractions. Electrophoresis with 7M urea. A - αS1-casein fractions; B - β-casein fractions; K - κ-casein fractions; samples labeled letter "C" are control casein (C – C is control c-casein, just C is control total casein).

For κ- and β-caseins, this method of concentration was useful: it was possible to obtain approximately 100 mg of κ-casein and 400 mg of β-casein from 2 L of initial milk. κ-casein was concentrated from 0.5 mg/ml to ~ 12 mg/ml, β-casein was concentrated from 2 mg/ml to 20 mg/ml. Further concentration of β-casein was considered dangerous due to the ability of this protein to micellization. For the αS form, attempts to concentrate the protein were shown to lead to its spontaneous aggregation and the impossibility of immobilization on the column.

After the procedure, the κ- and β-forms of casein were stored at -20°C.

Ammonium sulfate protein precipitation

The main mechanism of this method is the reduction of the hydrophilic potential of an aqueous solution in order to disrupt the structure of the protein, lead to the exposure of its hydrophobic areas on a molecule surface and, as a result, to provoke reversible non-specific aggregation. The presence of ammonium ions and sulfate ions causes the clustering of water around them and, as a result of competition, the destruction of hydrate clusters around protein molecules. This method is applicable to proteins with the same isoelectric point and allows storage of the protein in a salted suspension. In

our case, it was used for cleaning and storing proteins of the GroEL/GroES chaperonin complex.

Operations: Slowly add dry and crushed $(NH_4)_2SO_4$ to the protein solution at 4°C with constant stirring. After adding salt, incubate the suspension with stirring at the same temperature for at least one hour.

Expression and purification of GroEL/GroES proteins

The expression was carried out in the culture of *E. coli* strain W3110 with the plasmid pOF39. In this case, activation of the expression of target proteins was not required; therefore, the procedure included only injection of night bacteria culture to the expression medium up to D(600) = 0.1 and incubating these media for a day.

Then the cell culture was washed from the medium, lysed in a buffer (100 mM Tris-HCl, pH 8.1, 0.1 mM EDTA, 10 mM DTT and 0.2 mg/ml protease inhibitors) and destroyed by ultrasonic treatment with 6 pulses for 30 seconds with an amplitude of 40%. The obtained suspension was centrifuged and salted out from the supernatant (50 ml), first impurity proteins (30% of ammonium sulfate saturation), and then chaperonins (80%). From the suspension, chaperonins were dissolved (30 ml) in buffer B (50 mM Tris-HCl, pH 7.2, 9.1 mM EDTA, 2 mM DTT) and dialyzed from ammonium sulfate in two changes of 4 L buffer.

Then the proteins were purified by ion-exchange chromatography on a DEAE-Sepharose gel. The chaperonin solution was prediluted to a volume of 100 ml to reduce the effect of ammonium sulfate on binding. Protein was applied at a speed of 1 ml/min, washed at a speed of 3 ml/min with buffer B with 0.5 M NaCl (the buffers were changed using a linear gradient).

GroEL and GroES have a relatively low molar absorption coefficient, and it is difficult to detect them against the background of other bacteria proteins, therefore all fractions,

presumably containing chaperonins, were analyzed by SDS-electrophoresis. The fractions containing the target proteins were combined into 2 separate solutions.

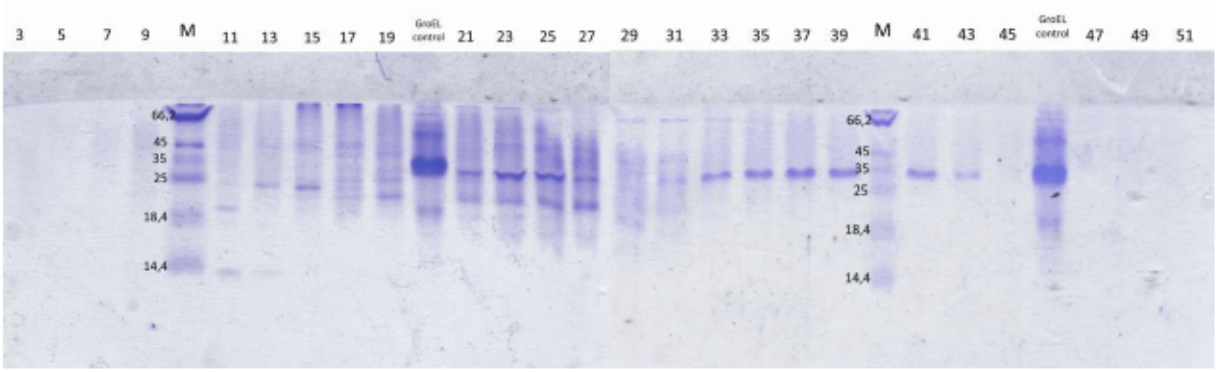


Fig. 24. Electrophoregram fractions of ion-exchange chromatography of chaperonins from a cell lysate. All fractions were collected using a linear NaCl gradient process.
The numbers mean the fraction collected and applied to the gel.

The GroEL solution was dialyzed against the same buffer B, and then subjected to another chromatography under the same conditions in order to achieve the maximum degree of purification. The new fractions containing GroEL were combined, and from the resulting volume the protein was salted out by adding $(NH_4)_2SO_4$ to a saturation degree 80%. In this form, the protein was stored in the refrigerator at +4°C. The GroES sample was also subjected to additional purification, but by a heat treatment method. The chromatographic fractions with GroES were incubated for 20 min at 60°C and 80°C. After each heating, the suspension was centrifuged at 11,000 g for 20 minutes, so that only thermostable GroES remained in the supernatant. Then the protein was salted out by adding ammonium sulfate to 80% of saturation. The purity of the preparations was checked using SDS electrophoresis.

As a result, 3 solutions were obtained:

GroEL - 4 mg/ml, purified.

GroEL - 5.3 mg/ml, an insignificant admixture of high molecular weight protein is noticed. Totally 60 mg.

GroES - 6.75 mg/ml. Totally 40 mg.

Glycation of beta-casein

At the initial stages of lysine with a monosaccharide interaction, substituted imines are formed (Schiff bases), which are then regrouped in the so-called "Amadori products". Later glycation products can undergo modifications, releasing other glycating agents, but the main result is formation of modified amino acids, for example, CM-lysine, which is fundamentally different in properties from lysine (this is an acidic amino acid), cross-linkages between amino acids (for example, lysyl-alanyl-lysin), but in addition - reactive oxygen species.

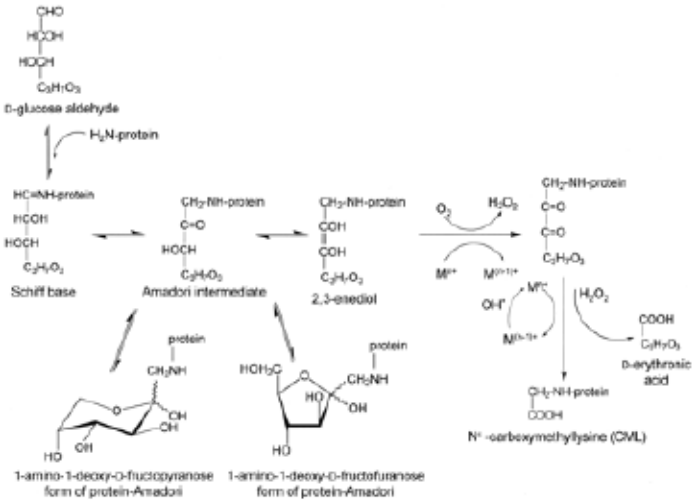


Fig. 25. One of potential ways of proteins glycation and the formation of products of this process.

Beta-casein (5 mg/ml) was incubated in 50 mM Tris-HCl buffer, pH 8.0, containing 0.01% sodium azide in the presence of 200 mM glucose at 37°C during 72 hours with gentle shaking. After the incubation during specified time intervals, the protein solution was purified from D-glucose and low molecular weight compounds using reverse-phase chromatography on a Sep-Pak 20 CC column containing 30 RPC gel (Amersham Biosciences, Piscataway, NJ). The column was equilibrated with water, containing 0.01% trifluoroacetic acid (TFA). Protein was eluted with 80% acetonitrile, containing 0.005% TFA, v/v). Then samples were dialyzed against 50 mM MES buffer, pH 6.7, concentrated using SpeedVac and frozen. In case of

methylglyoxal beta-casein solution (5 mg/ml) was incubated in 50 mM borate buffer, pH 8.0 in the presence of 200 mM methylglyoxal at 37°C during 1-24 hours with gentle shaking. Further purification from methylglyoxal and low molecular weight compounds was performed by dialysis against 20 mM MES buffer, pH 6.7. In both cases 35 mM sodium borohydride reduction was used if necessary.

PrP glycation

This experiment is also a model of *in vivo* process. Compared to the casein glycation, this process is performed under milder conditions due to high tendency of PrP to aggregate. The modification was carried out for 1 day at 37°C with stirring. The protein concentration was 5 mg/ml (192 μM); D-glucose was used as a glycating agent. Optionally, a reducing agent $NaBH_3CN$ was added to the samples before the glycation procedure to a concentration of 35 mM. In case of prion protein, reverse phase chromatography could lead to nonspecific aggregation, therefore, after the glycation procedure, PrP solution was dialyzed against MES buffer pH 6.7 to remove low molecular weight glycation products. In this case, the pH of the buffer was reduced in comparison with that for glycated beta-casein, to shift the conditions from isoelectric point of the protein (pI = 9.1-9.2) to avoid nonspecific aggregation. The products of the experiment were analyzed by SDS-electrophoresis for the degree of possible aggregation, by staining the samples with NBT, according to the number of remaining free amino groups by the ortho-phthalaldehyde method. The number of early glycation products was determined by the fructosamine method, the number of late products was determined by the intrinsic fluorescence of AGE.

Thermo-induced protein aggregation

This method is aimed at obtaining protein aggregates similar in properties obtained in native conditions, but in vitro and in less time. In our experiments, we adhered to maintaining the acidity of the medium in the near-physiological range,

varying the temperature. In addition, in some conditions, the ionic strength of solutions was increased by adding salts (for example, NaCl) to concentrations close to physiological (for example, in milk). After testing several protocols, the most versatile and efficient was chosen.

Incubation was carried out in 50 mM Na-phosphate or, more preferably (due to greater chemical inertness) MES buffer in pH 6.7. In addition, for the initiating "sub-deployment" of protein, the solution contained 0.1 M NaCl (approximately the same salt concentration is contained in milk). The incubation itself was carried out at 94°C for 1 hour. The samples obtained were preferably analyzed immediately, if this was not possible, they could be stored at + 4°C for a maximum of 1 week. Storage in a frozen state is possible, however, in this case, the structure of the aggregates in the sample changed under the influence of ice crystals, which led to the acceleration of the precipitation of protein particles from suspensions.

GAPDH denaturation and reactivation

The method allows for the gentle denaturation of the enzyme and further assessment reactivation degree (if it is possible for the enzyme) by its activity. Glyceraldehyde-3-phosphate dehydrogenase has easily measured activity and is capable of partial renaturation.

For denaturation, 120 μM of the enzyme (calculated per monomer) were incubated in a solution containing 4 M guanidine hydrochloride in 10 mM KH_2PO_4, pH 7.5, 1 mM EDTA, 2 mM mercaptoethanol for 15 minutes until complete inactivation of the enzyme (enzymatic activity was measured).

Reactivation of GAPDH was initiated by 200-fold dilution of the denatured enzyme to a final concentration of 0.6 μM per monomer (now the concentration of guanidine hydrochloride was 0.02 M). Spontaneous reactivation of GAPDH was carried out in a buffer of the following composition: 10 mM KH_2PO_4, pH 7.5, 1 mM EDTA, 5 mM

mercaptoethanol, 1.5 mM NAD^+. In the case of chaperonin-dependent reactivation, the GAPDH mixture also contained 2 mM ATP, 2 mM Mg^{2+}, 0.6 μM GroEL (14 s/u) and 1.2 μM GroES (7 s/u). In the process of reactivation, aliquots were taken from the reaction medium to determine the enzymatic activity of GAPDH and a plot of the dependence of GAPDH activity on the initial (in percent) on the reactivation time was complied. Enzyme reactivation was monitored for 2 hours.

Analytical methods

Protein concentration measurement by UV-spectrometry

The method is based on the absorption of light with a wavelength of 280 nm by three amino acids in proteins: tryptophan, phenylalanine and tyrosine. Due to the different content of these amino acids in proteins, the value of the molar extinction coefficient for different proteins is different; in addition, absorption is affected by the localization of these amino acids in the tertiary structure of the protein: tryptophan, located on the surface of the protein molecule, provides a much stronger absorption than the same residue in the depths of the globule. For β-lactoglobulin, α-lactalbumin and most casein isoforms, the absorption coefficient is 1 conventional unit of absorption for 1 mg/ml, but 1 mg/ml of β-casein solution has an absorption coefficient of 0.5. The absorption coefficient of sheep prion protein with a concentration of 1 mg/ml is much higher due to the relatively large percentage of absorbing amino acids, including in the unstructured domain, and is 2.7.

The measurements were carried out on a Hitachi U-2900 instrument at 3 wavelengths:

260 nm (to detect the presence of DNA and other substances whose presence can distort the measurements of the optical density of the solution caused by the protein);

280 nm (to measure protein concentration);

320 nm (to evaluate the contribution of dispersion resulting from, for example, protein precipitation or the formation of structures stable in solution. For caseins, the problem is particularly relevant because of their tendency to micellization in aqueous solutions).

If a dispersion was detected, the protein solutions were diluted or small amounts of detergents were added to them in order to destroy the micelles. Urea is often used as a detergent in concentrations up to 4 M.

Protein concentration measurement with bicinhoninic acid

The method is based on the reduction of the Cu^{2+} ion to Cu^{1+} during the interaction of Cu(II) and some amino acid residues: cysteine, tyrosine and tryptophan, and the further formation of a complex of Cu^{1+} and bicinchoninic acid. This complex has a maximum absorption at 562 nm, which allows to measure its content in the sample. This method is used to measure the protein concentration in solutions with a high content of impurities that absorb at 280 nm and thus interfere with the measurement of the absorption of proteins.

Procedures:

1. Dilute the protein solution to an estimated concentration of 0.25-1 mg/ml;
2. Mix the protein solution and assay reagent (purchased bicinchoninic acid + $CuSO_4$ preparations in a 50:1 ratio) in a 96-well plate;
3. Incubate the tablet for half an hour at 37°C
4. Measure the absorption of the samples in the plate at 562 nm.

Due to the dependence of the final absorption on the slightest changes in the process of preparing the reagent for the experiment, it is necessary each time to build a new calibration curve from samples with bovine serum albumin of known concentration.

Protein concentration measurement by Bradford

The method is based on the reaction of Coomassie Brilliant Blue G-250 dye with arginine and hydrophobic amino acid residues. The solution with the bound form of the dye has a blue color with an absorption maximum at 595 nm. The method allows to accurately determine the concentration of protein in the range from 2 μg/ml to 120 μg/ml.

In our case, the Bradford method was used being adapted for measurement in 96-well plates. A protein solution in 20 mM MES pH 6.7 was diluted with buffer to a concentration of less than 30 μg/ml, after which 150 μl of the resulting solution was added to the well. For an accurate measurement of protein concentration, it is recommended to make a calibration on the tablet from protein solutions of a previously known concentration, in our case in the range from 2.5 to 500 μg / ml. Then 75 μl of Bradford reagent was added to them and incubated at room temperature for 5-10 minutes. Colored samples are not stored for more than 1 hour regardless of storage temperature, since an insoluble precipitate of the colored product of the protein and reagent interaction begins to form in them.

Polyacrylamidic gel-electrophoresis

Electrophoresis in PAAG is one of the most common methods for analyzing the protein composition of solutions. In this method, electricity, which is applied to the boundaries of the gel, provides for the migration of proteins through the gel. The speed of protein molecules movement in the gel depends on the length of the protein chain and some other parameters, for example, the charge of the molecule. The effect of these parameters depends on the conditions of electrophoresis. In addition, the speed of movement of the protein depends on the concentration of acrylamide and, as a consequence, the density of the gel.

SDS-PAGE

Objective: to fractionate proteins by molecular weight;
Parameters of the gel: AA = 15%; SDS concentration = 0.1%; gel thickness 0.75 mm.

Urea-PAGE

Objective: to fractionate proteins by molecular weight and total charge of the molecule;
Parameters of the gel: AA = 12.5%; urea concentration = 6 M, gel thickness 0.75 mm.
Electricity: in the concentrating gel I = 10 mA, in the separating I = 20 mA on one gel.

Nat-PAGE

Objective: to fractionate proteins according to the size of the particles formed by them with minimal damage to their structure;
Parameters of the gel: AA = 10%; no additional reagents, gel thickness 0.75 mm.

Early glycation products measurement by fructosamine reaction

To determine Amadori products content (Yaylayan, Huyghues-Despointes, & Feather, 1994) 100µl of the tested solution, containing 0.2 – 2 mg/ml of protein, was added to 900 µl of a solution containing 0.25 mM nitro blue tetrazolium in 100 mM sodium carbonate, pH 10.8, and after 10 min of incubation at 50 °C, absorption of the sample at 530 nm was measured.

Detection of fluorescence of advanced glycation end-products (AGE)

Advanced glycation end-products (AGE) were detected using its characteristic fluorescence (R. Singh, Barden, Mori, & Beilin, 2001). Fluorescence measurement of protein solutions (0.5 mg/ml) in 50 mM Tris-HCl buffer, pH 7.5, was performed in 96-well plates and fluorescence was excited at 335 nm and registered at 410 nm (PerkinElmer 2030 Multilabel Reader Victor X5).

Determination of free amino groups using o-phthalaldehyde

Protein sample (0.1 mg/ml) was incubated in 50 mM sodium borate buffer, pH 9.3, containing 0.2% N,N-diethylethanolammonium chloride (DMMAC) as reducing

agent, and 5 mM o-phtalaldehyde during 10 minutes (Svedas, Galaev, Borisov, & Berezin, 1980). Absorbance was measured at 360 nm using spectrophotometer Hitachi U2900.

Dynamic Light Scattering (DLS)

DLS experiments (Merkus, 2009) were carried out by using a Zetasizer Nano-ZS apparatus (Malvern Instruments, Malvern, U.K.) equipped with 173° optics for detection of scattered light intensity. Size distribution by number was used for interpretation of results. Beta-casein (5 mg/ml) was glycated in 50 mM Tris-HCl buffer, pH 8.0, containing 0.01% sodium azide with 0.2 M glucose at 37°C during 48 hours with further purification using reverse phase chromatography and dialysis into 50 mM MES buffer, pH 6.7, containing 0,1 M NaCl. DLS measurements were carried out at 25 °C using 0.5 mg/ml protein in the same buffer. Each reported data point is an average of 7 runs, 15 s each. Data sets obtained were analyzed using the Malvern DTS software. Distribution of particles by number, volume and intensity were reported. Presence of soluble particles with the hydrodynamic diameter less than 1 μm was monitored.

Turbidimetric analysis

Usual turbidimetric detection of optical density at 320 nm was hindered by the presence of ThT in the samples, so wavelength was changed to 600 nm. Absorbance measurement of protein solutions (0.5 mg/ml) in 50 mM Tris-HCl buffer, pH 7.5, was performed using spectrophotometer Hitachi U-2900.

Curcular dichroism measurement

This method is based on the optical activity of protein molecule (Kelly, Jess, & Price, 2005). The protein solution is capable of absorbing polarized light in different directions to varying degrees. It is explained by the presence in the protein structure of α-helices, β-structures, and disordered regions of optically active molecules that

interact with light polarized in different ways. By itself, plane-polarized light can be divided into 2 equal components of right- and left-handed light. If plane-polarized light is transmitted through solutions containing optically active molecules, these two components will be absorbed differently depending on the type of structures that molecules form in solution. So α-helix have negative absorption peaks at 208 and 222 nm and positive at 192 nm. β-folded structures have a negative maximum at 215 nm and a positive at 198 nm.

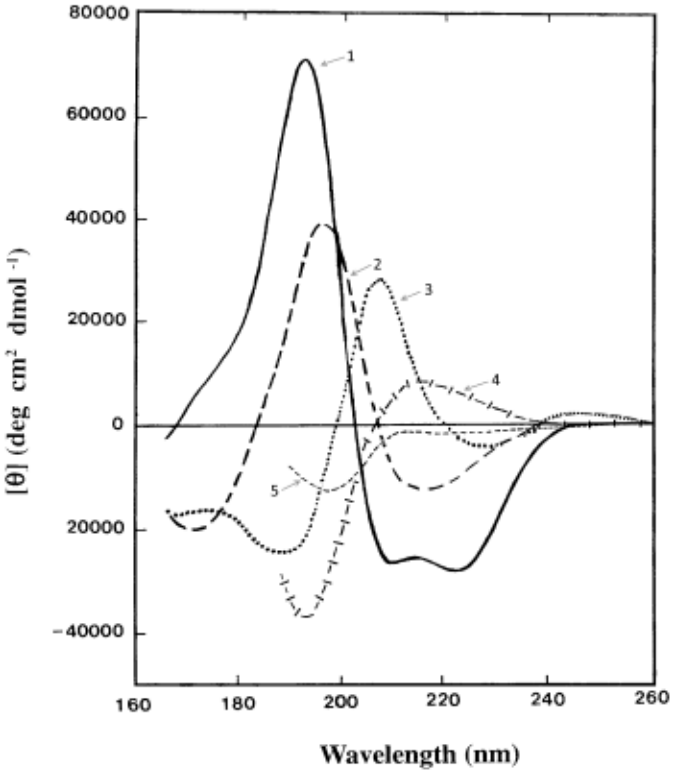


Fig. 26. Circular dichroism spectra of typical protein structures:
1 - α-helix; 2 - antiparallel β-strands; 3 - β-turns; 4 - polyproline helix; 5 - disordered protein chains.

The ratio between protein absorption of light with single wavelength, but different angle of polarization, is called molar ellipticity (Θ). The ratio of this parameter at 218 and 207 nm shows the change in the number of β-structures, for example, and samples of the aggregated prion are compared with it.

The measurements were carried out in samples with a protein concentration of 40 to 80 μM, made on the basis of 100 mM Na-acetate pH 4.0 and 20 mM MOPS buffers (Device Applied Photophysics Chirascan). The solutions were placed in quartz cuvettes with an optical path length of 0.1 mm and circular dichroism was measured in the range from 196 to 260 nm.

Thioflavin T fluorescence

The method is based on measuring the fluorescence spectra of dye thioflavin T in combination with amyloid structures (Nilsson, 2004). In its free form, thioflavin T has a fluorescence peak of about 480 nm, when linked to amyloid and, presumably, micellar structures, it shifts by 10–20 nm to the long-wavelength region, and the fluorescence intensity of the dye greatly increases.

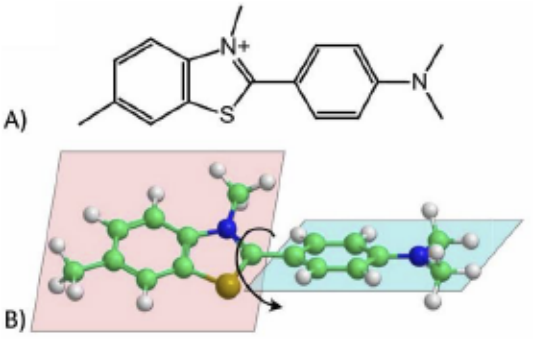


Fig. 27. The structure of the dye thioflavin T.
A - the flat structure of the dye; B - the volume structure of the dye with an indication of the bond along the axis of which rotation is possible (Biancalana & Koide, 2010).

A freshly prepared water solution of ThT was added to 0.1 mg/ml beta-casein sample in 20 mM Tris-HCl buffer, pH 7.5 at a molar ratio of 100:1. ThT was incubated avoiding light at room temperature for 20 min with protein samples before the measurements. Spectra of ThT fluorescence at 490-600 nm were acquired on Hitachi F 4500 spectrofluorimeter at 20°C using an excitation wavelength of 445 nm.

Measurement of ThT fluorescence intensity at the maximum point is informative too. The measurements were carried out in 96-well black plates for fluorescence (Device

PerkinElmer 2030 Multilabel Reader Victor X5). 50 µl of aggregated protein solution with a concentration of 40 µM was added to the well, then a dye solution was added to the well to a protein concentration of 20 µM and thioflavin 4 mM, then the sample was mixed by pipetting and incubated for 15 min at room temperature. The wavelength of the exciting light is ThT 435 nm (a light filter at 430 nm was used), the fluorescence intensity maximum was 490 nm (a light filter was used at 485 nm).

Fluorescence microscopy with thioflavin T

The fluorescence microscopy is the most informative way of protein samples stained with thioflavin T analysis. This method is especially useful for analyzing samples containing big colored particles, which prevents fluorescence intensity measurement by the classical way using a fluorometer. The method allows to estimate the fluorescence intensity of aggregates by the exposure time required for obtaining clear images of comparable brightness, as well as the structure and nature of aggregates, i.e. their size and packing density of molecules.

Conditions for fluorescence microscopy: c (protein) = 1.5 mg / ml, c (ThT) = 2 mM in Tris-HCl buffer 20 mM pH 7.5. λ (exc) = 440-480 nm, $\Delta\lambda$ (fluo)> 490 nm. The protein solution in a total concentration of 3 mg/ml is mixed with a solution of 4 mM thioflavin T in the same buffer as the protein, in a 1: 1 ratio. Mixing is possible in eppendorfs or directly on glass with mixing by a plastic tip of a pipette. The glasses 26x76x1 mm were used for microscopy, the size of cover glasses were 22x22 mm. Prepared samples is incubated for ~ 20 min in the darkness at room temperature. The preparations were studied under a microscope at an excitation wavelength of 445 nm, and a light filter for emitting fluorescence of 500 nm (parameters similar to fluorescence microscopy of preparations with the FITC tag). A Leica DMRD fluorescence microscope with a Nikon DS-1QM camera or a Carl Zeiss Axiovert 200M camera with a Hamamatsu Orca 2GF2 was used.

Congo red treatment

A freshly prepared water solution of Congo red (Frid, Anisimov, & Popovic, 2007) was added to protein solution (0.1 mg/ml) in 20 mM Tris-HCl buffer, pH 7.5 at a molar ratio of 100:1. Congo red was incubated for 15 min with protein samples before the measurements. Spectra of Congo red absorption were acquired at 490-520 nm using spectrophotometer Hitachi U-2900 (Japan) at 20 ºC with a 10-mm-path-length cuvette. The buffer spectrum was used as a blank and subtracted from all other spectra.

Immunofluorescence microscopy

Protein samples were applied onto slides (Shakes, Miller, & Nonet, 2012) with preliminarily fixed nitrocellulose membrane and air-dried during 10 min, then slides were washed five times with 25 мM Tris-HCl buffer, pH 7.5, containing 0.1% Tween-20 and 5 more times with 25 мM Tris-HCl buffer, pH 7.5. Next, slides were blocked with 1% BSA in 25 мM Tris-HCl buffer, pH 7.5, containing 0.1% Tween-20 for 40 minutes and washed with the same buffer. The slides were incubated with primary polyclonal rabbit antibodies against bovine beta-casein (50 µg/ml) for 1 hour. After washing, secondary monoclonal goat anti-rabbit antibodies with Alexa Fluor 555, ThermoFisher Scientific) were applied for 1 hour. Representative images were taken with a microscope Carl Zeiss Axiovert 200M combined with a camera Hamamatsu Orca 2GF2.

Transmission electron microscopy (TEM)

Samples of beta-casein (5 mg/ml) were glycated in 50 mM Tris-HCl buffer, pH 8.0, containing 0.01% sodium azide with 200 mM glucose at 37°C during 48 hours. The prepared samples were adsorbed on the Formvar film applied to the copper mesh (200 mesh) and stained with uranyl acetate for the negative contrast of the structures (Anderson & Webb, 2011). After air drying and carbon spraying, the samples were investigated using an Jeol JEM-1400 transmission electron microscope (Jeol, Japan) at an accelerating voltage of 100 kV.

Measurement of glyceraldehyde-3-phosphate dehydrogenase enzymatic activity

Enzyme activity was measured by the dynamics of NADH accumulation during the dehydrogenase reaction catalyzed by GAPDH.

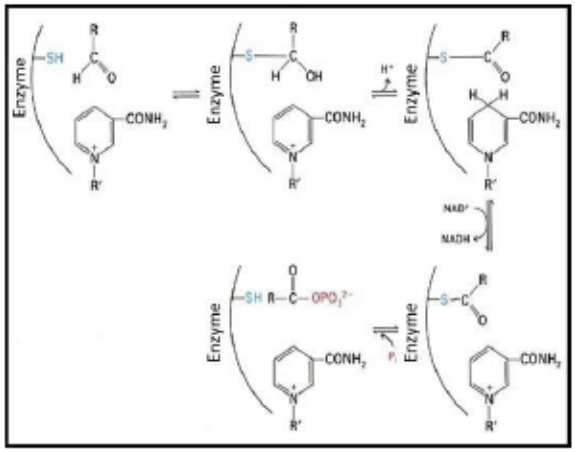


Fig. 28. The mechanism of reaction catalyzed by GAFD (Stryer, 1999).

The measurements were carried out on a Shimadzu UV-1601 spectrophotometer (molar extinction coefficient NADH ε (340) = 6.22 * 103 M-1*cm-1). The sample (volume 1 ml) contained 50 mM glycine, 2 mM EDTA, 100 mM KH_2PO_4, pH 8.9, 1 mM 3-PGA, 1 mM NAD^+, and 1 µg GAPDH from rabbit muscles, the activity of which was to be assessed. The reaction was initiated by the addition of 3-PGA. The growth of absorption was recorded within 1 minute. To calculate the specific activity, a linear area of the absorption curve was used, which was observed during the first 15 seconds of the reaction. The amount of enzyme that catalyzes the conversion of 1 µmol NAD + to NADH per minute was taken as unit of activity. The total activity was obtained by multiplying the enzymatic activity by 1 ml of solution by the total volume of the sample.

Results of experiments

Part 1. Coaggregation of prion protein and food proteins

Aggregation of prion protein in the presence of low-molecular potential inhibitors of amyloid conversion

At the first stage of the research, sheep prion protein was put in the forefront as a protein easily provoked on amyloid conversion and aggregation. The effect of low molecular weight ligands on its aggregation was evaluated without any modifications. The goal was to determine the most effective anti-amylolid ligands from 4 tested compounds. Curcumin, a well-known prion protein ligand capable of inhibiting amyloid aggregation, is considered to be known antiamyloid ligand (Maiti & Dunbar, 2018). An alternative ligand with a presumably high ability to inhibit amyloidization in our study was 3,4-dimethoxycinamic acid. In addition, two new potential inhibitors of amyloidization were included to the list: pentamidine isethionate, used as an antibiotic (Western, Perera, & Schultz, 1985) and an anti-cancer agent (Zerbini et al., 2014), and resveratrol, for which cardioprotective (Meng, Liu, & Du, 2014), and also anti-oncological (Carter, D'Orazio, & Pearson, 2014) effects were previously shown.

The data from this section were used in published articles (Tishina, S. A., Stroylov, V. S., **Zanyatkin, I. A.** et al., 2017; **Zanyatkin, I.**, Stroylova, Y., Tishina, S, 2017).

To obtain the most obvious results, we decided to accelerate the amyloidization of PrP and apply the protocol previously used for the amyloid conversion of β-lactoglobulin (Raynes et al., 2017), in which the prion protein also forms intermediate oligomers and goes through amyloid conversion more actively:

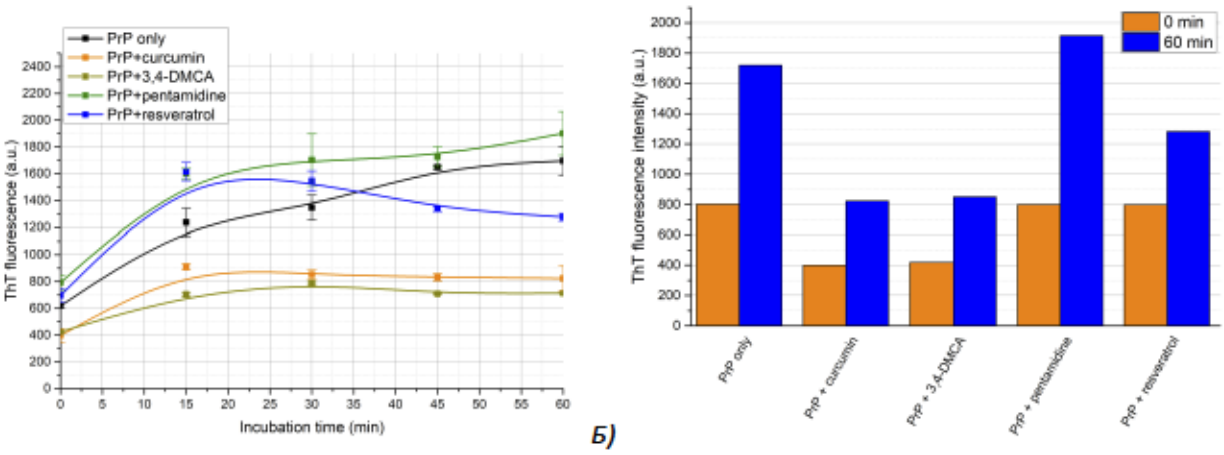
The protein concentration is 3 mg/ml.

Incubation conditions: MES 25 mM pH 6.7 with 0.1 M NaCl. Incubation was carried out for 1 h at 94°C.

Initially, the ligands were supposed to be introduced into the solution in a 5-fold excess relative to the protein concentration, but due to the low solubility of curcumin, this idea

was abandoned and in all cases the ligand excess against the protein concentration was only 3-fold.

The analysis of the samples started from fluorimetry with thioflavin T color.



А) *Б)*

Fig. 29. Fluorimetric analysis of prion protein aggregation in the presence of low molecular weight ligands. Thioflavin T coloration.

A - kinetics of fluorescence intensity; B - fluorescence intensity at peak before and after thermoaggregation. The ratio of protein: ligand = 1: 3, Incubation for 1 h at 94°C.

As expected, in the presence of curcumin and 3,4-DMCA the fluorescence intensity of the amyloid-sensitive dye decreased significantly, pentamidine did not show a decrease in the fluorescence intensity of thioflavin T, and resveratrol showed rather controversial results: on the one hand, the dye fluorescence during thermal incubation increased stronger than with curcumin or 3,4-DMCA, on the other - not so much as in the control sample of the protein or in the presence of pentamidine. Next, the samples were analyzed under a fluorescent microscope. Conditions for fluorescence microscopy: s (protein) = 1.5 mg/ml, s (ThT) = 2 mM in Tris-HCl buffer 20 mM pH 7.5. λ (exc) = 440-480 nm, Δλ (fluo)>490 nm.

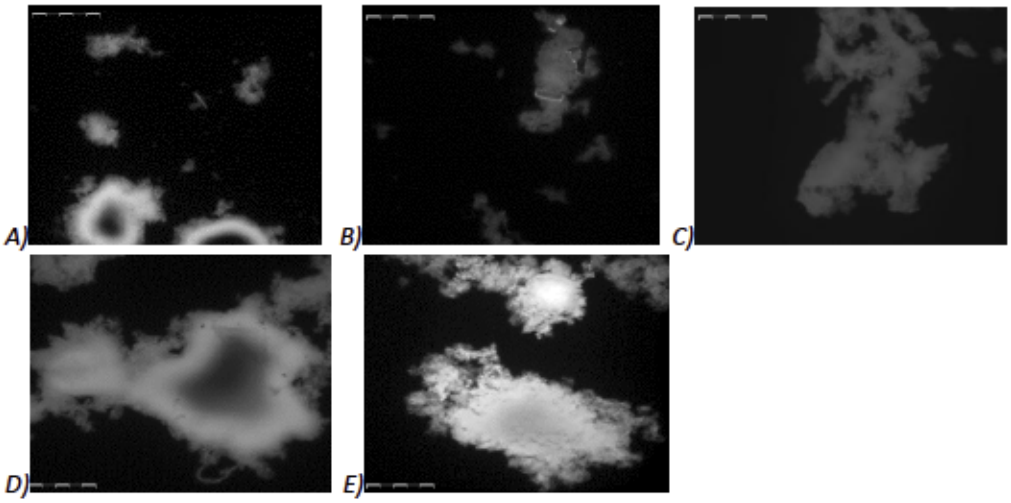


Fig. 30. Fluorescence microscopy of prion protein thermoaggregation with low molecular weight ligands. A - the isolated prion protein; B - protein with curcumin; C - protein with 3,4-dimethoxycinnamic acid; D - protein with pentamidine isethionate; E - protein with resveratrol. Exposure at 16 ms. Incubation 1 h at 94°C. Scale bar 50 microns.

Curcumin presence led to a strong decrease of fluorescence intensity and decrease in the size of the prion protein aggregates. 3,4-DMCA made the aggregates much less dense, but the fluorescence of the dye was less affected. Pentamidine almost didn't demonstrate significant effect, and the presence of resveratrol made the aggregates less dense, but unexpectedly their fluorescence intensity increased.

The ability of the most promising ligand of 3,4-dimethoxycinnamic acid to bind to the prion protein was further studied using the methods of circular dichroism and dynamic light scattering.

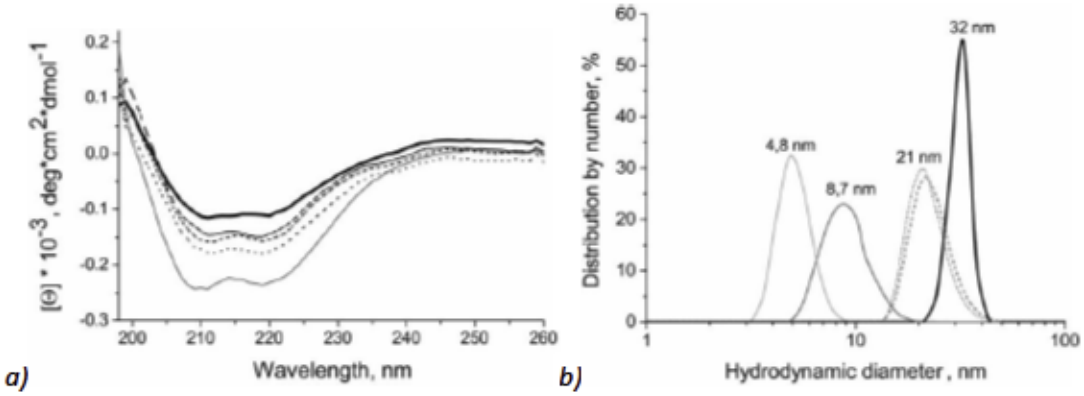


Fig. 31. A – hydrodynamic diameters of particles after PrP oligomerization in the presence of 3,4-DMCA; B - Circular dichroism spectra of native PrP and prion in oligomeric form in the presence of 3,4-DMCA. The

populations of particles from each experiment are shown on the same plot. Native 20-μM PrP in 20 mM MOPS buffer, pH 7.5, is shown by light gray solid line; 20-μM PrP oligomers formed by incubating at 60 °C for 1 h in 20 mM MOPS buffer, pH 7.5, are shown by black solid line; 20-μM PrP oligomers, formed in the presence of 50-μM 3,4-DMCA, are shown by gray dashed line; 20-μM PrP oligomers, formed in the presence of 100-μM 3,4-DMCA, are shown by gray solid line; 20-μM PrP oligomers, formed in the presence of 100-μ M curcumin, are shown by gray dotted line.

Circular dichroism spectra of PrP subjected to thermoaggregation with 3,4-DMCA was shown to become much closer to the CD spectrum of the native protein, than PrP thermoaggregated without ligands. Protein particles in the presence of 3,4-DMCA, according to DLS, also turned out to be smaller than oligomers, corresponding, rather, to protein dimers (hydrodynamic diameter is ~ 2 times larger than the size of monomers).

Coupled with the results of determining the parameters of binding the protein to the ligand and comparing the solubility of ligands in aqueous systems, it can be argued that 3,4-DMCA is the ligand, the most effective of the studied compounds that suppress amyloidization of the prion protein.

Analysis of the cytotoxicity of 3,4-DMCA on the SH-SY5Y neuroblastoma culture showed that the survival of cells in the presence of only ligand in small concentrations even increased. The ligand also had a positive effect on the survival of the culture in the presence of prion oligomers - the number of survived cells in the presence of 3,4-DICA increased by 15–20% compared with the sample with PrP^{Sc} oligomers and without ligand.

Co-aggregation of prion protein and some food proteins in the presence of low-molecular potential inhibitors of amyloid conversion

In the next experiment, joint incubation of PrP and food (mainly milk) proteins with ligands was carried out. The concentration of the ligand was determined by the concentration of prion protein and was 3-fold excess relative to PrP.

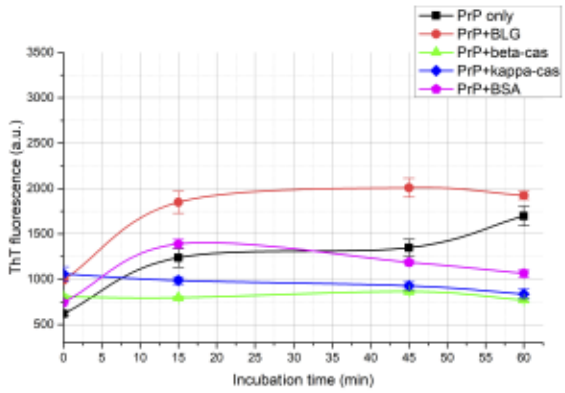
Table 2. Concentrations of proteins and ligands in samples intended for the joint incubation of prion protein, β-lactoglobulin, bovine serum albumin, κ- and β-caseins.

Sample name	Protein composition	Ligand concentration (if it was presented)
PrP isolated	PrP 130 uM	650 uM
PrP+BLG	PrP 65 uM + ß-lactoglobulin 83 uM	325 uM
PrP+β-casein	PrP 65 uM + β-casein 60 uM	325 uM
PrP+κ-casein	PrP 65 uM + κ-casein 57 uM	325 uM
PrP+BSA	PrP 65 uM + albumin 21uM	325 uM

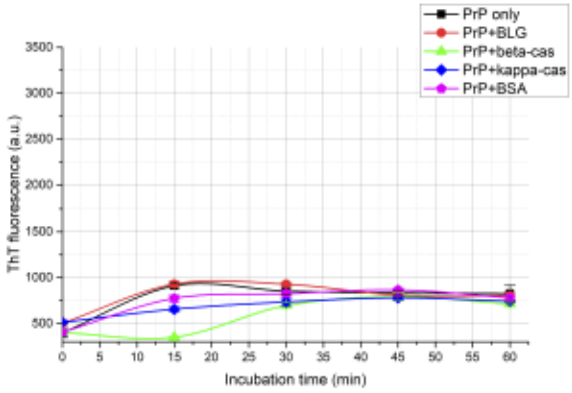
Conditions:

- Incubation time: 1 h.
- Temperature: 94°C.
- Buffer: 25 mM Na-phosphate pH 6.7 with 0.1M NaCl.

Analysis of the sampleswas started from fluorescence spectroscopy. The fluorescence intensity was measured for an hour at several points.



A)



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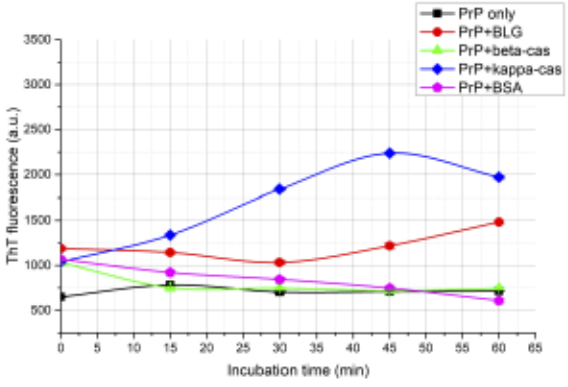

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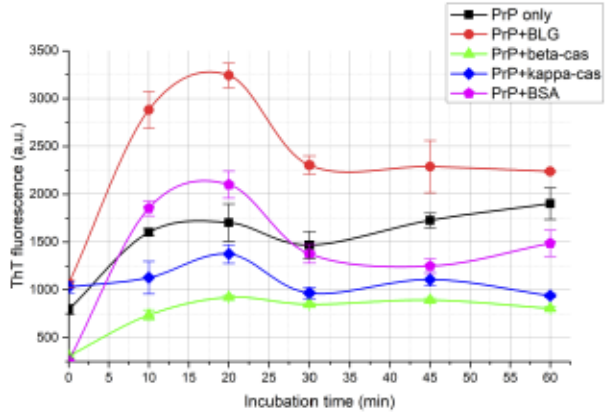
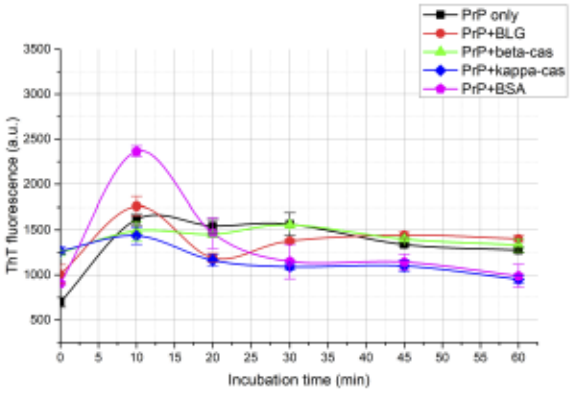
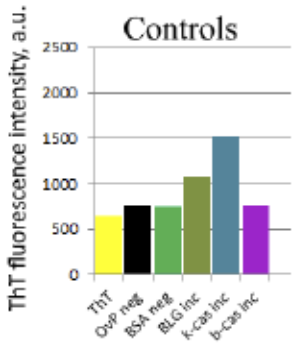


D)



E)



F)

Fig. 32. Fluorimetric analysis of coaggregation of prion protein, β-lactoglobulin, β- and kappa-caseins, as well as bovine serum albumin. Thioflavin T coloration.

A - aggregation without ligands; B - aggregation in the presence of curcumin; C - aggregation in the presence of 3,4-dimethoxycinnamic acid; D - aggregation in the presence of pentamidine isethionate; E - aggregation in the presence of resveratrol, F - maxima of fluorescence intensity at 500 nm in the control samples.

Accepted control measurements (F, from left to right): thioflavin T, non-aggregated PrP, non-aggregated bovine serum albumin, incubated with β-lactoglobulin at 94 ° C, thermo-incubated at 94 ° C, kappa-casein, thermo-incubated at 94 ° C, β-casein. In the graphs: the black line is the kinetics of thioflavin T fluorescence in samples of thermally incubated prion protein (c (PrP) = 3 mg/ml). The red line is the kinetics of thioflavin T fluorescence in samples of thermo-incubated prion protein and β-lactoglobulin (c (PrP) = 1.5 mg/ml, c (BLG) = 1.5 mg/ml). The light green line is the kinetics of thioflavin T fluorescence in samples of thermo-incubated prion protein and β-casein (c (PrP) = 1.5 mg/ml, c (k-cas) = 1.5 mg/ml). The blue line is the kinetics of thioflavin T fluorescence in samples of thermo-incubated prion protein and kappa-casein (c (PrP) = 1.5 mg/ml, c (k-cas) = 1.5 mg/ml). The pink line is the kinetics of thioflavin T fluorescence in samples of thermo-incubated prion protein and bovine serum albumin (c (PrP) = 1.5 mg/ml, c (BSA) = 1.5 mg/ml). Incubation 1 h at 94°C.

As a result, according to the fluorimetric analysis of the effect of ligands on these proteins protein interactions, the following observations were made:

- Curcumin - fluorescence of samples of all proteins combinations is significantly reduced, although it slightly increases soon after the start of thermal incubation.
- 3,4-dimethoxycinnamic acid is capable of suppressing the fluorescence of the dye in preparations, but, unlike curcumin, not in all combinations of proteins. Unexpectedly bright fluorescence of thioflavin T was detected in a sample containing prion protein, DMCA and kappa-casein.
- Pentamidine isethionate - fluorescence has become even brighter than without ligand. The mixture of prion protein and β-casein is practically unchanged.

- Resveratrol - fluorescence intensity was approximately equal in all samples, but at the level of aggregated prion protein.

Because of the active precipitation in the samples, it was decided to examine the samples with a fluorescence microscope.

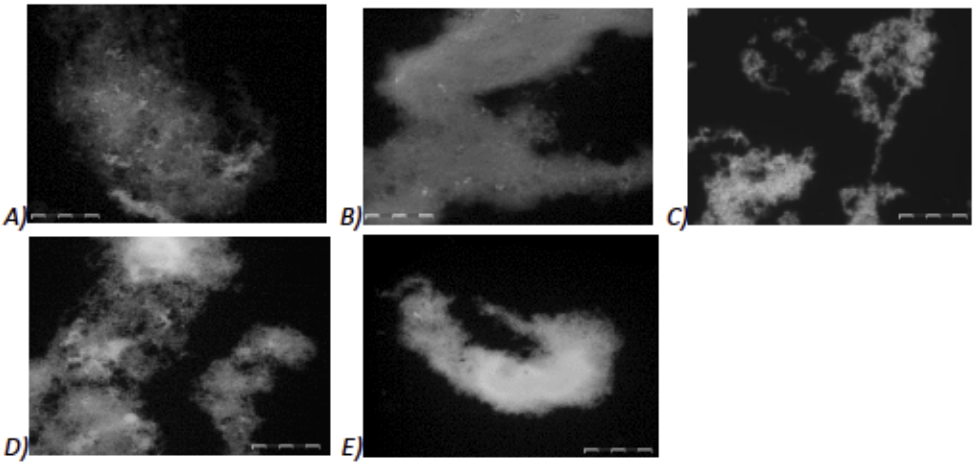
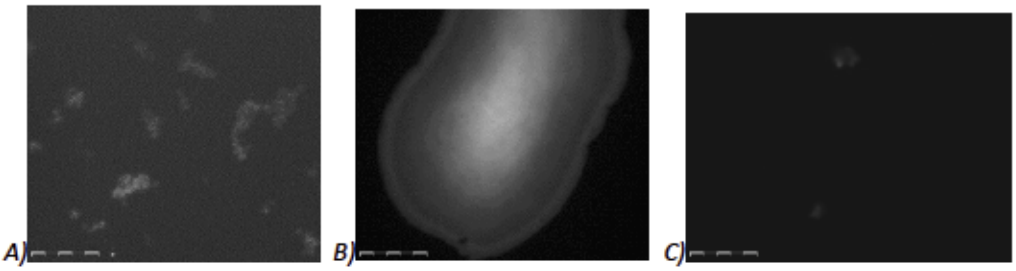


Fig. 33. Fluorescence microscopy of co-aggregation of prion protein and β-lactoglobulin with low molecular weight ligands. Thioflavin T coloriation.

A is a prion protein and β-lactoglobulin; B - proteins with curcumin; C - proteins with 3,4-dimethoxycinnamic acid; D - proteins with pentamidine isethionate; E - proteins with resveratrol. Exposure at 16 ms. Scale length 50 microns.

As a result of thermoaggregation, prion protein and β-lactoglobulin themselves form rather loose aggregates consisting of many entangled filaments. In the presence of curcumin, the aggregates became unexpectedly more dense, with the DMCA the aggregates decreased in size, but seemed to become compacted. Pentamidine led to a slight increase in the size of the aggregates, without changing their structure; with resveratrol the aggregates became unexpectedly dense and with quite bright fluorescence of the bound dye.



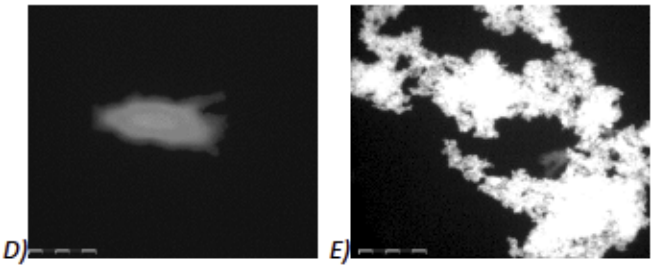


Fig. 34. Fluorescence microscopy of co-aggregation of prion protein and β-casein with low molecular weight ligands. Thioflavin T coloration.

A - prion protein and β-casein (200 ms exposure); B - proteins with curcumin (600 ms exposure); C - proteins with 3,4-dimethoxycinnamic acid; D — proteins with pentamidine isethionate (600 ms exposure); E - proteins with resveratrol (24 ms exposure). Scale bar 50 microns.

Even after thermal incubation, the number of prion protein aggregates in the presence of β-casein remained small, and the aggregates themselves were small. In the presence of curcumin, the fluorescence of the aggregates became even lower, but their size and density increased significantly. The combined action of DMCA and β-casein almost completely suppressed both the formation of aggregates and the fluorescence of amyloid-sensitive thioflavin T in the sample. In the presence of pentamidine isethionate, the aggregates of the prion protein and β-casein are similar to those obtained in the presence of curcumin, but they are smaller, although they have slightly brighter fluorescence. Resveratrol unexpectedly led to a very bright fluorescence of the aggregates, negating the anti-amyloid effect of β-casein.

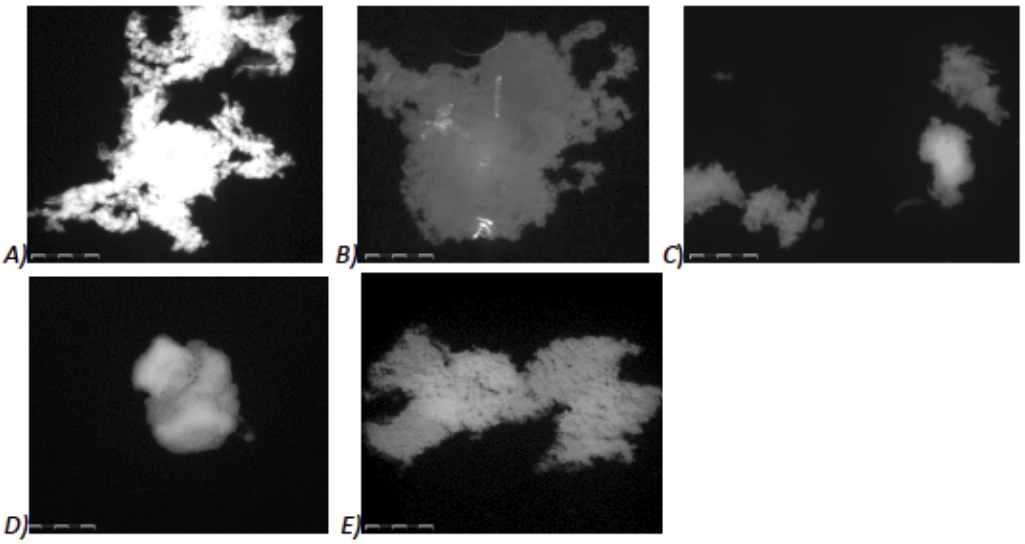


Fig. 35. Fluorescence microscopy of co-aggregation of prion protein and kappa-casein with low molecular weight ligands. Thioflavin T coloration.

A - prion protein and kappa-casein (11 ms); B - proteins with curcumin (33 ms); C - proteins with 3,4-dimethoxycinnamic acid (33 ms); D - proteins with pentamidine isethionate (103 ms); E - proteins with resveratrol (11 ms). Scale bar 50 microns.

Aggregates of thermally incubated prion protein and kappa-casein showed very bright fluorescence and rather high density. The presence of curcumin did not lead to significant disassembly of these aggregates, but the fluorescence intensity became significantly lower. 3,4-DMCA led to a significant decrease in the aggregates size and a decrease in ThT fluorescence, slightly less significant than curcumin. In the presence of pentamidine isethionate, the aggregates became small, but dense, and the dye in them fluorescently dim. Finally, in the presence of resveratrol, aggregates became looser, but the dye fluorescence decreased slightly.

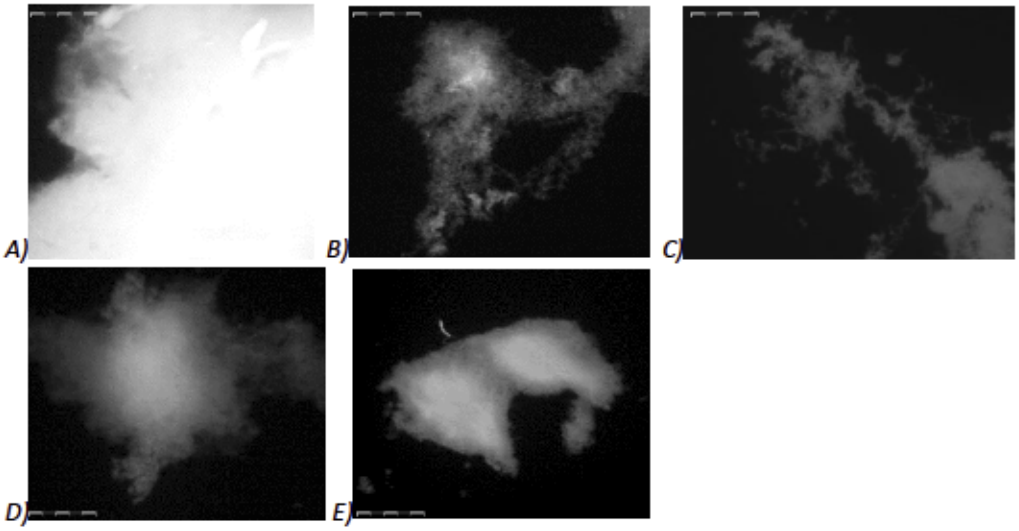


Fig. 36. Fluorescence microscopy of co-aggregation of prion protein and bovine serum albumin with low molecular weight ligands. Thioflavin T coloration.

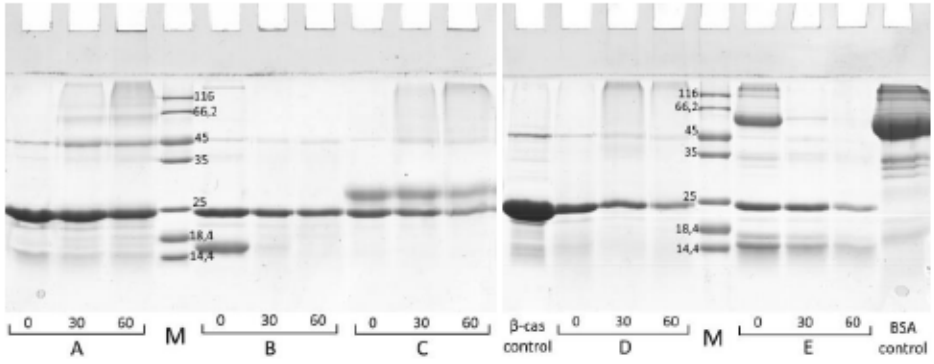
A - prion protein and bovine serum albumin (extract 4 ms); B - proteins with curcumin (16 ms); C - proteins with 3,4-dimethoxycinnamic acid (20 ms); D - proteins with pentamidine isethionate (4 ms); E - proteins with resveratrol (4 ms). Scale bar 50 microns.

Thermal incubation of prion protein and bovine serum albumin led to the formation of a huge conglomerate (compared with the other ones) with very bright fluorescence. This is particularly noteworthy in the light of the fact that individual proteins did not aggregate so rapidly. All low molecular weight ligands used led to loosening, a decrease in the size of the aggregates and fluorescence intensity of the dye associated with them.

Summarizing the results of fluorescent microscopy, the following conclusions can be made about the effect of the tested ligands:

- Curcumin - the fluorescence intensity has decreased significantly.
- 3,4-dimethoxycinnamic acid - a decrease in the intensity of fluorescence, but not as powerful as in the case of curcumin. Instead, a somewhat higher aggregation suppression effect.
- Pentamidine isethionate - the fluorescence decreased slightly, but the aggregates became denser.
- Resveratrol - the formed aggregates became practically identical in structure, morphology and fluorescence intensity for all studied protein combinations.

Then, all samples were analyzed for the size and stability of the aggregates by the methods of native and denaturating electrophoresis.



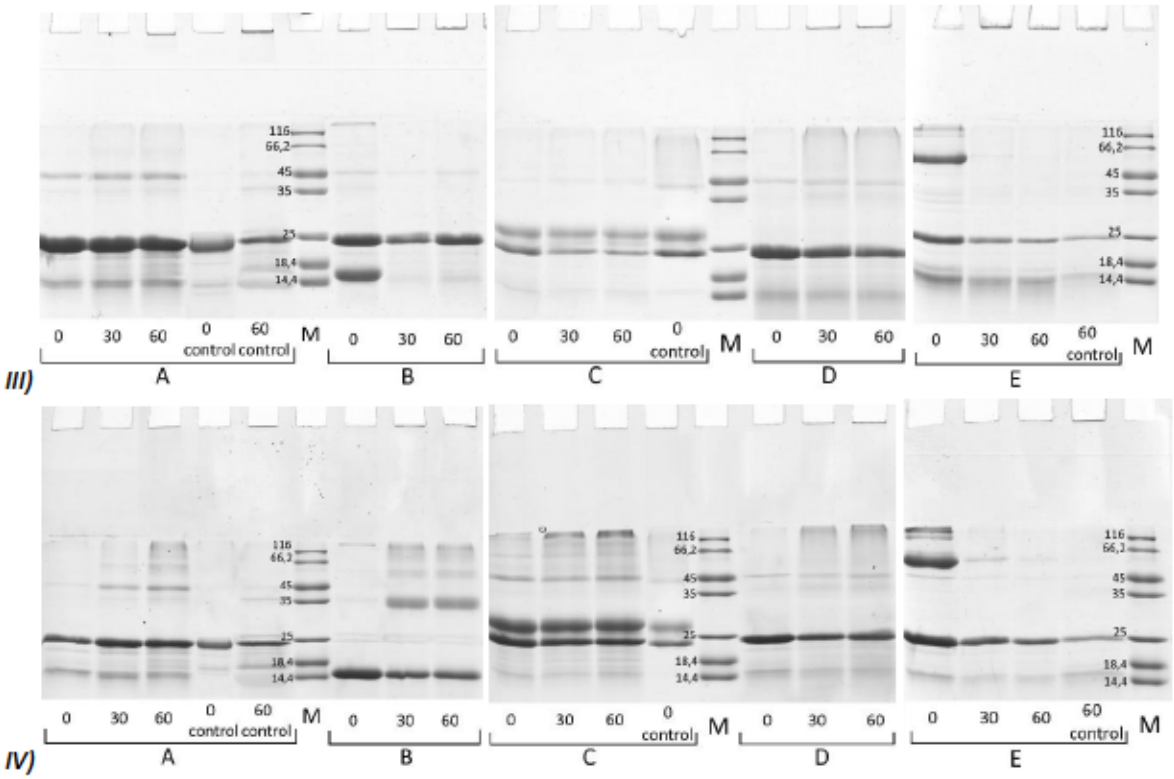


Fig. 37. Analysis of coaggregation of prion protein, β-lactoglobulin, β- and kappa-caseins, as well as bovine serum albumin by SDS-electrophoresis:

I - aggregation with curcumin (s (PrP): c (cur) = 1:5), II - aggregation with 3,4-DMCC (s (PrP): c (DMCA) = 1:3), III - aggregation with pentamidine isethionate (c (PrP): c (pen) = 1:3), IV - aggregation with resveratrol (c (PrP): c (res) = 1:3).

A is an isolated prion protein, B is a prion protein and β-lactoglobulin; C is a prion protein and β-casein; D - prion protein and kappa-casein; E - prion protein and bovine serum albumin. The ratio of proteins in all samples is 1:1.

2% concentrating gel, 10% separating gel, the amount of protein per well - 10 μg. Before the experiment, the samples were heated at 96 ° C for 2-3 minutes.

According to the results of the experiments, the following observations were made:

- Curcumin - the fluorescence intensity has decreased significantly.
- 3,4-DMCC - is particularly effective against PrP and its combination with β-casein, in which both aggregation and amyloidization are suppressed. With other proteins, the effect is less.
- Pentamidine isethionate - the amount of monomer of the prion protein becomes larger, the aggregation of other proteins is affected by little. The fluorescence of thioflavin T in samples varies little from the ligand.

- Resveratrol - the formation of aggregates, including large ones that are not included in the concentrating gel, was observed in all samples, including those with β-casein. The aggregation parameters become similar, regardless of the protein composition.

Interaction of prion protein and chaperons

After detection of β-casein antiaggregation effect on prion protein, it was decided to test a similar effect for true chaperonins, in particular, GroEL/GroES complex. The experiment involved native prion protein, intermediate oligomers, amyloid immature "protofibrils" and formed fibrils.

The data from this section were used in published articles (Kudryavtseva SS, Stroylova YY, **Zanyatkin** IA et al. 2017)

The interactions between different forms of PrP and the chaperonin complex were determined by the method of enzyme immunoassay on a sorbent (ELISA) with staining by primary mouse monoclonal antibodies $Q^{220}YQRES^{225}$ and secondary anti-mouse antibodies conjugated with horseradish peroxidase. The PrP control preparations were applied first for sorption on the tablet surface, the GroEL / GroES chaperonin complex was added to the experimental samples for sorption, and then PrP was added to bind to the complex, and its presence was measured by the absorption intensity of the o-phenylenediamine oxidation product by hydrogen peroxide (the reaction was catalyzed by peroxidase), at a wavelength of 492 nm.

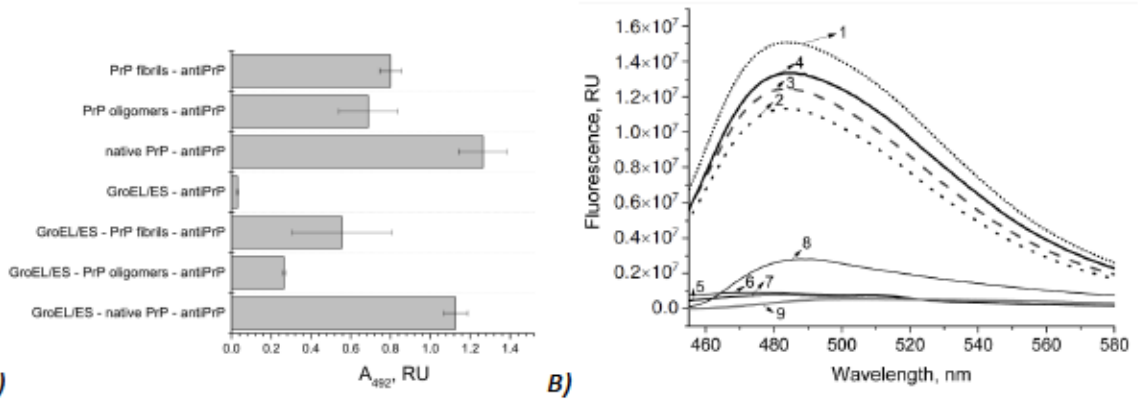


Fig. 38A (left). ELISA analysis of the binding of the prion protein and the chaperonin complex GroEL/GroES. Immunochemical staining with mouse monoclonal antibodies against $Q^{220}YQRES^{225}$ epitope

and secondary antibodies conjugated with horseradish peroxidase.

Fig. 38B (right). Fluorescence analysis of the interaction of the prion protein and the GroEL/GroES chaperonin complex. Thioflavin T staining.

Curves: 1 — GroEL/GroES chaperonin complex after interaction with native PrP^c 2 — GroEL14/GroES7 chaperonin complex after interaction with oligomers; 3 - GroEL/GroES chaperonin complex after interaction with PrP "protofibrils"; 4 - GroEL/GroES chaperonin complex after interaction with PrP fibrils; 5 - native prion protein PrPc; 6 - PrP oligomers; 7 - PrP "protofibrils"; 8 - PrP fibrils; 9 - isolated chaperonin complex GroEL/GroES.

A 0.6 μM prion protein was incubated in the presence of 0.6 μM GroEL, 1.2 μM GroES in medium containing 10 mM KH_2PO_4, 1 mM EDTA, 5 mM beta-ME, 1.5 mM NAD +, 2 mM ATP, 2 mM Mg^{2+}, pH 7.5.

ThT fluorescence and consequently percent of amyloid structures has only increased from the presence of GroEL/GroES chaperonin complex.

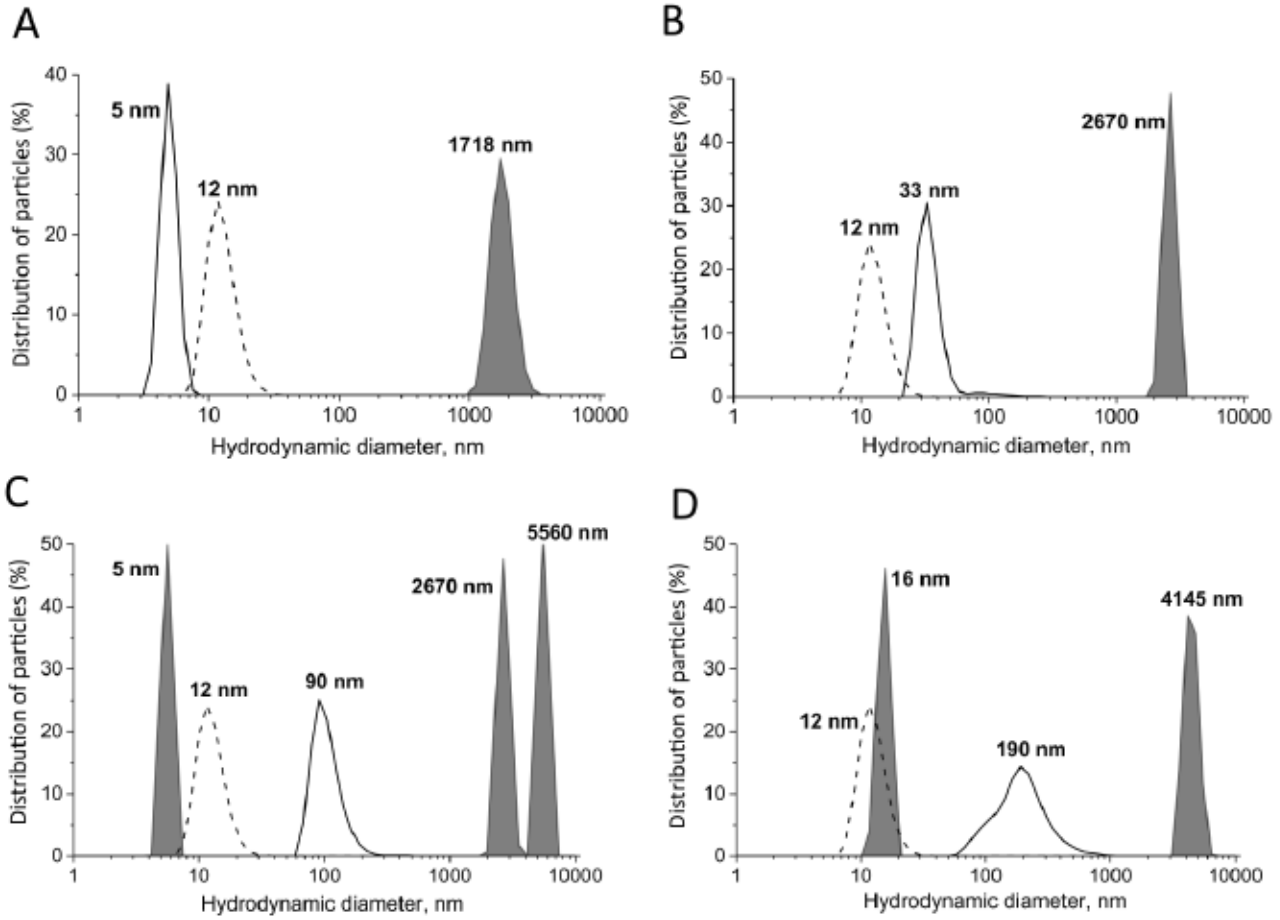


Fig. 39. Particle size distribution of the prion protein and the GroEL/GroES chaperonin complex. Detection by dynamic light scattering.

Protein combinations: A - 0.6 μM native PrP with 0.6 μM GroEL/GroES complex; B — 0.6 μM PrP oligomers with 0.6 μM GroEL/GroES complex; B — 0.6 μM PrP "protofibrils" with 0.6 μM GroEL/GroES complex; G - 0.6 μM PrP fibrils with 0.6 μM GroEL/GroES complex.

The peaks of the solid lines are isolated prion protein, the peaks are dotted - the isolated GroEL/GroES complex, the gray peaks are a mixture of PrP and the chaperonin complex.

Co-incubation was carried out in a medium containing 10 mM KH_2PO_4, 1 mM EDTA, 5 mM beta-ME, 1.5 mM NAD^+, 2 mM ATP, 2 mM Mg^{2+}, pH 7.5.

The aggregates formed in a mixture of proteins are much larger in size than the aggregates of isolated proteins, even prion fibrils.

Part 2. Influence of glycation on β-casein properties

Research of glycation effect on the properties of casein is still at the initial stage, therefore, first, experiments were carried out to select the optimal modification conditions, i.e. least time-consuming, but with the result as close as possible in vivo.

Modification of beta-casein by glucose and methylglyoxal

Firstly, we studied the efficacy of beta-casein glycation by glucose varying incubation conditions - temperature, pH, incubation time and concentration of protein and modifier. After the modification of beta-casein was completed, we performed the removal of glucose and low-molecular intermediate glycation products by reverse phase chromatography. The efficacy of modification of beta-casein was evaluated monitoring the loss of free amino groups of the protein in reaction with ortho-phthalic aldehyde. We also carried out reductive amination with sodium cyanoborohydride in order to fix the products of modification at the stage of Schiff base formation.

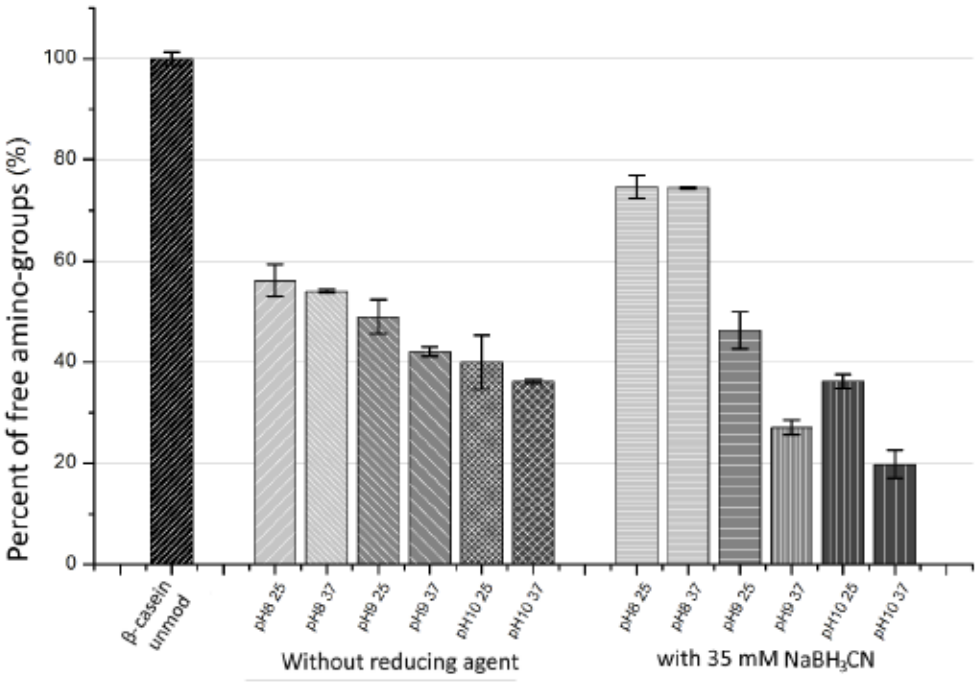


Fig. 40. The effect of pH, temperature and the addition of sodium cyanoborohydride during glycation of glucose β-casein on the content of free amino groups. O-phthalaldehyde analysis.

Glycation was carried out at 25°C or at 37°C at pH 8, 9, or 10 for 3 days in the presence or absence of a reducing agent of sodium cyanoborohydride (CN). The content of all available amino groups in a protein that has not undergone modification (theoretically 17 amino groups) is taken as 100%.

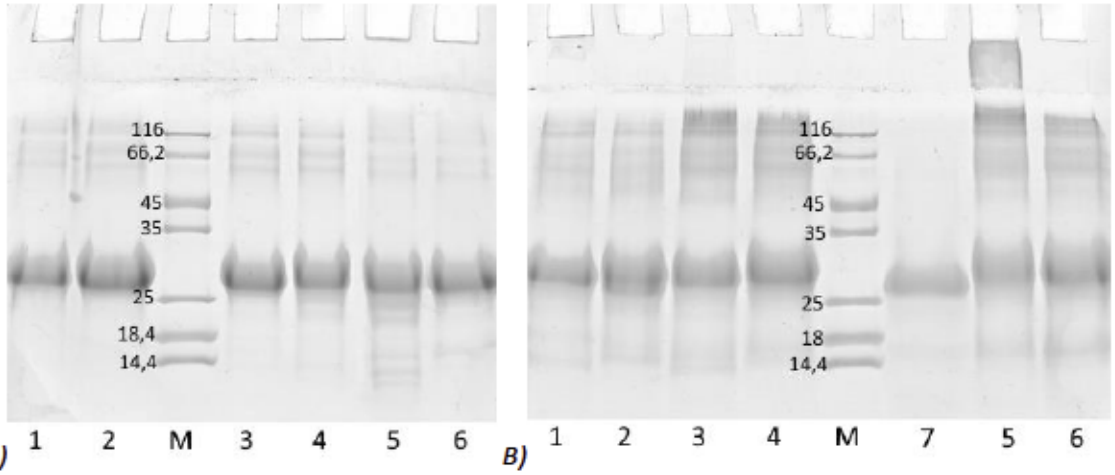


Fig. 41. Analysis of thermoaggregation of β-casein after its glycation. SDS electrophoresis.

A - samples after glycation at 25°C, B - samples after glycation at 37°C.

1-2 - samples after glycation at pH 8.0; 3-4 - samples after glycation at pH 9.0; 5-6 - samples after glycation at pH 10.0. Control: 7 - unmodified β-casein.

Glycation was performed at a protein concentration of 5 mg / ml, 0.2 M D-glucose. 3% concentrating gel, 15% separating gel, the amount of protein per well - 10 µg. Before the experiment, the samples were heated at 96 ° C for 2-3 minutes.

Figure 40 shows the dependence of the number of unmodified amino groups of beta-casein as a function of the temperature and pH, as well as the presence of sodium cyanoborohydride. As follows from presented data, the efficacy of modification of beta-casein rises with pH shift from 8 to 10, and also rises slightly with temperature increase from 25 to 37°C. Essential effect on the modification sodium cyanoborohydride demonstrated, reducing its level at pH 8.0. At the same time, the content of the modified groups increases in the presence of sodium cyanoborohydride at pH 9.0 and 10.0 and at a temperature of 37°C.

A further temperature rise to 60° C at pH 8.0 not only leads to an increase in the number of modified groups (from 43% to ~ 60% of the glycated groups for modification without a reducing agent), but also to aggregation of beta-casein. It should also be noted that the degree of aggregation rises with increasing pH to 9.0 and 10.0.

The aggregation can be observed not only by the increase in the turbidity of protein solutions but also by the data of electrophoresis in the presence of sodium dodecyl sulfate. As can be seen from the Figure 41, after glycation of beta-casein, a lot of additional bands appear, probably corresponding to the modified polypeptide chains of the protein, as well as its oligomeric forms. After glycation at high temperature and/or alkaline pH values, the content of oligomers and aggregates not entered in the gel significantly increases. This is especially noticeable when upon glycation at pH 10.0 and 37°C, a significant portion of the protein was found in the form of aggregates that did not enter the separating gel. The addition of cyanoborohydride reduces the content of oligomers and aggregates, at least at a temperature of 37°C and at pH 10.0.

Considering that the increase in the incubation temperature and the pH of the medium leads to an enhancement of beta-casein aggregation, for further experiments

we selected conditions leading to sufficiently effective glycation (modification of 40-50% amino groups of the protein) and minimal aggregation of beta-casein at a temperature of 37°C. We incubated beta casein for 48 or 72 hours at a temperature of 37°C in 50 mM Tris-HCl buffer, pH 8.0. The samples of beta-casein (5 mg/ml) were glycated by 0.2 M glucose without a reducing agent or in the presence of 35 mM $NaBH_3CN$.

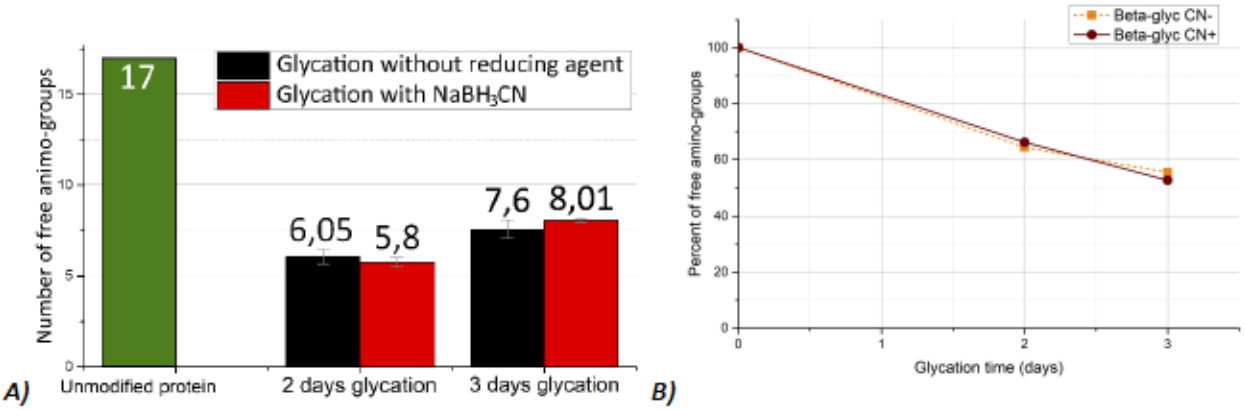


Fig. 42. Analysis of β-casein glycation degree by the number of early glycation products. Fructosamine method.

The amount of early products of β-casein glycation carried out for 2 or 3 days was compared at a concentration of 5 mg / ml versus 0.5 M D-glucose and optionally 35 mM NaBH3CN (CN- label means no reducing agent, CN + its presence) at 37 ° C . And the number of glycated groups per molecule of β-casein; B - the percentage of free amino groups in one β-casein molecule. The β-casein molecule has 12 Lys, 4 Arg + 1 α-amino groups.

The degree of glycation of beta-casein in the obtained preparations was additionally determined to monitor the amount of early glycation products by NBT-assay. The content of early products after 48 and 72 hours of incubation of beta-casein with glucose without the addition of cyanoborohydride is 6.1 ± 0.4 and 7.6 ± 0.5 moles per mole of protein, respectively. There are 17 free amino groups in the beta-casein molecule (12 lysine residues, 4 arginine residues, and one 1 alpha-amino group), and consequently, about 36% and 45% of the available amino groups undergo modification at 48 and 72 hours of incubation, respectively. These results are in good agreement

with the data obtained by reaction with ortho-phthalic aldehyde: after 72 hours of incubation, about 54% of the amino groups remain free under the same conditions.

The incubation of beta-casein with glucose also results in the accumulation of advanced glycation end-products (AGEs) that have been identified from the spectra of their own fluorescence. As follows from the results given in Table 1, AGEs are formed even in the presence of $NaBH_3CN$, although in this case, their content is reduced after 72 hours of incubation.

Glycation of beta-casein was also carried out with methylglyoxal, which is a highly reactive compound and allows to modify proteins at lower concentrations of the reagent and significantly faster. In addition, methylglyoxal modifies the amino groups of the amino acid residues of the proteins mainly resulting in carboxymethyl-lysine formation, whereas the various products formed by modification with glucose are difficult to identify precisely. Moreover, methylglyoxal is formed as one of the compounds in the cascade of glycation reactions, including glycation of proteins by glucose.

It was shown that the glycation of beta-casein with methylglyoxal at a concentration of 0.2 M at 37°C in 50 mM Tris-HCl buffer, pH 8.0, after only 1 day resulted in the modification of 12.8 ± 0.3 amino groups of the protein, that is 75% of their total number. Addition of sodium cyanoborohydride as the reducing agent significantly decreases the number of modified groups to 10.2 ± 0.1 per mole of protein. At the same time, as expected, the amount of AGEs is significantly lower upon modification by methylglyoxal comparing with glucose (Table 3).

Tab. 3. Analysis of β-casein glycation degree by the intrinsic fluorescence of late glycation products (AGE). The protein concentration in the samples was 0.5 mg/ml. λ (exc) = 335 nm, λ (fluo) = 410 nm. Fluorescence was measured in samples prepared on a 50 mM Tris-HCl buffer pH 7.5.

	ThT fluorescence intensity in β-casein samples

	Without reducing agent	With 35 mM $NaBH_3CN$
Native β-casein	4936	-
β-casein unmodified, 48 h	6765	-
β-casein unmodified, 72 h	6913	-
β-casein, 0,2M glucose, 48 h	18765	20222
β-casein, 0,2M glucose, 72 h	32812	26574
β-casein, 0,2M methyl-glyoxal, 24 h	15058	12387

Influence of glycation on beta-casein anti-aggregation properties

For the initial assessment of glycation effect on the properties of β-casein, it was decided to evaluate its ability to influence refolding of enzymes, whose activity is easy to measure. Rabbit GAPDH was chosen as the object of experiments because of its availability, easily measured enzymatic activity and the ability to partially renature and reactivate. In order to obtain more reproducible results, the time taken to measure the activity of the enzyme at each point was increased from 1 to 5 minutes. Denaturation and reactivation of the enzyme were carried out according to the method described in chapter "Materials and methods". In the process of reactivation, aliquots were taken from the reaction medium to determine the enzymatic activity of GAPDH and a plot of the dependence of GAPDH activity on the initial (in percent) on the reactivation time was constructed. Enzyme reactivation was monitored for 2 hours.

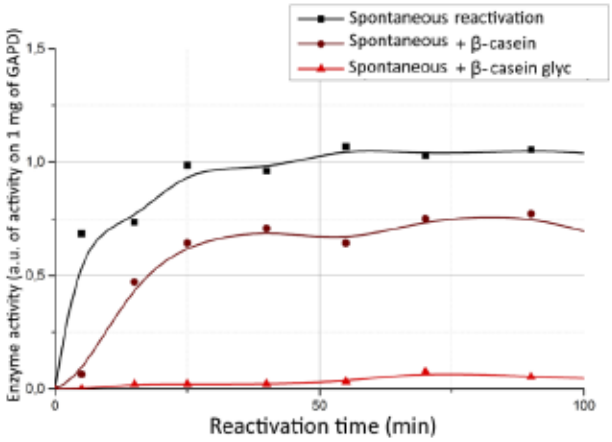


Fig. 43. influence of native and glycated casein on the spontaneous reactivation of GAPDH.

As a result, spontaneous reactivation of GAPDH in the presence of even native β-casein was found to be not so effective as in the case of isolated GAPDH. This is probably due to the fact that β-casein itself is able to bind other proteins, preventing them from misfolding, but does not have the activity of true chaperone capable of providing the correct folding of other proteins due to ATP energy. Glycated β-casein still has an ability to bind other proteins, however, its chaperone-like properties completely disappeared due to modification, which led to complete inactivation of GAPDH and blocking its independent recovery of activity.

At the next stage, it was decided to compare the effect of true chaperones and β-casein on the GAPDH refolding. GroEL/GroES bacterial chaperonins were chosen as a comparative refolding system. In addition, β-casein and the GroEL/GroES complex were added to the same sample in order to assess the possibility of competition between refolding systems. Similar samples with glycated β-casein were also presented in order to confirm the disappearance of its anti-aggregation properties and to study its competition with the GroEL/GroES complex for the GAPDH refolding.

To increase the efficiency of chaperonins in the next experiment, we changed the conditions of preincubation of chaperonin complexes in order to provide more effective refolding: if earlier the reactivation buffers were heated (with glycated casein,

where necessary) for 1 hour at 37°C, now they have assumed that protein-protein interactions will be sufficient and less time, but increased temperature leads to β-mercaptoethanol degradation only, the decomposition products of which inhibit the activity of enzymes. Thus, then the preincubation was carried out for just 5 minutes and at room temperature 26°C. In addition, for the purity of the experiment, $MgCl_2$ and ATP in identical concentrations were now added to all samples for reactivation, and not only to the complexes containing GroEL/GroES.

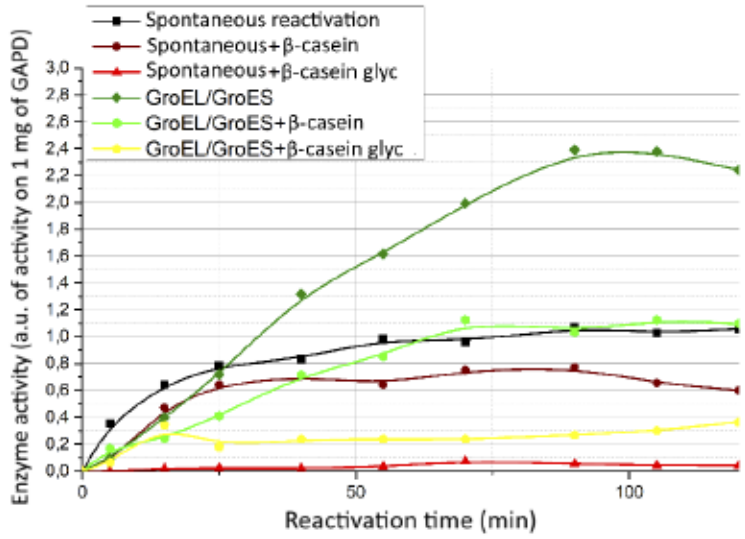


Fig. 44. Comparison of effect of glycated and non-glycated caseins on the spontaneousand chaperon-induced reactivation of GAFD.

As a result, it was shown that in the presence of the GroEL/GroES chaperonin complex, GAPDH reactivation activity proceeded more efficiently than with the isolated protein; At the same time, in a mixture of casein and chaperones GAPDH reactivation was still continuing by the end of incubation, in contrast to spontaneous reactivation, which had already exhausted its potential by that time. There is an assumption that the activity of GAPDH in the presence of casein may continue to grow, however, the competition of casein with the GroEL/GroES complex for binding of GAPDH slows down refolding in comparison with spontaneous reactivation.

In contrast, glycated casein almost completely suppressed the spontaneous reactivation of GAPDH. Addition a chaperonin complex to this mixture led to a slight reduction in the activity of the enzyme, but the final indicators of the activity of the enzyme were still significantly lower than that even for spontaneous reactivation.

Result: native β-casein slows down GAPDH reactivation, the level of reactivation is compensated for by the GroEL/GroES chaperonin complex.

Result: glycated β-casein almost completely suppresses GAPDH reactivation, which even the GroEL/GroES complex cannot abort; the modified protein completely loses its anti-aggregation properties.

The effect of casein glycation on GAPDH aggregation in the presence of the GroEL/GroES chaperonin complex

It has been suggested that the enzymatic activity of GAPDH should correlate with the refolding of the enzyme and, therefore, inversely correlate with non-specific protein aggregation. To prove this proposition, an experiment was conducted under conditions similar to the reactivation of GAPDH, but not the enzyme activity was measured by increasing the concentration of NADH, but protein aggregation by increasing the absorption of the solution at 320 nm. To compare the degree of aggregation of GAPDH, the experiment was set at a concentration of 0.6 μM, and they also tried to reduce the concentration of casein to the ratio of GAFD: casein = 1:2.

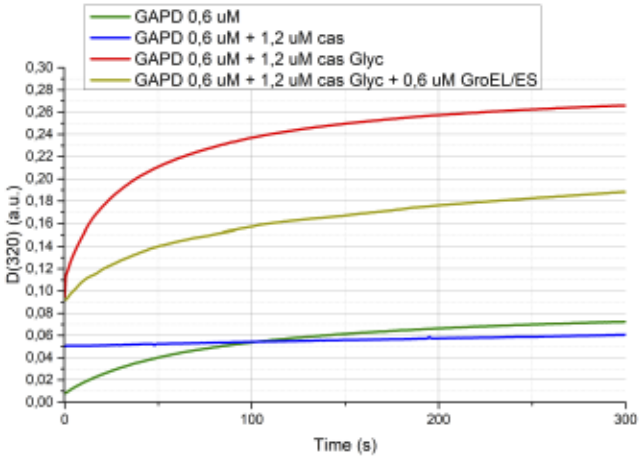


Fig. 45. Effect of glycated casein presence on the GAFD aggregation in the presence of GroEL/GroES complex.

Concentrations with the most vivid results were selected. c (GAPDH) = 0.6 μM, c (Beta-cas Gl) = 1.2 uM, c (GroEL) = 0.6 uM per complex. Incubation 5 min at 37 ° C.

In the absence of casein GAPDH was shown to aggregate slowly and gradually. In the presence of unmodified β-casein, the turbidity of the GAPDHH solution almost doesn't increase during time, which may indicate the antiaggregation effect of this milk protein. At the same time, the presence of glycated casein leads to a noticeably stronger aggregation of GAPDH than in the control sample. The presence of the GroEL/GroES complex in the medium resulted in a significant decrease in the optical density of the solution at 320 nm compared with the same breakdown without chaperonins, but did not rule out coaggregation.

In addition, it was shown that optical density depends on the concentrations of both components — an increase in the concentration of GAPDH or glycated β-casein led to an increase in the turbidity of the solution.

Part 3. Unique interaction between glycated β-casein and thioflavin T

The effect of glycation on beta-casein thermal aggregation

In order to detect the possible formation of amyloid-like structures by glycated beta-casein, we performed its thermal aggregation, followed by the detection of amyloid structures with ThT. It was shown that the incubation of all glycated casein

preparations at 94°C leads to its slight aggregation, which can be observed by increasing turbidity at 320 nm (the growth was an average of 0.1 a.u. in a solution with beta-casein concentration of 3 mg/ml). There is also no significant thermal aggregation of unmodified beta-casein - the optical density of the samples at 320 nm did not exceed 0.02 a.u. in a solution with the same concentration.

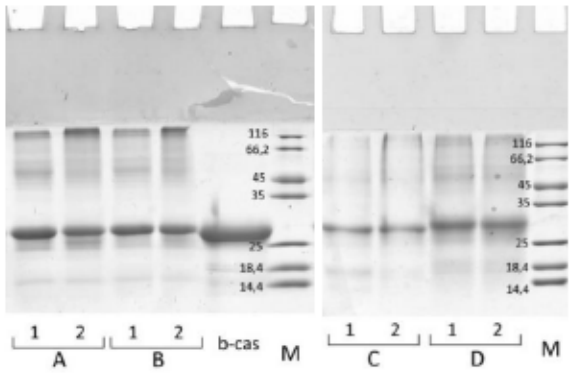


Fig. 46. Analysis of thermoaggregation of glycated β-casein. SDS electrophoresis
A, B - glycation without reducing agent; C, D - glycation with reducing agent.
A, C - glycation for 48 h; B, D - glycation for 72 h. β-cas - unmodified β-casein. 3% concentrating gel, 15% principal gel, the amount of protein per well - 10 μg. Before the experiment, the samples were heated at 96 ° C for 2-3 minutes.

It was shown by SDS-PAGE that the mobility of beta-casein in the gel under denaturing conditions decreases after modification (the bands of glycated beta-casein are located just above the native protein), and SDS-stable oligomeric particles slow down at the very beginning of the separating gel after heat-induced aggregation (Fig. 46).

Conclusion: glycation increases the propensity of β-casein to aggregate.

Aggregation of glycated β-casein with thioflavin T

In order to study the nature of the aggregation of glycated β-casein and to verify whether it has an amyloid nature, analysis was begun from fluorescence spectroscopy with staining of samples by thioflavin T.

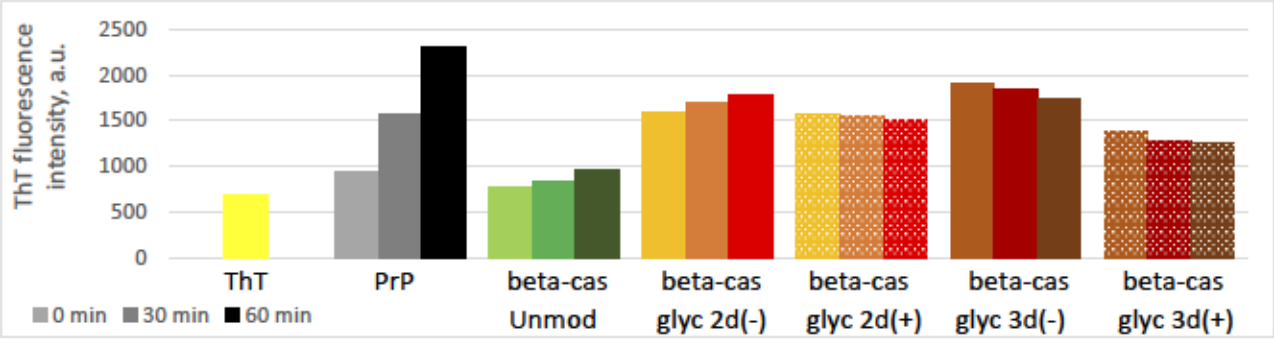


Fig. 47. Thermal aggregation of glycated β-casein, analysis by fluorimetry with thioflavin T.
Incubation during 0 min, 30 min and 1 h at 94oC. λ (exc) = 445 nm, Δλ (fluo) = 500 nm.
Samples used: ThT - negative control of the dye; PrP - 3 mg / ml prion protein, subjected to thermoaggregation (as a protein that is known to undergo amyloidization and cause thioflavin T fluorescence); β-casein - β-casein; β-cas. Glyc. - glycated β-casein: 2d(-) - the protein was glycated for 2 days without reducing agent; 3d(-) - protein was glycated for 3 days without reducing agent; 2d(+) - protein was glycated for 2 days with reducing agent; 3d(+) - protein was glycated for 3 days with reducing agent.

The fluorescent parameters of bound ThT also changed during the heat-induced aggregation of beta-casein, and in the case of glycated protein, the fluorescence intensity increased more than of native protein. However, the degree of increase in the fluorescence intensity of ThT did not exactly correspond to the characteristic for dye complexes with beta-sheets of amyloid structures. Instead of a 5-10-fold raise, it increased only 3.5-fold. Presented data showed that ThT interacts with beta-casein, especially with its glycated forms, but this interaction may not be due to the formation of its complexes with beta-sheets. We used optical, fluorescent and transmission electron microscopy to identify the structures, formed by glycated beta-casein.

When trying to analyze the presence of amyloid structures in glycated beta-casein, it was found that upon addition of ThT, the solution rapidly (within 5-30 seconds) became turbid. This fact was confirmed by the method of turbidimetry. In this case, we had to use the absorption of the sample at 600 nm instead of the usual 320 because ThT itself absorbs very intensively at 320 nm (Fig. 48).

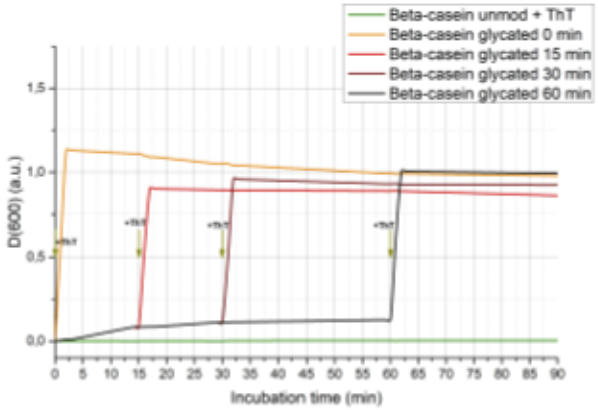


Fig. 48. Solution turbidity of unmodified and glycated β-casein. Measurement at 600 nm.
The following were compared: β-casein nemod. + ThT - non-modified β-casein, stained with thioflavin T, β-casein glyc. - β-casein, glycated under various conditions, which at the end of heat treatment was stained with thioflavin T. Measurements were carried out on a protein subjected to thermoaggregation at 94 ° C for 0, 15.30 and 60 minutes. The instrument was normalized according to the thioflavin T solution without protein.

In order to assess changes in protein structure from glycation and thioflavin T addition, a measurement of circular dichroism was used.

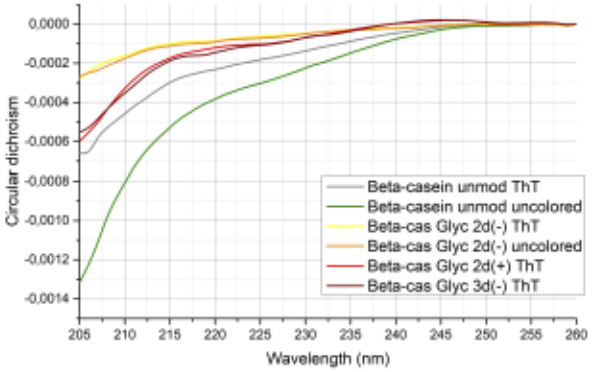


Fig. 49. Circular dichroism spectra of solutions of glycated β-casein in the presence of thioflavin T.
Protein modification tags: nemod. - non-glycated protein; glyc - glycated protein.
Dye presence labels: ThT - sample with 2 mM thioflavin T.
Labels of glycation conditions: 2d(-) - glycated for 2 days without β-casein reducing agent; 2d(+) - glycated for 2 days with NaBH3CN β-casein; 3d(-) - glycated for 3 days without β-casein. Glycation was carried out at 37 ° C in solution with a pH of 8.0. Measurement of CD was carried out in a buffer with pH 6.7 and 0.1 M NaCl.

As a result, it was found that the addition of thioflavin T to even unmodified β-casein led to a decrease in the peak of unstructured protein. The glycated protein had a

significantly smaller proportion of unstructured regions, regardless of the presence or absence of thioflavin T. In addition, some positive drug absorption was observed in the 245 nm region. Such absorption is characteristic of β-structural sites, but it is difficult to say this because the main negative absorption peaks in the area at 218 and 207 nm, characteristic of β-structures, and 212 nm, characteristic of α-helices, were difficult to distinguish.

The presence and properties of beta-casein aggregates, both unmodified and glycated, were investigated using dynamic light scattering in the presence of ThT. According to the DLS in the preparations of unmodified beta-casein before heating, there are particles about 25 nm in size, characteristic for the micelles of this protein.

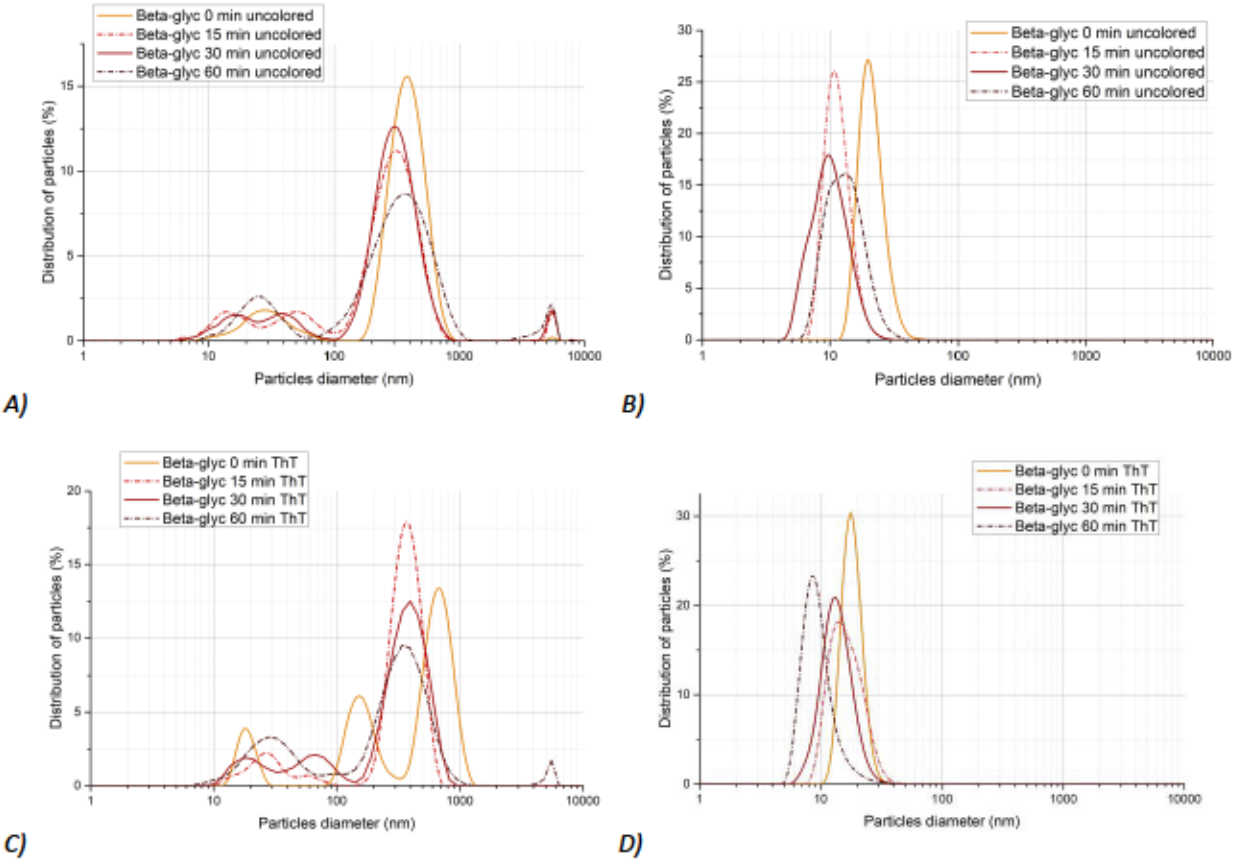


Fig. 50. The distribution of aggregates of glycated β-casein in particle size. Detection by dynamic light scattering.

A, B - unpainted samples, C, D - samples with thioflavin T.

A, B - distribution of populations by intensity; B, G - distribution by population size. The measurements were carried out in samples subjected to thermal incubation at 94 o C for 15, 30 minutes and 1 hour.

After glycation, the main fraction of the particles of the modified beta-casein decreases slightly in size to 22 nm (the minor fraction, apparently, consists of monomers with a hydrodynamic diameter of 8 nm), with the formation of huge particles of sizes 5-6 microns in the course of heat-induced aggregation, which precipitate in the form of pellet. The addition of ThT to samples without heat-induced aggregation of unmodified and glycated beta-caseins stabilizes the main fraction with a hydrodynamic diameter of 20 nm for native protein or 18 nm for glycated one (Fig. 50). Among the large particles, a stable fraction with a diameter of 400 nm in the samples of both unmodified and glycated proteins is found. However, in the case of unheated sample the addition of ThT leads to the splitting of this fraction by two, with hydrodynamic diameters of ~ 160 and 700 nm.

Analysis of the distribution of particles by intensity allows to evaluate the properties of large, but relatively few aggregates; analysis of the distribution of particles by volume of their populations allows to determine the hydrodynamic diameter of the prevailing fraction.

After heating particles with diameters of 20 nm, corresponding to micelles were found in samples of both samples, and a fraction of monomeric protein that disappears upon addition of ThT was also observed. In comparison with the fraction of large aggregates of unmodified beta-casein, which retained a diameter of 400 nm, large particles of glycated beta-casein slightly decreased in size to 350 nm. In addition, a fraction of huge aggregates with a diameter > 5 μm appeared.

Thus, an addition of ThT to the preparations of native and glycated beta-casein provokes a change in the size, which was detected by DLS, as well as the effect of ThT on the heat-induced aggregation of beta-casein.

Formation of spiral structures upon addition of thioflavin T to glycated beta-casein

In order to identify amyloid-like structures in the preparation of glycated beta-casein during its heat-induced aggregation, we used the treatment with ThT, followed by fluorescence microscopy.

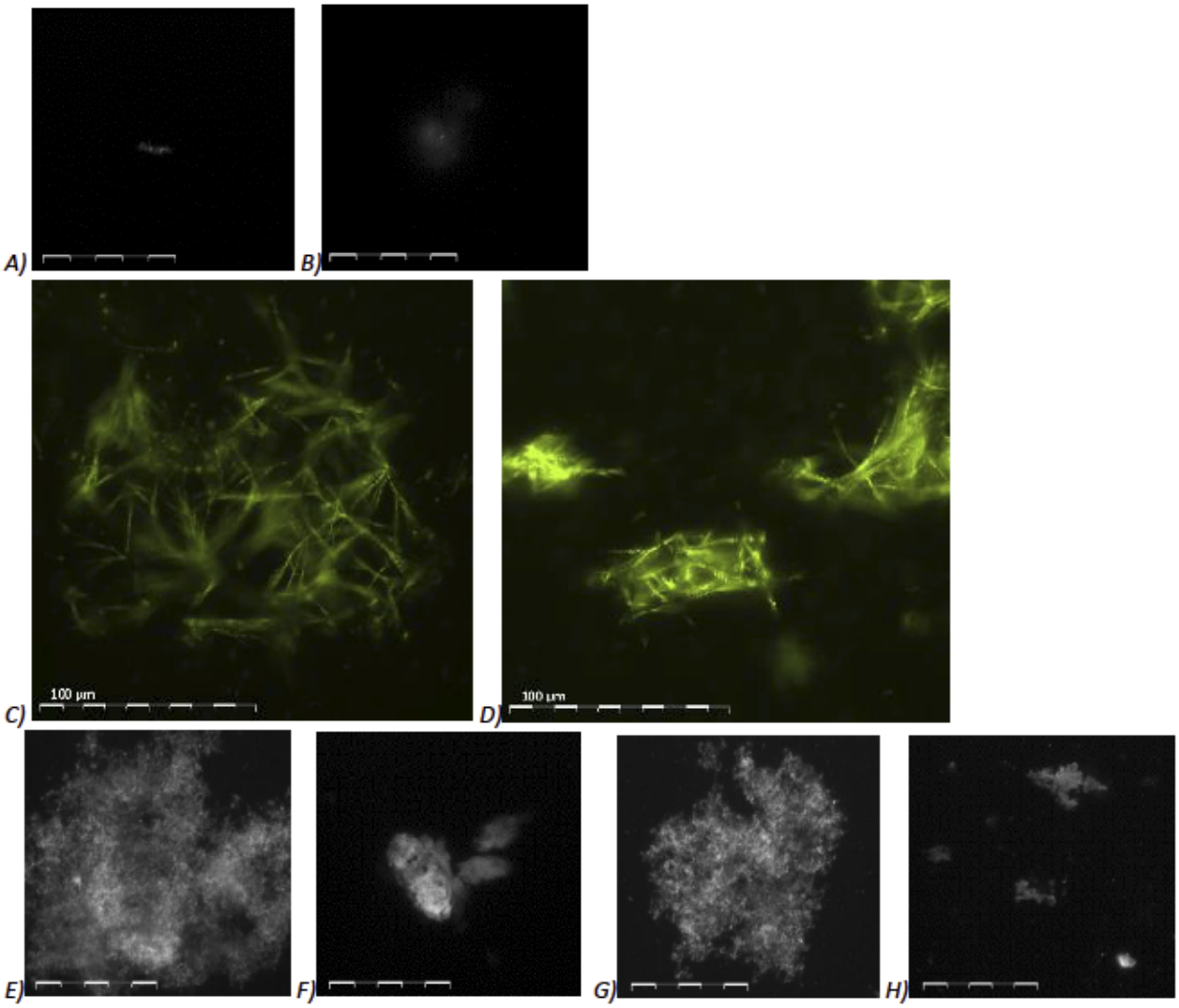


Fig. 51. Fluorescence microscopy of aggregation of glycated β-casein. *A, C, E, G - samples prior to thermal incubation; B, D, F, H - samples after 1 hour of thermal incubation. A, B - non-glycated protein, C, D - protein glycated for 2 days without reducing agent, E, F – protein glycated for 2 days with 35mM NaBH3CN, F, H - protein glycated for 3 days without reducing agent. Incubation 1 h at 94oC. Scale bar 100 microns.*

As follows from photos on Figure 51, native beta-casein possess a weak fluorescence with ThT before heat-induced aggregation (Fig. 51, A). At the same time,

according to the light microscopy, before heat-induced aggregation in preparations of unmodified beta-casein, protein aggregates were practically absent. After heat-induced aggregation of unmodified beta-casein during 1 hour at 94°C, the amorphous aggregates which were formed in a small amount, gave a weak fluorescence of bound ThT (Fig. 51, B).

A different situation was observed with all the investigated preparations of beta-casein, glycated with 0.2 M glucose for 48 or 72 hours with or without sodium cyanoborohydride. First of all, it should be noted that after the addition of ThT to glycated beta-casein, preparations contained a large amount of protein aggregates with notable fluorescence, both before and after heat-induced aggregation (Fig. 51, C-H). In case of beta-casein preparations, glycated for 48 hours without sodium cyanoborohydride, unusual structures looking as spiral-like aggregates that adhere to one another after incubation at 94°C during 60 minutes were discovered (Fig. 51, C). The aggregates were formed within ~ 5–10 minutes after the addition of the dye, and after thermal incubation they stuck together into larger conglomerates in a random fashion. Upon glycation under same conditions and subsequent heating beta-casein showed a further increase in ThT fluorescence intensity and a change in the structure of the aggregates (Fig. 51, D).

Thus, the presence of unusual spiral aggregates in preparations of glycated under certain conditions beta-casein was demonstrated. It is noteworthy that addition of reducing agent (Fig. 6, E, F) or increasing the glycation time up to 72 hours (Fig. 6, G, H) led to the absence of unusual structures.

With the colorant congo red, also binding to amyloid structures and because of this changing its absorbance properties, such aggregates were not formed. Since Congo red binds to protein structures differently than thioflavin T, it can be assumed that the binding of thioflavin T to glycated casein is specific.

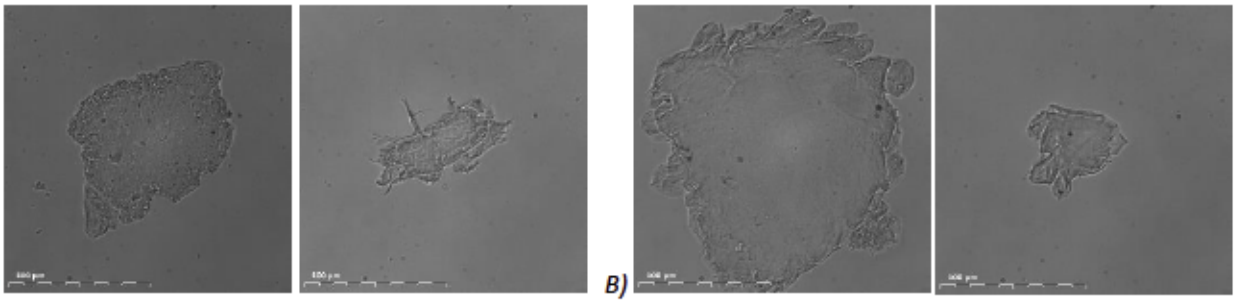


Fig. 52. Optical microscopy of glycated β-casein aggregation after staining with Congo red. In each pair of photos, the first one is protein before thermal incubation, the second one after 1 hour of thermal incubation. B - the protein is glycated for 2 days with 35 mM NaBH3CN, B - the protein is glycated for 3 days without a reducing agent. Incubation 1 h at 94 ° C, staining with 1 mM Congo red. Scale bar 100 µm, shutter speed 20 ms.

For further experiments, confirmation, that these spiral-shaped structures are not an artifact of thioflavin T itself, was necessary. In order to check whether isolated thioflavin T can form similar structures, a preparation with 2.5 mM dye solution in 50 mM MES pH 6.7 with 0.1 M NaCl left for 2 days at + 4°C, allowing it to dry at the edges of the coverslip so that thioflavin T forms crystals. Then this sample was examined in the same conditions as the preparations with protein.

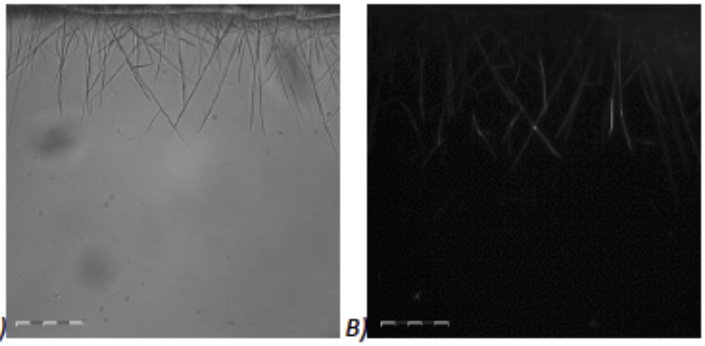


Fig. 53. Microscopy of thioflavin crystals obtained in a dried sample.
And - optical microscopy of the drug at the edge of the cover glass; B - fluorescence microscopy of the same view of the drug at the edge of the cover glass (exposure time 500 ms). Scale bar 50 microns.

However, no spiral structures were found, and the fluorescence of the crystals was significantly lower than that of the reaction of ThT with glycated beta-casein.

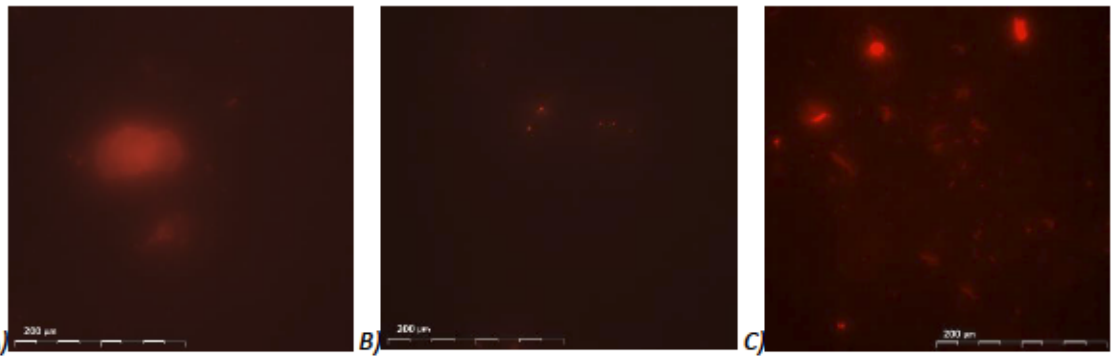
In addition, it was necessary to confirm that β-casein is indeed involved in helical aggregates, and not only necessary for their appearance. For a preliminary assessment

of the drugs were stained with ninhydrin. The presence of β-casein in aggregates and helical structures was first proved by staining with ninhydrin. Immunochemical staining was used for confident detection of protein in all types of aggregates. Initially, the aggregates on the slide were immobilized with buffer containing 3.7% formaldehyde, but under such conditions, with further washing from excess albumin and antibodies, thioflavin T was removed as well. in the sample, the experiment was performed on a nitrocellulose membrane.

Conditions for fluorescence microscopy: c (protein in applied drops) = 3 mg / ml, c (ThT) = 2 mM in Tris-HCl buffer 20 mM pH 7.5. The experiment was performed on a nitrocellulose membrane attached to a glass slide. λ (exc) = 530-595 nm, Δλ (fluo)> 615 nm.

A) B) C)

Fig. 54. Fluorescence microscopy of control preparations stained with antibodies.
A - glycated β-casein, stained with serum anti-casein rabbit antibodies and anti-rabbit antibodies FB555; B - prion protein, stained with anti-casein rabbit antibodies from serum and anti-rabbit antibodies FB555; C - anti-casein rabbit antibodies stained with anti-rabbit antibodies FB555. The scale bar is 200 microns.



A) B) C)

Fig. 55. Fluorescence microscopy of preparations of glycated casein and its helical aggregates stained with anti-casein antibodies.

Amorphous aggregates of glycated Β-casein before thermal incubation (A) and spiral-shaped structures of glycated Β-casein before thermal incubation (B) and after 60 minutes of aggregation at 37 ° C (C) in the presence of thioflavin T, stained with anti-casein rabbit antibodies and anti-rabbit antibodies FB555. Used Β-casein, glycated for 2 days without reducing agent. Thermoaggregation of drugs was not carried out. The scale bar is 200 microns.

As a result, the preparations detected the binding of secondary antibodies on the aggregates, in the form corresponding to amorphous (found without thioflavin T) and helical aggregates. Thus, the presence of β-casein was confirmed in both amorphous and specific aggregates.

Conclusion: glycated β-casein forms spiral-like aggregates with thioflavin T.

Determination of parameters of specific β-casein-thioflavin T aggregates and the process of their formation

To determine the parameters of the spiral aggregates, the experiment was repeated many times with their preparation. The resulting aggregates were analyzed by fluorescence microscopy with the highest possible magnification. A Carl Zeiss Axiovert 200M microscope device with a Zeiss AxioCam camera, a CCD camera was capable of using a lens with a magnification of 100x, an additional magnification of Tubulens 2.5x. So the total increase in the image entering the camera was 250x.

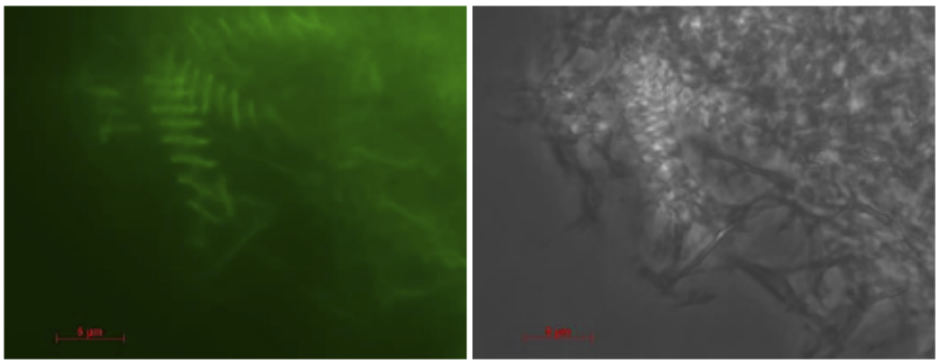


Fig. 56. Fluorescence microscopy of spiral aggregates of glycated Β-casein in high magnification. On the left is a micrograph of the fluorescence of the drug, on the right is a micrograph of the same angle in the visible range. Scale bar 5 microns.

According to the micrographs obtained, the pitch of the helix is ~ 1 μm, the helix can be either right or left curved. The diameter of the helix is ~ 3 microns.

The final method of studying spiral aggregates was transmission electron microscopy. The goal was to find similar ordered structures in smaller aggregates. A Jeol JEM-1400 microscope was used. As a result, ordered aggregates were detected by this method. The length of the ordered sections was 250 nm, and the diameter was about 50.

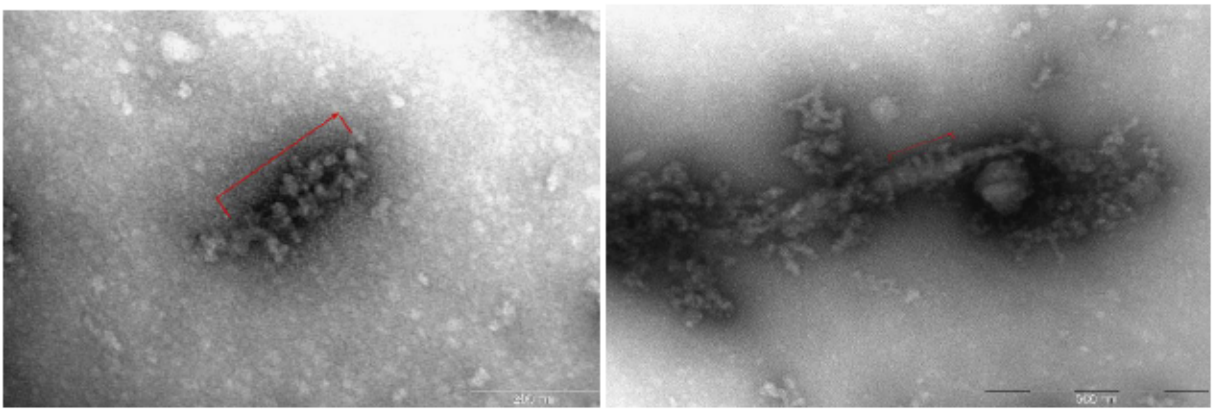
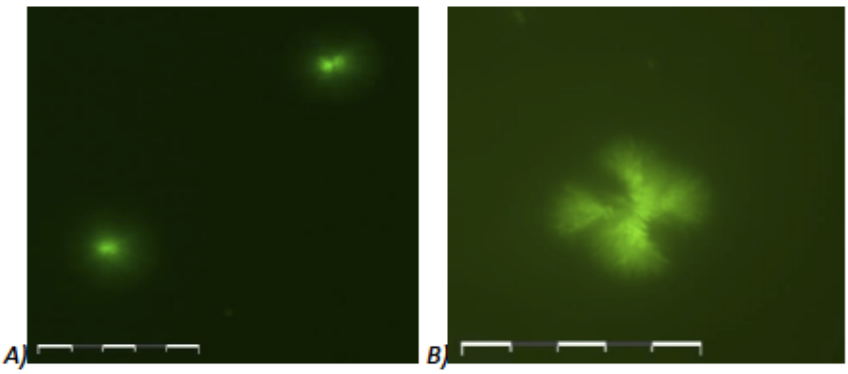


Fig. 57. Transmission electron microscopy of the preparation of glycated β-casein after the addition of thioflavin T.

In the experiments, β-casein, glycated for 2 days without reducing agent, was used; thermoaggregation of the protein was not carried out.

Results from multiple replications of the aggregation of glycated β-casein were also systematized in order to identify the different stages of aggregate formation.



A) *B)*

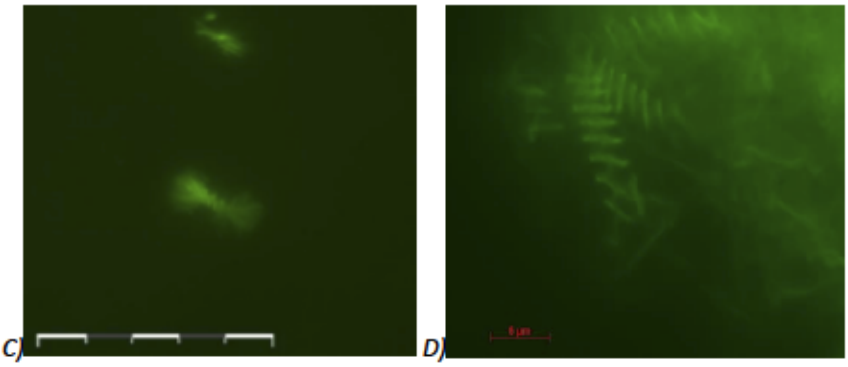


Fig. 58. The sequence of structures found in samples of β-casein bound to thioflavin T. The scale bar in the first 3 micrographs is 50 microns, in 4 - 5 microns. Heat incubation was carried out at 94 o C for 1 h.

As a result, at the initial stages of aggregation, stellate aggregates were found, and at later stages, aggregates that are intermediate in form between stellate and spiral ones. On the basis of these observations, assumptions were made that star-shaped aggregates are the initial state, and the spiral-like structures are formed from them by twisting along one or several axes.

Determination of the necessary components for the formation of specific aggregates of glycated β-casein

The question arises whether spiral structures are detected with the help of ThT or they are caused particularly by the interaction of ThT with glycated beta-casein. In favor of the latter assumption, the above-mentioned data on the effect of ThT on the aggregation of glycated beta-casein testify. In order to find out what components are necessary for the formation of spiral-like aggregates, attempts were made:

1) Replace the dye (used thioflavin S instead of thioflavin T).

2) Replace the glycating agent (methyl glyoxal was used instead of glucose)

3) Replace glycated protein

1) By replacing thioflavin T with thioflavin S, which also contains benzothiazoline cores in the structure, we also failed to detect any unusual structures, only amorphous aggregates were present in the samples.

2) An attempt was made to carry out glycation with another substance, which is basically different in structure than glucose. Methylglyoxal was chosen as the most active glycating agent. In view of its high activity, conditions of glycation were changed - the incubation time was reduced to 1 day. After modification, the beta-casein was purified by reverse phase chromatography and then heated for 1 hour, 15 hours or 24 hours at temperature 94°C in 25 mM MES buffer, pH 6.7, containing 0.1M NaCl.

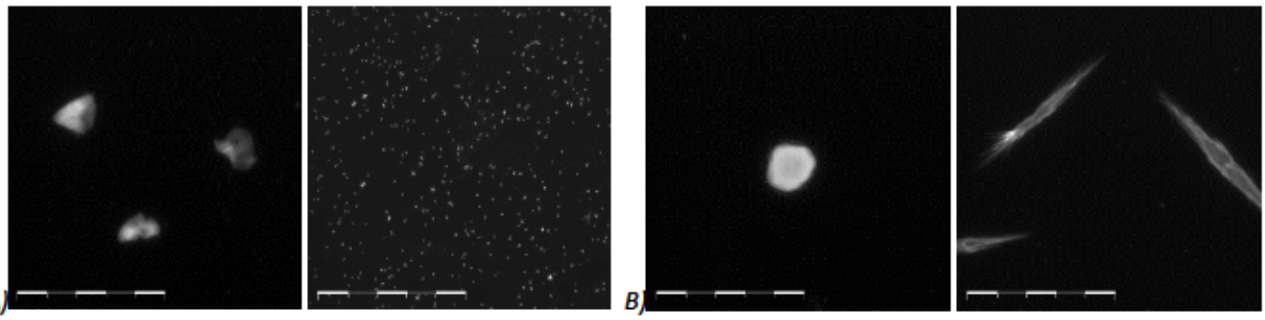


Fig. 59. Fluorescence microscopy of thermoaggregation of β-casein glycated with methyl glyoxal for different times.

If 2 fractions were present in the sample, they are presented in 2 photos under one letter. A - β-casein, glycated for 1 h prior to thermoaggregation; B - β-casein, glycated for 1 h after thermoaggregation; B - β-casein, glycated for 24 h prior to thermoaggregation; G - β-casein, glycated for 24 h after thermoaggregation. Scale bar 100 microns.

It was found that structures of a specific shape were formed in some samples (for example, in Fig. 59B, right), but there were no spiral aggregates. In addition, glycation of beta-casein by methylglyoxal at short time intervals (up to 1 hour) led to the formation of a number of relatively small particles with a diameter of about 570-600 nm (Fig. 59A, right) along with large aggregates.

Thus, it was shown that spiral structures form only certain forms of glycated beta-casein, and their formation is induced by the addition of ThT.

3) Finally, an attempt was made to replace the protein subject to modification. As a comparative object of the experiment, β-lactoglobulin and prion protein were taken, as proteins initially susceptible to amyloid conversion, and therefore potentially more easily forming some specific aggregates.

Protein glycation was performed under conditions as close as possible to β-casein:

c (protein) = 5 mg / ml, c (gluc) = 0.2 M, s (NaBH3CN) = 35 mM. Buffer 50 mM Tris-HCl pH 8.0.

The incubation time is 3 days, the temperature is 37°C.

After modification, both proteins were divided into 2 parts, one of which was also purified, and the other was involved in subsequent experiments without purification. This was done to assess the effect of low molecular weight glycation products on protein and thioflavin aggregation.

Conditions for fluorescence microscopy: s (protein) = 1.5 mg / ml, s (ThT) = 2 mM in Tris-HCl buffer 20 mM pH 7.5. λ (exc) = 440-480 nm, Δλ (fluo)> 490 nm.

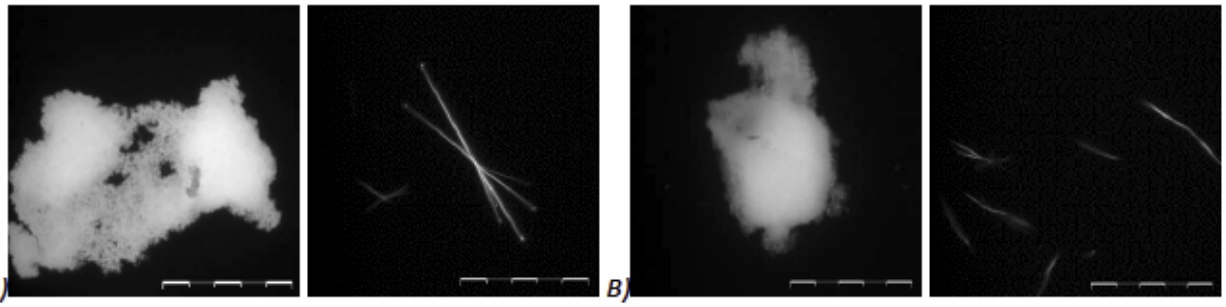


Fig. 60. Fluorescence microscopy of glycated β-lactoglobulin and prion protein after thermoaggregation. In each sample, 2 aggregate populations were found, and therefore micrographs are grouped in pairs. And - glycated β-lactoglobulin; B - glycated prion protein without purification from low molecular weight modification products. The samples presented were not cleared of low molecular weight modification products. Incubation 1 chpri 94oS. Scale bar 100 µm, shutter speed 50-80 ms.

As a result, some structures of a specific form were obtained, for example, glycated β-lactoglobulin, not purified from low molecular weight products, formed some very smooth needle structures with fluorescent thioflavin T-rich tips (in Figure 60A), i.e.

Thioflavin T interacted with other glycated proteins, however, again spiral-shaped aggregates were not found.

Conclusion: only glycated D-glucose β-casein and thioflavin T are able to form specific helical aggregates.

Part 4. Coaggregation of glycated beta-casein and unmodified prion protein

Coaggregation of glycated β-casein and prion protein

After studying the effect of glycation on the properties of β-casein itself, it was decided to study in more detail the changes in cross-aggregation of glycated β-casein with other proteins, for example, with amyloidogenic prion protein. Previously, it was shown that native β-casein was able to suppress amyloid conversion and aggregation of prion protein, and together with low-molecular-weight anti-amyloid ligands, to minimize these pathogenic processes.

The materials of the section were presented at the poster session of the Biomembranes-2018 conference (Dolgoprudny, 2018).

Thermoaggregation conditions remained practically the same.

- Incubation time: 1 h.
- Temperature: 94°C.
- Buffer: 25 mM MES pH 6.7 with 0.1M NaCl.

To compare the effect of both proteins on the coaggregation pathway, 3 different ratios of prion protein and glycated β-casein were tested:

- Sample 1: c (PrP) = 2.4 mg/ml (104 μM), c (β-cas) = 0.6 mg/ml (33 μM). Ratio PrP: β-cas = 4:1
- Sample 2: c (PrP) = 2 mg/ml (87 μM), c (β-cas) = 1 mg/ml (55 μM). Ratio PrP: β-cas = 2:1
- Sample 3: c (PrP) = 1.5 mg/ml (65 μM), c (β-cas) = 1.5 mg/ml (82.5 μM). Ratio PrP: β-cas = 1:1

First the samples were analyzed for the number of amyloid structures by the fluorescence of thioflavin T.

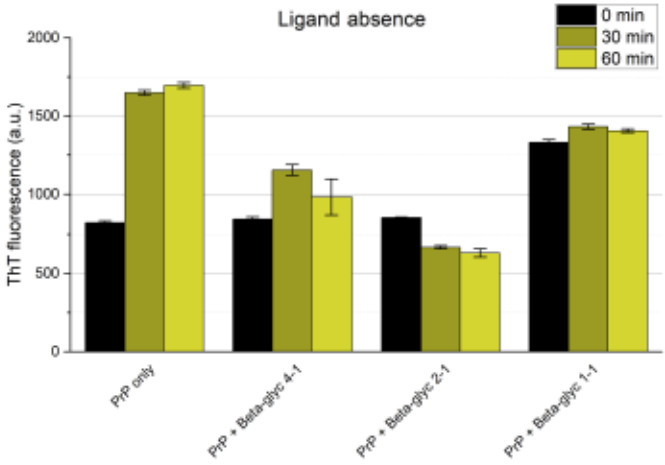


Fig. 61. Fluorescence spectroscopy of coaggregation of prion protein and glycated β-casein in different ratios with thioflavin T.

Used prion protein and glycated for 3 days without reducing β-casein. The total protein concentration in the samples is 3 mg / ml. Incubation 1 h at 94°C.

As a result, the measured fluorescence in the samples of prion protein and glycated β-casein, regardless of proteins ratio, became lower than the control one in the preparation of isolated prion protein, but adding one of the proteins to the sample with other ready-made components resulted in instantaneous turbidity of the sample until protein precipitation. Probably, it was the precipitation of proteins that complicated the measurement of the fluorescence of the dye and led to a decrease in the measured fluorescence of thioflavine T in the sample with a 2:1 protein ratio. In this regard, the samples were analyzed in more detail by the method of fluorescence microscopy.

Conditions for fluorescence microscopy: s (total protein) = 1.5 mg/ml, s (ThT) = 2 mM in Tris-HCl buffer 20 mM pH 7.5. λ (exc) = 440-480 nm, Δλ (fluo)> 490 nm.

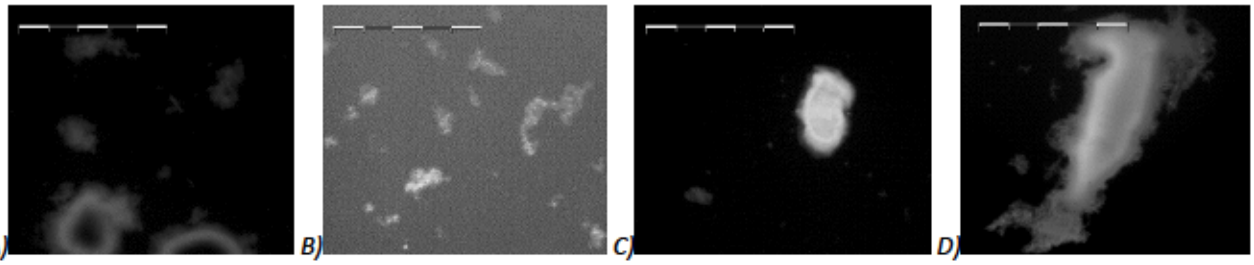


Fig. 62. Fluorescence microscopy of coaggregation of prion protein and glycated β-casein in a 4: 1 ratio. A (exposure 16 ms) - insulated thermo-aggregated PrP; B (200 ms) - co-aggregated PrP and β-casein; B (16 ms) - a mixture of PrP and glycated β-casein before thermoaggregation; G (120 ms) - thermally aggregated PrP and glycated β-casein. Incubation 1 h at 94oC. Scale bar 100 microns.

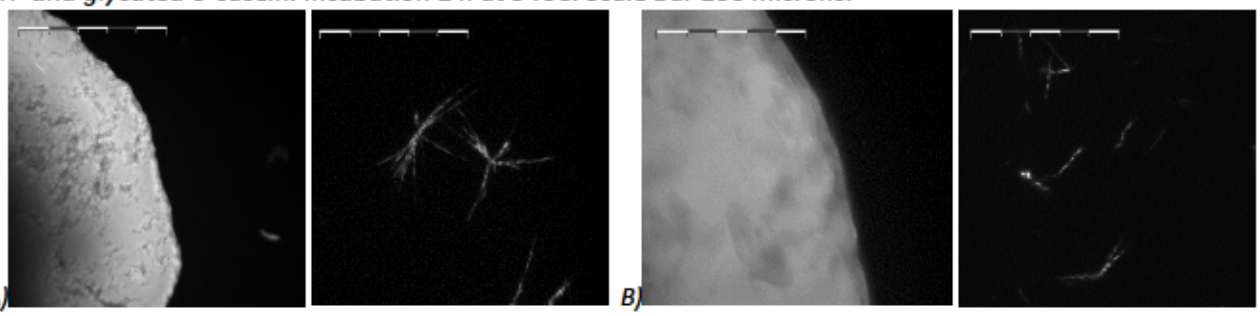


Fig. 63. Fluorescence microscopy of coaggregation of prion protein and glycated β-casein in a 2: 1 ratio. In each sample, 2 aggregate populations were found, and therefore micrographs are grouped in pairs. A (exposures of 12 and 50 ms) is a mixture of PrP and glycated β-casein prior to thermoaggregation; B, (5 and 45 ms) - thermo-aggregated PrP and glycated β-casein.
Incubation 1 h at 94°C. Scale bar 100 microns.

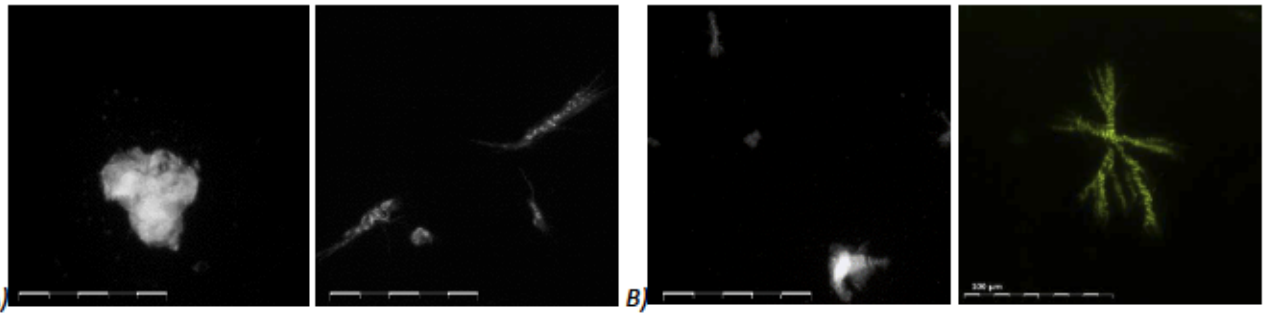


Fig. 64. Fluorescence microscopy of coaggregation of prion protein and glycated β-casein in a 1: 1 ratio. In each sample, 2 aggregate populations were found, and therefore micrographs are grouped in pairs. A (exposures 33 and 68 ms) - a mixture of PrP and glycated β-casein before thermoaggregation; B, (50 and 38 ms) - thermo-aggregated PrP and glycated β-casein.
Incubation 1 h at 94°C. Scale bar 100 microns.

Fluorescence microscopy revealed that the samples contained two fractions of aggregates: large amorphous, with poorly visible fibrous peripherical structures, and specific spiral-like ones, with the length of the latter increased by 1.5-2 times in comparison with those of isolated glycated β-casein. Thioflavin T fluorescence in all

types of aggregates is higher than with isolated glycated β-casein. The number of spiral-like aggregates in the presence of PrP increased compared with isolated β-casein, however, an excess of PrP made specific aggregation by an overwhelming process — specific glycated β-casein did not form spiral structures.

As in case of isolated glycated β-casein, a test for the need for thioflavin T to form specific structures was made.

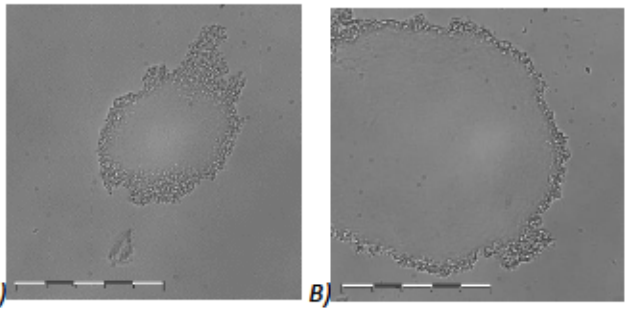
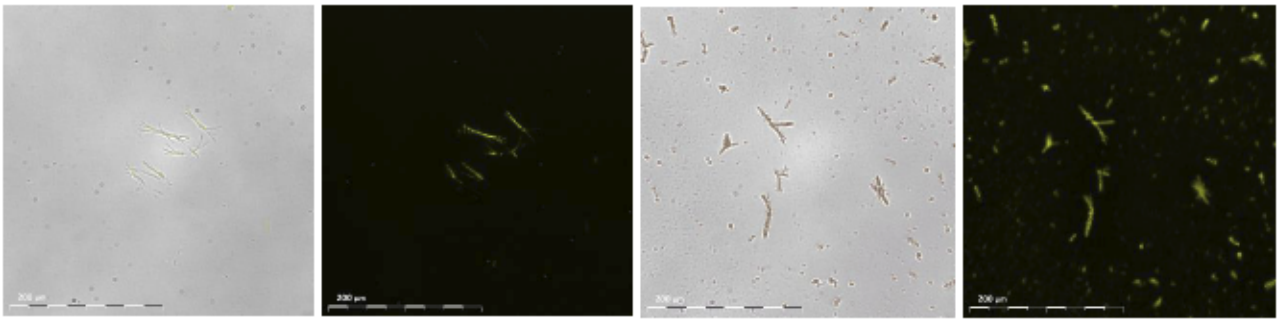


Fig. 65. Optical microscopy of coaggregation of prion protein and glycated β-casein in a 1: 1 ratio. Coloration was not used.

A - test with casein, glycated for 2 days without reducing agent, B - test with casein, glycated for 3 days without reducing agent. Scale bar 100 microns.

As in the case of glycated β-casein, that spiral-like aggregates were shown to be formed only in the presence of thioflavin T. In unstained samples, only large amorphous aggregates, whose morphology depended little on the degree of casein modification, were found.

To confirm the presence of protein in spiral-like aggregates, the samples were stained with 1% ninhydrin. Staining was performed by addition of a drop of dye to microscopy samples, which was already stained with thioflavin T. For the experiment β-casein was used, which was glycated for 3 days with sodium cyanoborohydride.



A B C D

Fig. 66. Light (A and C) and fluorescent (B and D) microscopy of spiral aggregates of glycated β-casein after staining with ninhydrin.

Glycated β-casein at a concentration of 3 mg / ml was thermally incubated for 1 h at 94 ° C, stained with thioflavin T at a concentration of 2 mM (A and B), and then 1% ninhydrin solution to a final dye concentration of 0.3% (C and D). The scale bar is 200 microns.

After staining with ninhydrin, the spiral aggregates retained their structure, as well as the fluorescence of thioflavin T associated with them. In addition, in optical microscopy mode, the aggregates acquired a pale purple hue and became more tightly twisted. Probably, ninhydrin contributes to more dense packing of the spirals due to stacking interactions with amino acid residues, both containing cyclic groups initially, and as a result of glycation.

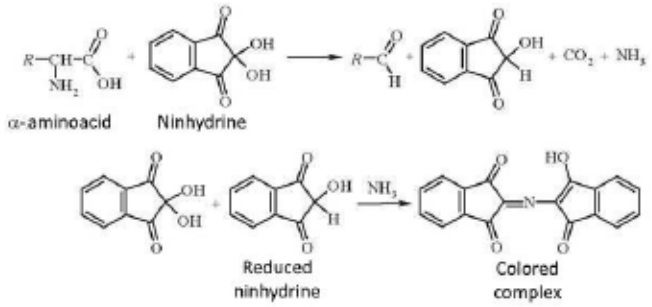


Fig. 67. The structure of ninhydrin dye and the mechanism of its reaction with protein α-amino acid, followed by formation of a colored product.

Immunochemical staining was used again to confidently confirm the presence of protein in all types of aggregates. Due to the need to conduct laundering of unbound antibodies with preservation of thioflavin T, in the experiment, the experiment was performed on a nitrocellulose membrane attached to a glass slide. The composition of the work applied to the membrane remained the same as that used

for fluorescence microscopy with thioflavin T: c (total protein) = 1.5 mg/ml, c (ThT) = 2 mM on Tris-HCl buffer 20 mM pH 7.5. Primary polyclonal rabbit antibodies against bovine milk β-casein, obtained manually by immunizing a rabbit, were used for staining. To visualize their binding, goat anti-rabbit FB555 secondary antibodies from Thermofisher were used. λ (exc) = 530-595 nm, Δλ (fluo)> 615 nm. After confirming the required specificity of the antibodies, a target experiment was carried out with staining of aggregates of glycated β-casein, previously bound to thioflavin T.

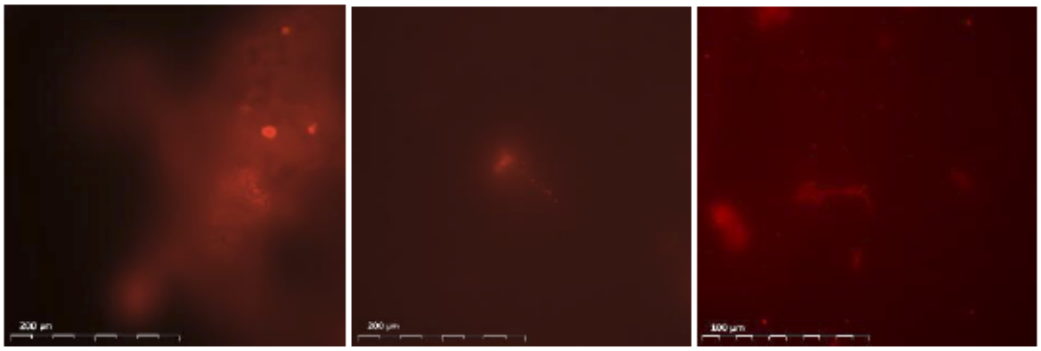


Fig. 68. Fluorescence microscopy of thermo-aggregated glycated β-casein and prion protein stained with antibodies.

Coaggregated glycated β-casein and prion protein stained with anti-rabbit antibodies. Thermally incubated proteins before immunochemical staining for 1 hour at 94oC. The scale bar is 200 microns in the first two photographs and 100 microns in the third.

As a result, aggregates stained with anti-casein antibodies were obtained. And it concerned not only the amorphous fraction, but the ordered too. Based on this, one could confidently assert that β-casein is present in both amorphous and specific aggregates.

The study of the properties of small (less than 1 μm) protein aggregates was carried out by the method of dynamic light scattering.

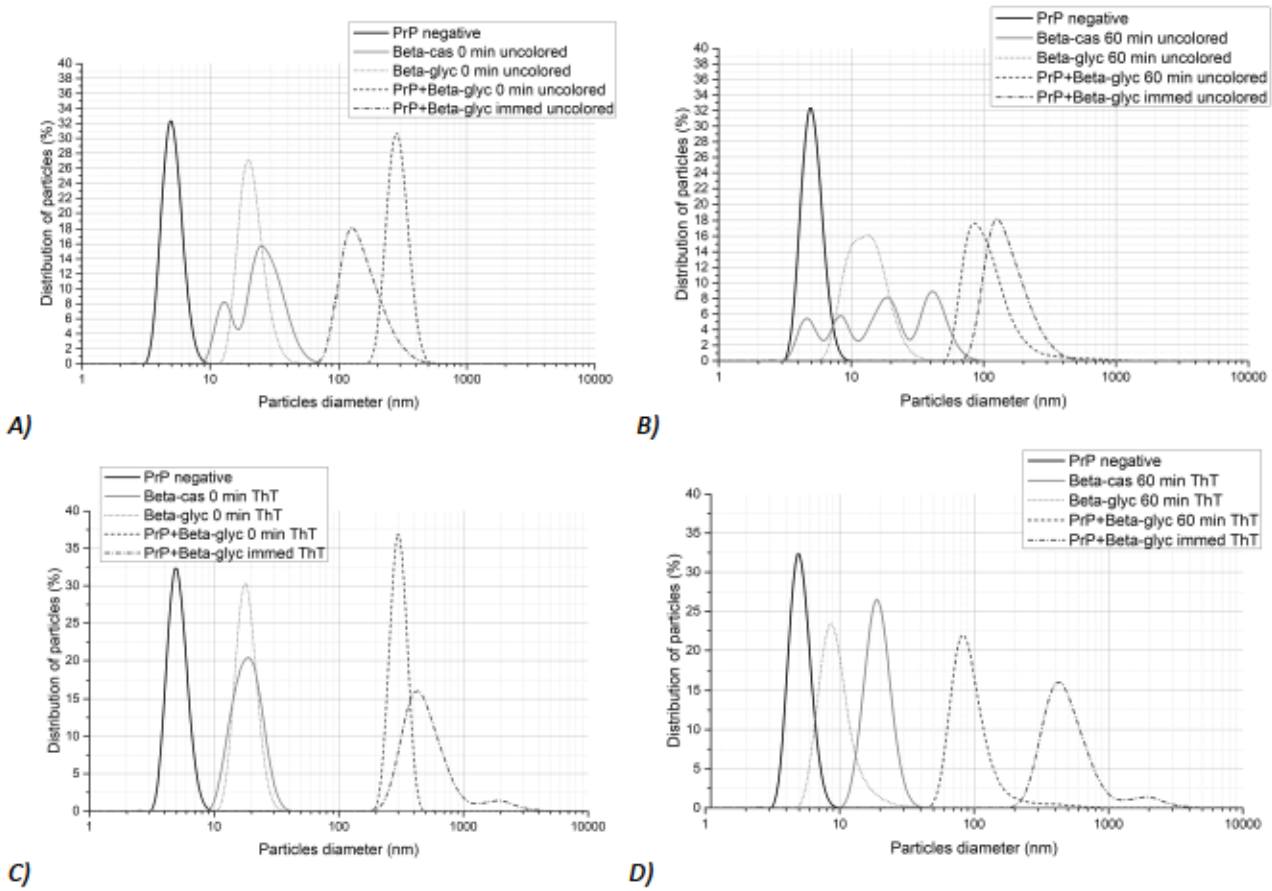


Fig. 69. The distribution of coaggregation products of glycated β-casein and prion protein by particle size. Detection by dynamic light scattering.

A, B - uncolored samples, C, D - samples with thioflavin T.

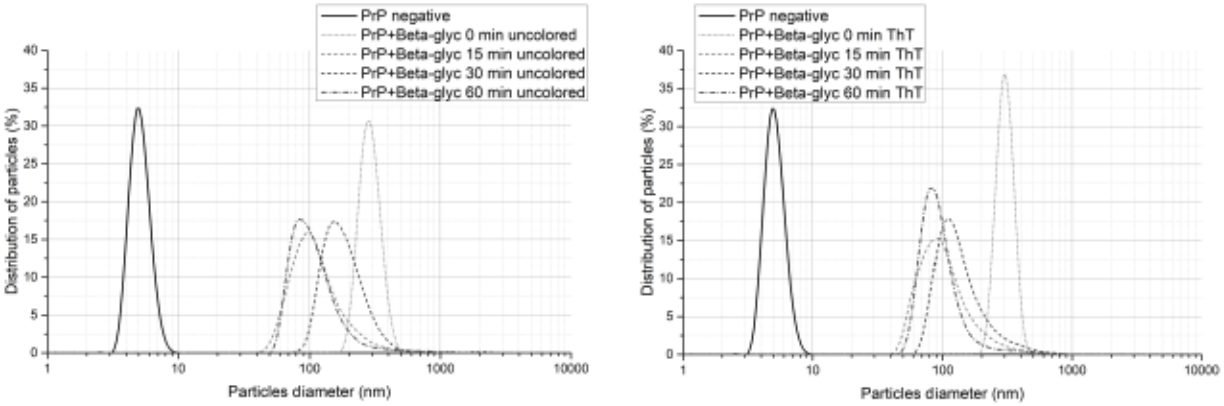
A, B - distribution of populations by intensity; B, G - distribution by population size. Used β-casein, glycated for 2 days without reducing agent. The calculation of the results was carried out according to number of populations.

The formation of very homogeneous aggregates population with a hydrodynamic diameter of about 300 nm was observed in a mixture of prion protein and glycated casein. During thermal incubation, this peak is blurred and shifted to a diameter of 90 nm, typical of PrP^{Sc} protofibrils.

In addition, it was considered noteworthy a sharp turbidity of the solution immediately after adding both proteins to the mixture, therefore, under the same conditions, a sample was analyzed with the addition of prion protein to the otherwise already prepared sample directly in the cell and with an immediate analysis of particle populations. A predominant population of aggregates was discovered with a

hydrodynamic diameter of about 140 nm, growing to ~ 400 nm upon the addition of thioflavin T. This is probably due to the fact that thioflavin T was added to this sample even before all the proteins were found.

Due to noticeable changes in the size of aggregates in the coaggregation sample of prion protein and glycated β-casein, the process was studied by the DLS method more detailed, with 4 time points: before incubation, after 15 min, 30 min, and 60 min of incubation.



A) *B)*

Fig. 70. The distribution of coaggregation products of glycated β-casein and prion protein by particle size. Detection by dynamic light scattering.

A - distribution of particles on hydrodynamic diameter in an unpainted coaggregation sample of prion protein and glycated β-casein. B - distribution of particles along the hydrodynamic diameter in a sample of coaggregation of prion protein and glycated β-casein stained with thioflavin T. Incubation took place at 94°C for 15, 30 and 60 minutes. The calculation of the results was carried out according to the number of populations.

As a result, a smooth transition of the major fraction of the aggregates from PrP specific for coaggregation and glycated β-casein 200 nm to the ones, typical for protofibrils PrP^{Sc}, was shown.

Additionally the influence of these small aggregates in absorbance was estimeted.

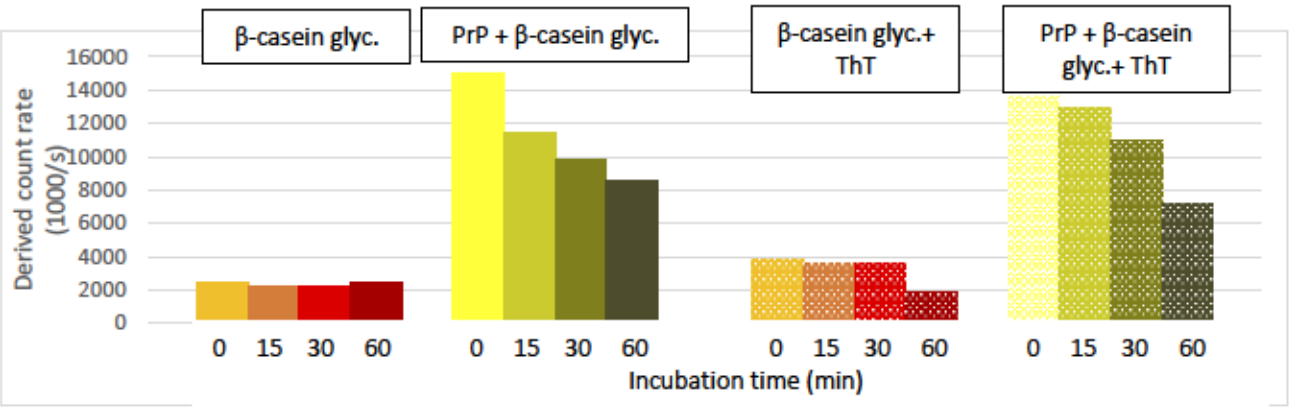


Fig. 71. Absorption of glycated β-casein and prion protein microparticles suspension; measurement by dynamic light scattering.

The measurements were carried out in samples subjected to thermal incubation at 94°C for 15, 30 minutes and 1 hour.

As a result, the expected sharp increase in the turbidity of the solutions was detected immediately after all the components were added to the sample. During thermal incubation turning turbidity gradually decreased. This is probably due to a decrease in the number of aggregates due to their adhesion into large conglomerates.

Conclusion: the prion protein accelerates the formation of β-casein spiral-like aggregates.

Determination of necessary components for the formation of glycated β-casein, prion protein and thioflavin T specific aggregates

As it was shown previously, the probability of formation of spiral structures by glycated β-casein increased in the presence of a prion protein, i.e. PrP activated the specific aggregation of β-casein, then decided to analyze the conditions under which these specific structures are formed more actively.

They decided to start with different ways of glycation of β-casein. The incubation time (2 or 3 days) and the presence / absence of a reducing agent (sodium cyanoborohydride) varied.

Thermoaggregation conditions remained practically the same.

- Incubation time: 1 h.

- Temperature: 94°C.
- Buffer: 25 mM MES pH 6.7 with 0.1M NaCl.

The ratio of proteins 1:1 was chosen.

To compare the distribution of aggregates by size and their number, the samples were analyzed by denaturing electrophoresis.

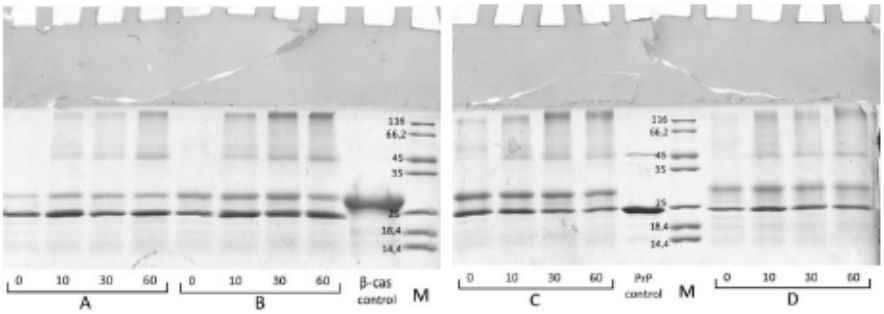


Fig. 72. Thermal aggregation of prion protein and β-casein glycated in different ways, analysis by SDS-electrophoresis:

0, 10, 30, 60 - time of thermal incubation in minutes. In all samples, the prion protein was unmodified. A - samples with β-casein glycated for 2 days without reducing agent; B - samples with β-casein glycated for 3 days without reducing agent. C - samples with β-casein glycated for 2 days with sodium cyanoborohydride; D - samples with β-casein glycated for 3 days with sodium cyanoborohydride. with (NaBH3CN) = 35 mM. Low molecular weight glycation components were removed by dialysis before incubation. Heat incubation for 1 hour at 94oC. 3% concentrating gel, 15% separating gel, the amount of protein per well - 10 μg. Before the experiment, the samples were heated at 96 ° C for 2-3 minutes.

According to the results of electrophoresis, thermoaggregation of prion protein with glycated for 3 days β-casein innon-reducing conditions was the most active.

In addition, to confirm the amyloid nature of the aggregates, an assessment of the stability of the aggregates to proteolysis with proteinase K was used. To detect the effect, the proteolysis conditions were somewhat altered to increase the percentage of proteolysis: c (PrK) = 0.2 μg/ml, c (CaCl2) = 5 μM. Incubation time was 15 minutes at 37°C.

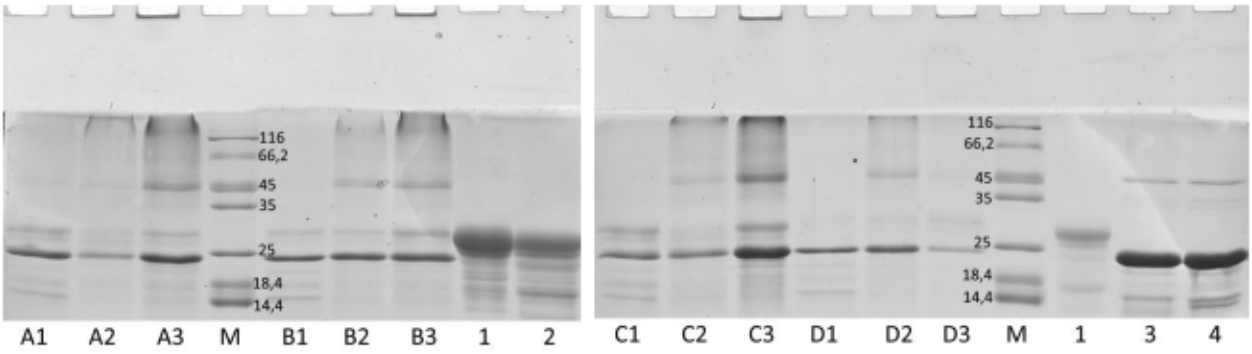
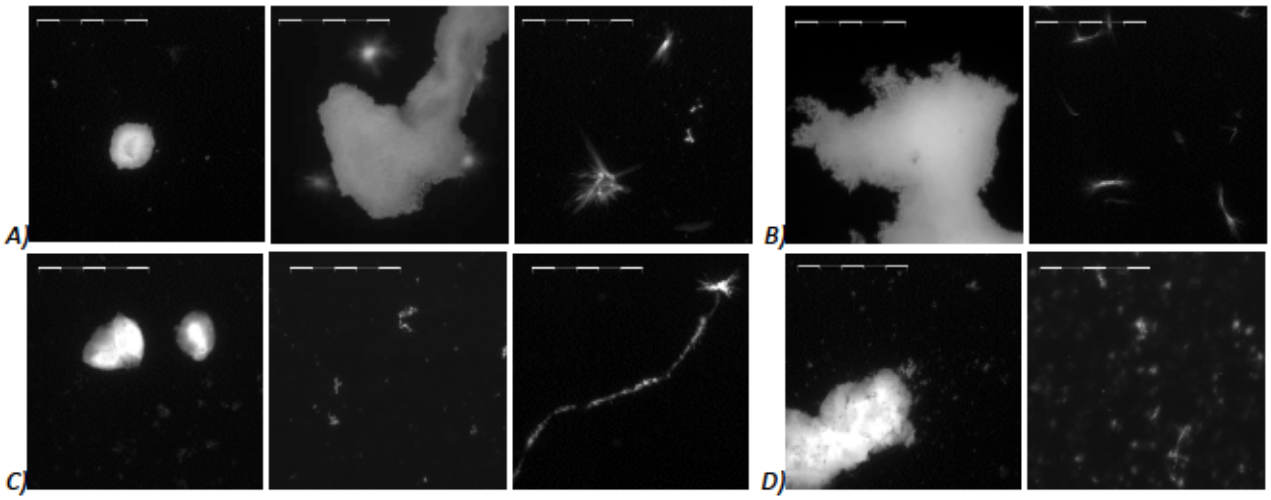


Fig. 73. Stability of thermoaggregated prion protein and β-casein glycated in different ways for proteinase K degradation, analysis by SDS electrophoresis:

Control samples: 1 - unmodified β-casein; 2 - β-casein, subjected to proteolysis. 3 - unmodified prion protein, 4 - prion protein, subjected to proteolysis. Experimental samples: A1-A3 - prion protein with β-casein, glycated for 2 days without reducing agent; B1-B3 is a prion protein with β-casein glycated for 3 days without reducing agent. C1-C3 - prion protein with β-casein, glycated for 2 days with sodium cyanoborohydride; D1-D3 is a prion protein with β-casein glycated for 3 days with sodium cyanoborohydride. Thermal incubation for 1 hour at 94°C. For proteolysis: s (PrK) = 0.2 μg/ml, s (CaCl2) = 5 μM. The time of proteolysis is 15 minutes at 37 ° C. 3% concentrating gel, 15% separating gel, the amount of protein per well - 10 μg. Samples were heated at 96°C for 5 minutes to block the activity of proteinase K.

As a result, the stability of prion protein and glycated β-casein aggregates was shown to be somewhat increased than that of individual proteins.

Conditions for fluorescence microscopy: s (protein) = 1.5 mg / ml, s (ThT) = 2.5 mM in Tris-HCl buffer 20 mM pH 7.5. λ (exc) = 440-480 nm, Δλ (fluo)> 490 nm.



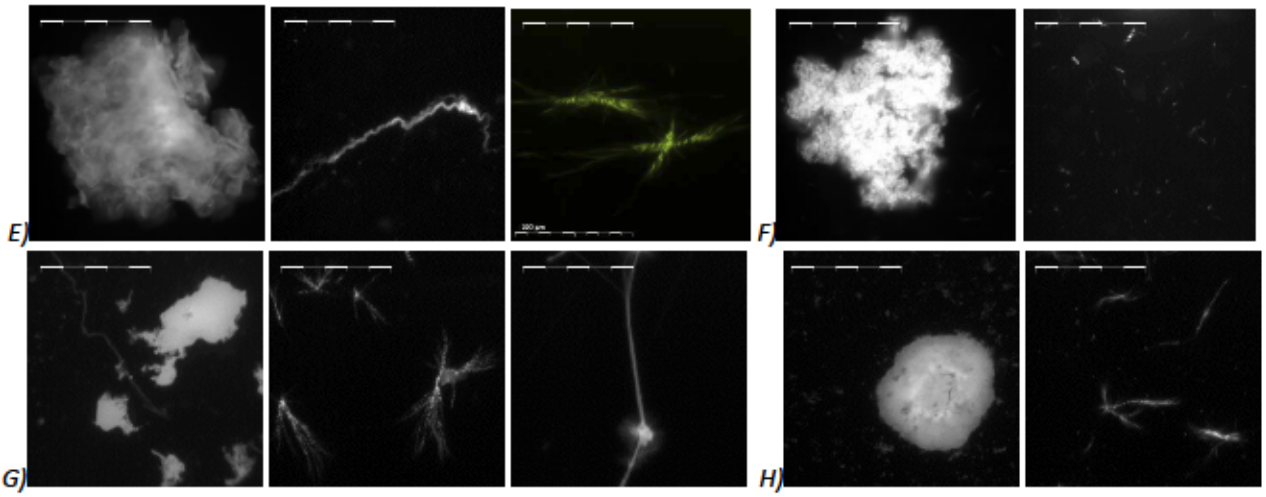


Fig. 74. Fluorescence microscopy of co-aggregation of prion protein and β-casein glycated in different ways.

If 2 or more fractions were present in the sample, they are presented in 2 or more photos under the same letter. A, B - 2 day glycation without reducing agent, C, D - 2 day glycation with 35 mM NaBH3CN; E, F - 3 day glycation without reducing agent, G,H - 3 day glycation with 35 mM NaBH3CN. A, C, E, G — samples before thermal incubation; B, D, F, H — samples after thermal incubation. Incubation 1 h at 94°C. Scale bar 100 µm, exposition 100 ms.

According to results of the experiment, the presence of prion protein contributes to the formation of spiral-like structures in a wider range of conditions – the β-casein glycation way now has almost no effect. Moreover, the aggregates become much longer, especially if the coaggregation took place with β-casein glycated with sodium cyanoborohydride — very long strands with a spiral structure were found in such samples. Co-aggregation with casein modified under non-reducing conditions led mainly to the formation of branched aggregates with a spiral structure.

In addition, structures were observed in the samples, in which a brightly fluorescent core emerged, from which the threads emanated, first straight, then twisting in spirals along 1 or more axes, as aggregates of isolated glycated β-casein. Apparently, these stellate structures are precursors of helical aggregates.

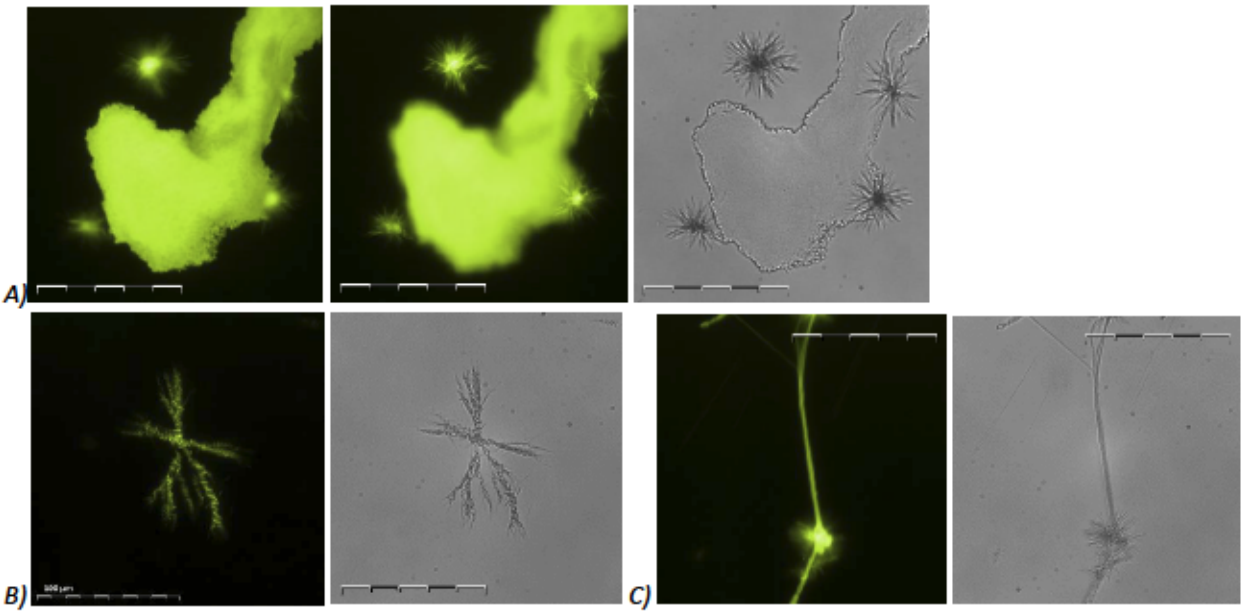


Fig. 75. Microscopy of specific structures from prion protein and glycated by various methods β-casein coaggregation.

For each preparation, micrographs are presented, obtained by methods of both fluorescent and classical optical microscopy. A — glycated 2 days without reducing agent, protein with a single cleaning before thermal incubation; B - glycated 2 days with $NaBH_3CN$ protein with a single purification after thermicinubation; B - glycated for 3 days with $NaBH_3CN$ protein with a single purification prior to thermal incubation; Incubation 1 h at 94°C. Scale bar 100 μm, exposition 100 ms.

In prion protein samples with glycated and β-casein samples colored by congo red, micelle-like structures were found prior to their joint thermoaggregation, which disappeared during heating. Some of them even had a mild triangular shape. Spiral aggregates, as in the case of isolated glycated β-casein, were not found.

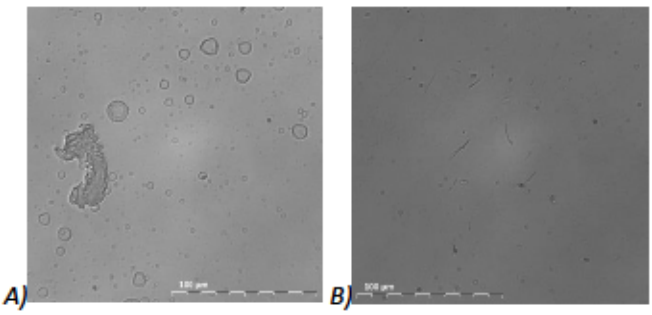


Fig. 76. Optical microscopy of coaggregation of prion protein and glycated and additionally purified β-casein.

And – sample before heat treatment; B – sample after heat treatment. Incubation 1 h at 94°C. Color 2 mM Congo red. Scale bar 100 microns.

An increase of specific β-casein aggregates number in the presence of a prion protein allowed us to suggest that such conditions would facilitate the formation of similar

aggregates for other conditions, in particular, a different glycating agent. As an alternative and agent already tested for glycation and subsequent aggregation of β-casein, methyl glyoxal was selected.

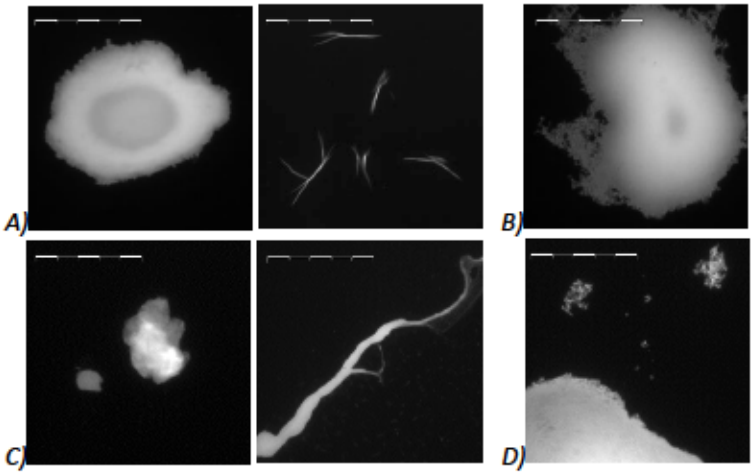


Fig. 77. Fluorescence microscopy of prion protein and glycated with methyl glyoxal for different times ϐ-casein co-aggregation.

If 2 fractions were present in the sample, they are presented in 2 photos under one letter. A, B - ϐ-casein, glycated for 1 h; C, D - ϐ-casein, glycated for 24 hours prior to thermoaggregation. A, C - samples before thermal incubation, B, D - glycation after thermal incubation. Scale bar 100 μm, exposition 50-80 ms.

As a result, some structures of a specific form were obtained (for example, in Fig. 77A and B), however, morphologically distinct spiral-shaped aggregates were not found, and the longer the time spent on glycation of β-casein, the fewer were there any specific forms. ThT fluorescence in this case was very bright.

At the end of this stage of work, it was decided to study the instant co-aggregation of PrP and glycated β-casein. Visually, from the very first experiments on the coaggregation of these proteins, it was observed that the addition of any of the proteins to the rest of the finished sample led to a turbidity of the solution within a few seconds. Moreover, large amorphous aggregates, seen in the sample with the PrP: β-cas = 2: 1 ratio, were also formed almost instantaneously. Therefore, using fluorescence microscopy, we studied the process of instant coaggregation of prion protein and glycated β-casein.

To study the process of instant aggregation, one of the proteins was added to the otherwise finished sample directly on a microscope slide.

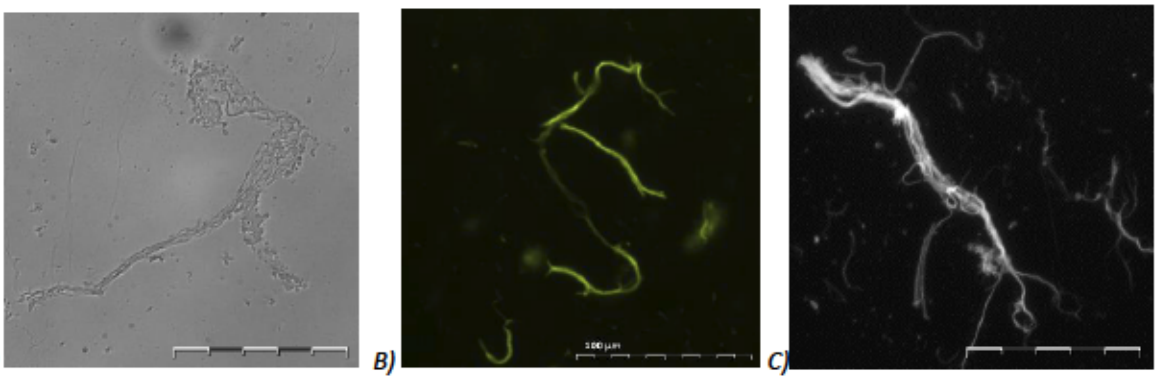


Fig. 78. Fluorescence microscopy of coaggregation of prion protein and glycated β-casein in a 1:1 ratio. A - a sample studied immediately after prion protein addition the and not undergoing staining; B - sample, studied immediately after prion protein addition and futher staining with thioflavin T to concentration 2 mM; B — a sample studied on 12 hours after adding the prion protein and staining with thioflavin T to concentration of 2 mM; for 12 hours the sample was stored at + 4°C. The scale bar is 100 µm, the shutter speed for photos with fluorescence is 75 ms.

Fibrillar structures not previously seen in samples incubated with eppendorfs were found in these samples. Over time, the structure slightly increased in size. At the same time, they were not found in the samples studied several minutes after mixing the proteins. Probably, over time, these threads interact between themselves folds into larger conglomerates.

A turbidimetric measurement of the solutions dispersion was also used to analyze instant coaggregation. On samples of prion protein and glycated β-casein mixture, incubated for at least more than 10 minutes, that was difficult because of the rapid formation of large aggregates that introduced heterogeneity in the measurements, therefore the prion protein was added just before the measurement. At the same time, the effect of thioflavin T on such aggregation was tested compared with similar conditions without a dye. Similarly, we estimated the contribution of the smallest particles (less than 1 micron) to the absorption of samples according to the DLS results.

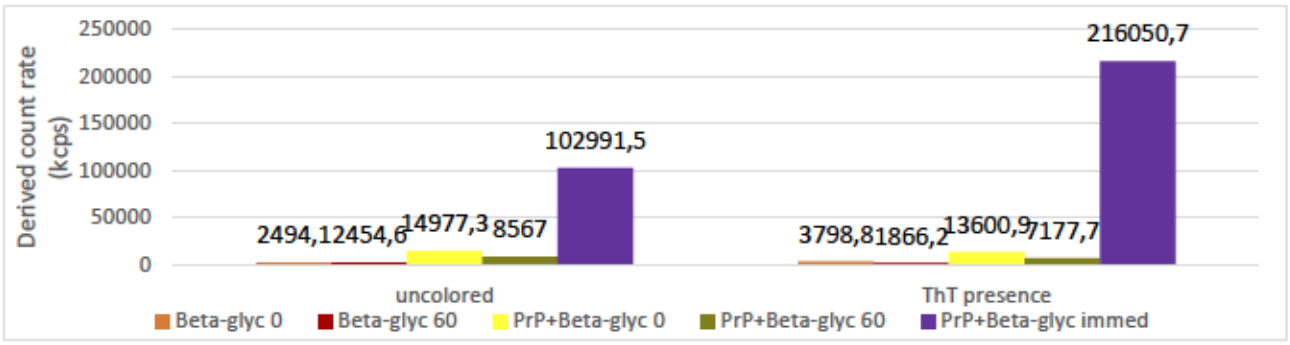


Fig. 79. Absorption of a suspension of microparticles of glycated β-casein and their mixture, just prepared and subjected to thermoaggregation; measurement by dynamic light scattering.
Uncolored - preparations without the addition of a fluorescent dye; ThT presence - preparations stained with thioflavin T. Beta-glyc - a sample with glycated β-casein. PrP + Beta-glyc - a sample with coaggregation of prion protein and glycated β-casein, PrP + Beta-glyc immed - a sample that was not subjected to thermal incubation, in which measurements were performed immediately after preparation. Heat incubation was carried out at 94°C for 1 h.

As a result, fast aggregation of prion protein and glycated β-casein in the mixture was confirmed, and, according to the turbidity measurements, aggregation in the mixture was much more active than isolated glycated β-casein with a dye. With thioflavin T, turbidity initially increased due to specific aggregation, it decreased slightly with time, which is probably due to the formation of large aggregates, which leads to a decrease in their number.

Conclusion: the presence of prion protein extends the range of conditions in which glycated β-casein forms helical aggregates.

Conclusion: only glycation of β-casein with D-glucose ensures the formation of helical aggregates, and the prion protein does not cancel this requirement.

Coaggregation of prion protein and glycated β-casein in the presence of certain anti-amyloid ligands

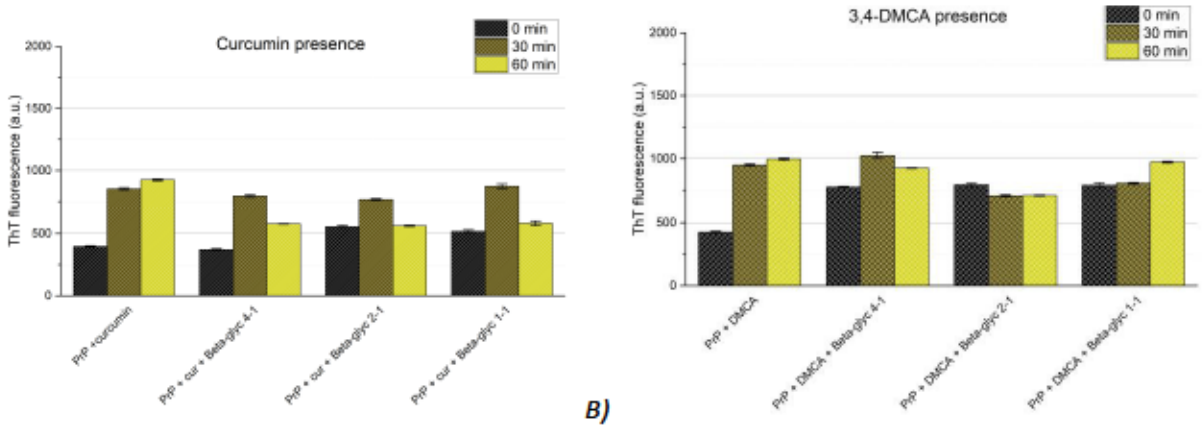
After confirming the mixture of the studied proteins to form specific aggregates, we decided to determine the effect of curcumin and 3,4-dimethoxycinnamic acid as anti-amyloid ligands on the coaggregation of prion protein and glycated β-casein and the formation of unique structures by them.

Thermoaggregation conditions remained the same.

Combinations of prion protein and glycated β-casein remained the same as they were when studying the coaggregation of these proteins without ligands:

- Sample 1: c (PrP) = 2.4 mg/ml (104 μM), c (β-cas) = 0.6 mg/ml (33 μM). The ratio of PrP: β-cas = 4:1
- Sample 2: c (PrP) = 2 mg/ml (87 μM), c (β-cas) = 1 mg/ml (55 μM). The ratio of PrP: β-cas = 2:1
- Sample 3: c (PrP) = 1.5 mg/ml (65 μM), c (β-cas) = 1.5 mg/ml (82.5 μM). The ratio of PrP: β-cas = 1:1

First of all, the kinetics of the process was analyzed by the method of fluorimetry with thioflavin T staining.



A) *B)*

Fig. 80. Fluorescence spectrometry of prion protein and glycated β-casein in different ratios coaggregation with thioflavin T and optionally antiamyloid ligands.

A - samples containing proteins and curcumin in the ratio PrP:cur = 1:3 during thermoaggregation; B - samples containing proteins and 3,4-dimethoxycinnamic acid in PrP:DMCA = 1:3 ratio during thermoaggregation. Used combinations of prion protein and glycated β-casein: isolated prion protein; PrP:β-cas glyc = 4:1 PrP:β-cas glyc = 2:1; PrP:β-cas glyc = 1:1. The total protein concentration in the samples is 3 mg / ml. Incubation 1 h at 94°C.

As a result, it was shown that the measured fluorescence became lower than in the preparation of isolated prion protein, but adding one of the proteins to the sample with other ready-made components still showed instantaneous turbidity of the sample until protein precipitation, i.e. it was not possible to completely exclude aggregation.

Further, the aggregation process was studied in more detail by the method of fluorescence microscopy with the same thioflavin T. Conditions for fluorescence microscopy remained unchanged.

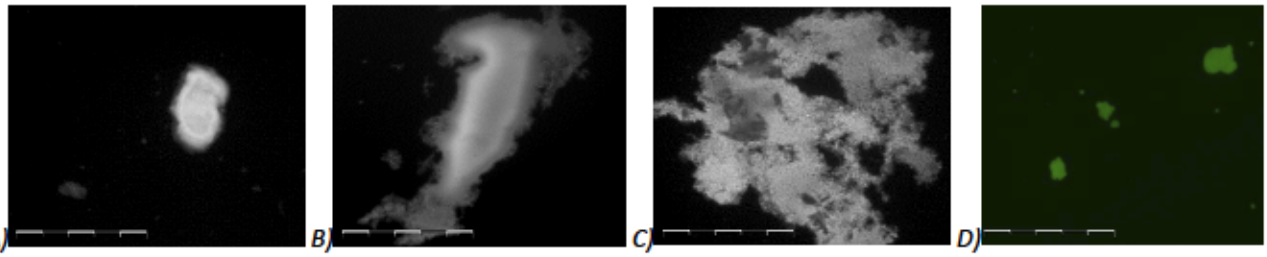


Fig. 81. Fluorescence microscopy of coaggregation of prion protein and glycated β-casein in a 4:1 ratio.
A (16 ms exposition) - a mixture of PrP and glycated β-casein before thermoaggregation; B, (120 ms) - thermally aggregated PrP and glycated β-casein; B (600 ms) - thermo-aggregated PrP and glycated β-casein with curcumin in the PrP ratio: cur = 1: 3; G - thermoset PrP and β-casein with 3,4-dimethoxycinnamic acid in the PrP: DMCA = 1: 3 ratio.
Incubation 1 h at 94oC. Scale bar 100 microns.

It was shown that in the presence of curcumin, the protein aggregates did not become smaller, but they became significantly less dense. The opposite situation was observed in the presence of 3,4-DMCA: the aggregates became much smaller, but retained a high density.

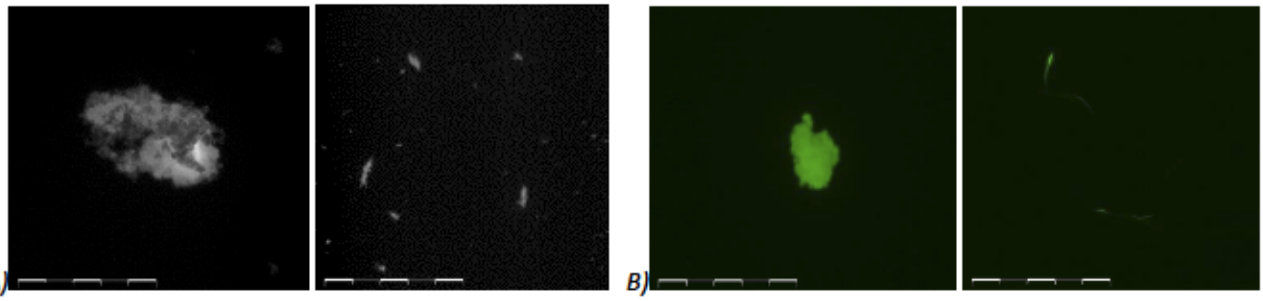


Fig. 82. Fluorescence microscopy of coaggregation of prion protein and glycated β-casein in a 2:1 ratio.
In each sample, 2 aggregate populations were found, and therefore micrographs are grouped in pairs. A (77 and 48 ms) - thermo-aggregated PrP and glycated β-casein with curcumin in the PrP ratio: cur = 1:3; B - thermoset PrP and β-casein with 3,4-dimethoxycinnamic acid in PrP: DMCA = 1:3 ratio. Incubation 1 h at 94°C. Scale bar 100 microns.

The presence of anti-amyloid ligands radically affected the coaggregation of prion protein and glycated β-casein in a 2:1 ratio. First, it was possible to prevent the formation of huge aggregates, the remaining conglomerates were smaller (especially with DMCA), and much more dispersed (with curcumin). In addition, the spiral

aggregates in the presence of ligands either became finer and deformed, or, as in the case of cinnamic acid, practically did not form.

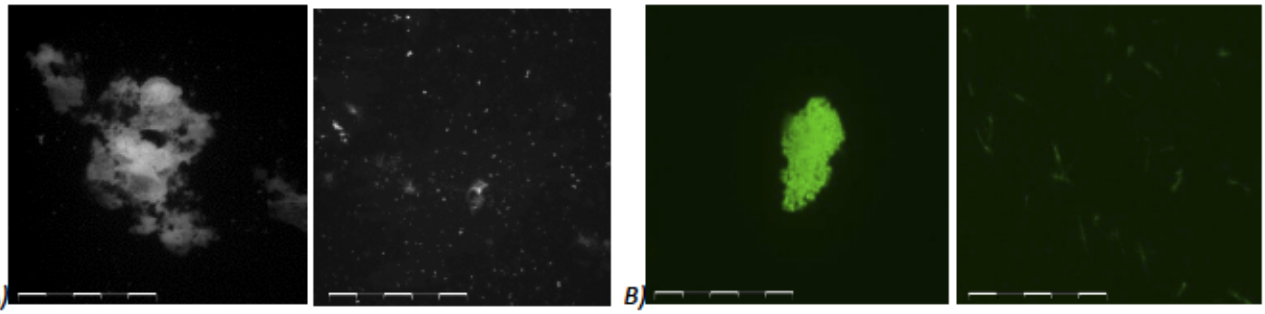
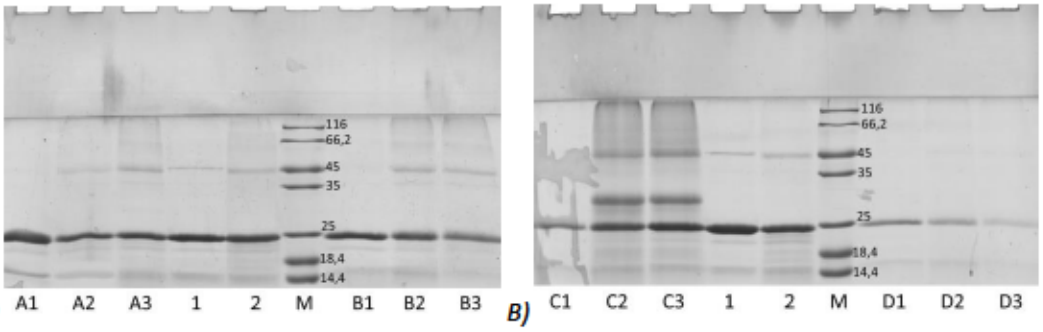
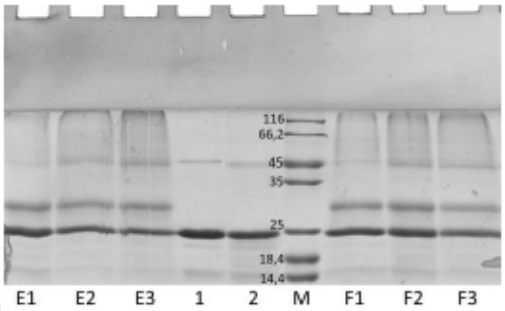


Fig. 83. Fluorescence microscopy of coaggregation of prion protein and glycated β-casein in a 1: 1 ratio. In each sample, 2 aggregate populations were found, and therefore micrographs are grouped in pairs. A (22 and 113 ms) - thermo-aggregated PrP and glycated β-casein with curcumin in the PrP ratio: cur = 1:3; B - coaggregated PrP and β-casein with 3,4-dimethoxycinnamic acid in the PrP: DMCA = 1:3 ratio. Incubation 1 h at 94°C. Scale bar 100 microns.

A similar situation is observed in the aggregation of prion protein and glycated β-casein in a 1: 1 ratio. Curcumin leads to loosening of dense aggregates and leads to deformation of spiral structures, 3,4-DMCA almost completely suppressed the formation of spiral aggregates, however, in its presence large aggregates turned from large dense lumps into conglomerates from small (~ 800 nm in diameter) balls.

In addition, protein aggregation was analyzed by denaturing electrophoresis.





C)

Fig. 84. Analysis of prion protein and glycated β-casein coaggregation in the presence of curcumin. SDS-electrophoresis analysis.

Gel A is the PrP: β-cas glyc = 4:1 protein ratio: A1-A3 samples are incubated without ligands, B1-B3 are incubated with curcumin. B - PrP: β-cas glyc = 2:1 protein ratio: C1-C3 samples - incubation without ligands; D1-D3 - incubation with curcumin. B - PrP: β-cas glyc = 1:1 protein ratio: E1-E3 samples - incubation without ligands, F1-F3 - incubation with curcumin. In each group with alphanumeric designation, samples 1,2,3 correspond to incubation of 0, 30 minutes or 1 hour at 94°C. Samples indicated only by numbers: 1 - isolated prion protein before heat treatment, 2 - isolated prion protein after heat treatment. The total protein concentration in the samples is 3 mg/ml.

The presence of curcumin led to a slight decrease in the intensity of the bands of small aggregates, but they failed to suppress the formation of large aggregates unable to enter the concentrating gel.

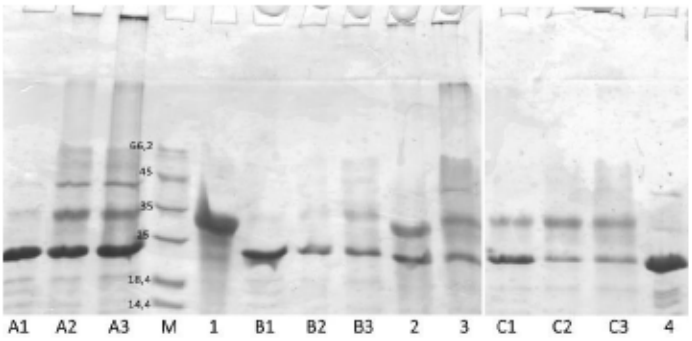


Fig. 85. Analysis of prion protein and glycated β-casein coaggregation in the presence of 3,4-dimethoxycinnamic acid. SDS-electrophoresis analysis.

Samples containing 3,4-DMCA: A1-A3 - the ratio of PrP proteins: β-cas glyc = 4: 1, B1-B3 - the ratio of PrP proteins: β-cas glyc = 2: 1; C1-C3 - PrP: β-cas glyc = 1: 1 protein ratio. In all samples with alphanumeric designation, the numbers 1,2,3 correspond to incubation for 0, 30 minutes or 1 hour at 94oC. Samples with numerical designation: 1 - unmodified β-casein, 2 - prion protein and glycated β-casein in a 1: 1 ratio before thermal incubation, 3 - prion protein and glycated β-casein in a 1: 1 ratio after 1 h of heat treatment; 4 - unmodified prion protein.

The presence of DMCA resulted in a decrease of large aggregates unable to enter the gel number, but instead of that, the percentage of dispersed fraction moving uniformly

along the separating gel, i.e. consisting of aggregates smaller in size than large particles, but larger than monomeric protein, increased.

Conclusion: curcumin suppresses aggregation of proteins as a whole better; 3,4-DMCC prevents aggregation of prion protein better, increasing the share of glycated β-casein reduces its effectiveness.

Conclusion: The presence of anti-amyloid ligands prevents the formation of helical aggregates.

Part 5. The effect of glycation on the properties of sheep prion protein

Determination of the degree of prion protein glycation

After experiments to determine influence of glycation on the properties of a protein capable of suppressing pathogenic aggregation, we decided to investigate what effect the same modification would have on amyloidogenic protein. A recombinant sheep prion protein was chosen as a model object, which was previously subjected to glycation to determine the potential formation of helical structures by other glycated proteins when interacting with thioflavin T, and which did not form them.

It was decided to apply the glycation conditions similar to those used for β-casein, but with a reduced protein concentration, in view of the tendency of PrP to undergo aggregation not related to glycation. For the same reasons, the glycation time was reduced to 1 day.

Before glycation, the prion protein was dissolved in 20 mM sodium acetate buffer, since it is under these conditions that the protein dissolves readily and is practically not inclined to spontaneously aggregate. Then it was transferred by gel filtration method into 50 mM sodium borate buffer pH 8.0, and in this form it was used in further experiments. The protein was dissolved in this way immediately prior to the experiment, in order to avoid random aggregation.

Glycation conditions:

c (protein) = 1 mg/ml, c (D-gluc) = 0.2 M, s ($NaBH_3CN$) = 35 mM. Buffer 50 mM Na-borate pH 8.0.

The incubation time is 1 day, the temperature is 37°C.

After modification, the entire prion protein was purified from low molecular weight glycation products. Due to the fact that the reverse phase chromatography of PrP was hampered by the probability of aggregation, it was necessary to use dialysis against 25 mM MES pH 6.7. The resulting solution was concentrated in centricons with pores permeable to particles with a modular mass of not more than 10 kDa (the molecular weight of the prion protein was 23 kDa) up to 3 mg / ml, and this solution was already used to prepare samples for thermoaggregation.

At the first stage of work, the degree of PrP glycation was estimated. The number of early glycation products was analyzed by the fructosamine reaction.

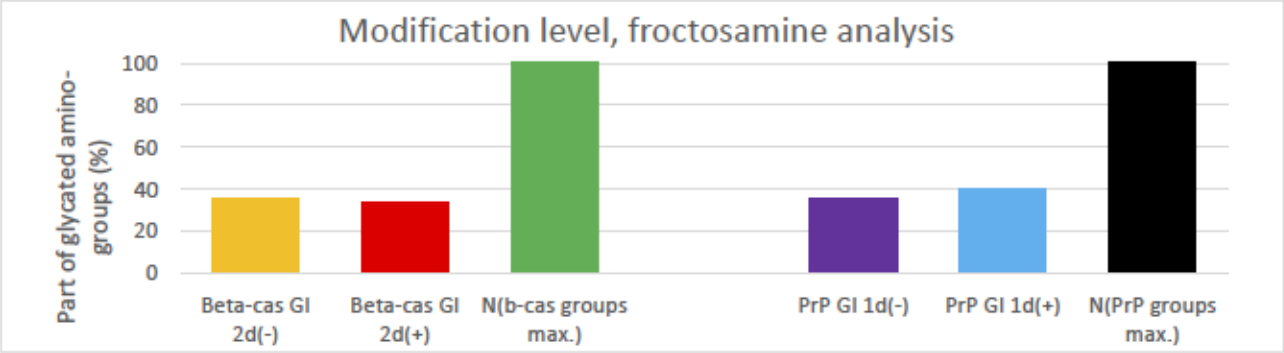


Fig. 86. Comparison of early glycation products amount after β-casein and prion protein modification by the fructosamine method.

And the absolute value of the number of glycated groups in the protein; B - the percentage of modified amino groups in the protein relative to the total (17 groups in β-casein, 24 groups in the prion protein). Samples marked "groups max" indicate the maximum possible glycation level of the corresponding protein.

The level of prion protein glycation reached approximately the same values as that for glycated β-casein, but after 1 day instead of 2. In absolute terms, the number of glycated groups in the prion protein reached approximately the same values as β-

casein, after 3 days of modification (8-9 groups per 1 protein molecule). Probably, glycation sites in the prion protein are more accessible due to the microenvironment.

The amount of late glycation products (AGE) was measured by their own fluorescence.

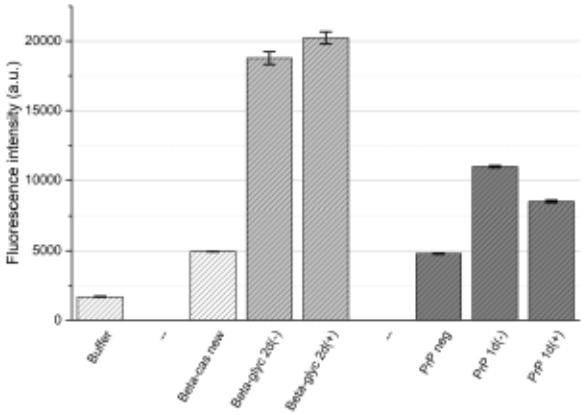


Fig. 87. Comparison of late glycation products (AGE) amount after β-casein and prion protein modification by their own fluorescence.
Samples involved: buffer control; native β-casein; glycated 2 days β-casein; unmodified prion protein; 1 day glycated prion protein (label "+" means the presence of sodium cyanoborohydride reducing agent).

The amount of late glycation products in the case of the prion protein is predictably less than in the sample with β-casein, since the modification time was much shorter.

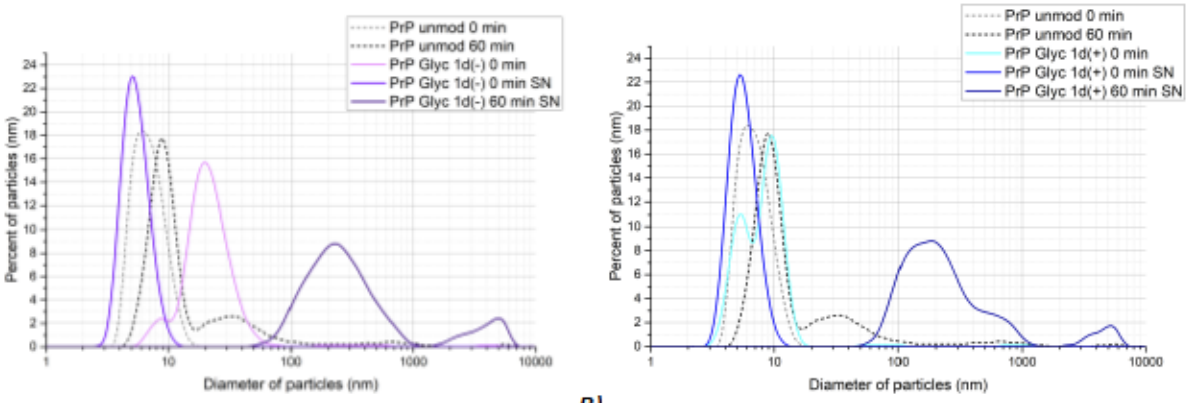
Conclusion: the prion protein is successfully glycated with D-glucose under selected conditions.

The study of glycated prion protein aggregation

Glycated prion protein was subjected to exactly the same heat-treatment procedure as β-casein.

The conditions of thermoaggregation remained the same: incubation time up to 1 h, temperature: 94°C. Buffer: 25 mM MES pH 6.7 with 0.1M NaCl.

Already visually, it was possible to noticeably turbidity of the samples after thermal incubation, so the analysis of changes in them as a result of heat treatment began with dynamic light scattering.



A) *B)*

Fig. 88. Distribution of glycated prion protein aggregates by particle size. Detection by dynamic light scattering.

The distribution of the volume of particles. Grouping pictures by protein in samples: A - prion protein, glycated for 1 day without reducing agent. B - prion protein, glycated for 1 day in the presence of sodium borohydride.

A glycated prion proteinloses stability and slightly aggregates already in solution for thermal incubation, but the monomer fraction still prevails in it (~ 4.8 nm), also a dimer fraction (9 nm) is present. Protein, glycated under reducing conditions, shows a slightly smaller part of aggregates than protein, which has undergone modification without cyanoborohydride. After thermoaggregation, in contrast to the unmodified protein, which forms a fraction with a diameter about 30 nm, glycated PrP forms particles ~ 200 nm. The presence of a reducing agent leads to some reduction in the size of the aggregates.

After that, it was decided to estimate the total aggregation of the protein by samples turbidity, and suspicions arose that the turbidity could be added up with specific absorption. In this regard, turbidity analysis was performed on a spectrophotometer, measuring the optical density not at a point, but at a segment of wavelengths. At the same time, it was possible to compare the efficiency of turbidity assessment of samples using two protocols at two wavelengths: 320 and 600 nm.

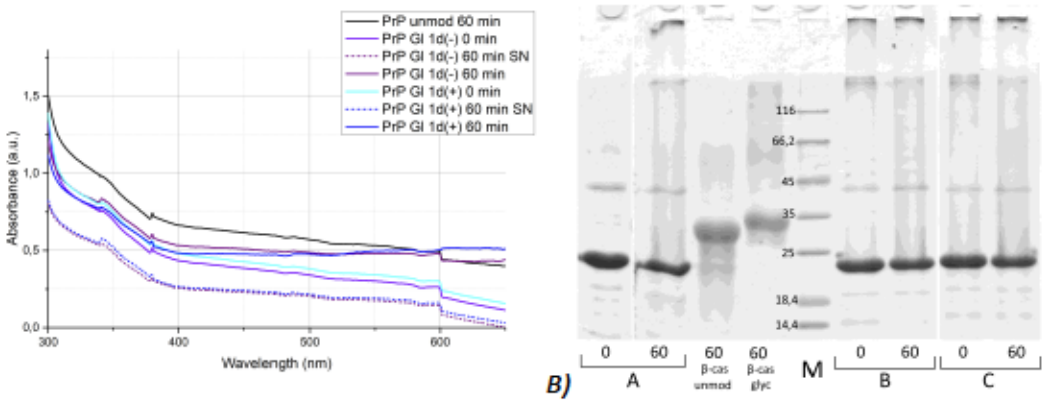


Fig. 89A. Absorption spectra of glycated prion protein samples before and after thermal incubation. 0 min - samples before thermal incubation, 60 min - after thermal incubation. The label "+" means the presence of 35 mM $NaBH_3CN$ during glycation, "-" - its absence. PrP unmod is an unmodified protein. Incubation 1 h at 94°C.

Fig. 89B. Thermo-aggregation of glycated prion protein, SDS-electrophoresis analysis. A - unmodified PrP, B - PrP, glycated without reducing agent, C - PrP, glycated with $NaBH_3CN$. 0 and 60 - time of thermal incubation. Incubation 1 h at 94°C.

Indeed, a slight increase in optical density was observed in the long-wavelength zone of the samples, which was not found for solutions prior to thermal incubation. The shape of the absorption spectra in the region of 320 nm for all samples was approximately the same. In general, it was noticeable that after thermal incubation, turbidity of the samples increased markedly, but if the solutions were subjected to even a brief centrifugation (within 5 min), the absorption was greatly reduced. So it can be argued that the abnormally high solution absorption at ~ 600 nm is associated with large protein aggregates.

The tendency of the protein to aggregate was checked by denaturing electrophoresis. Active protein aggregation was observed even before heat treatment, part of the bands of intermediate oligomers disappeared after incubation. The principal difference between a protein glycated with or without a reducing agent is not seen.

Finally, the most informative method for assessing the nature of aggregation was used - fluorescence microscopy with thioflavin T coloration.

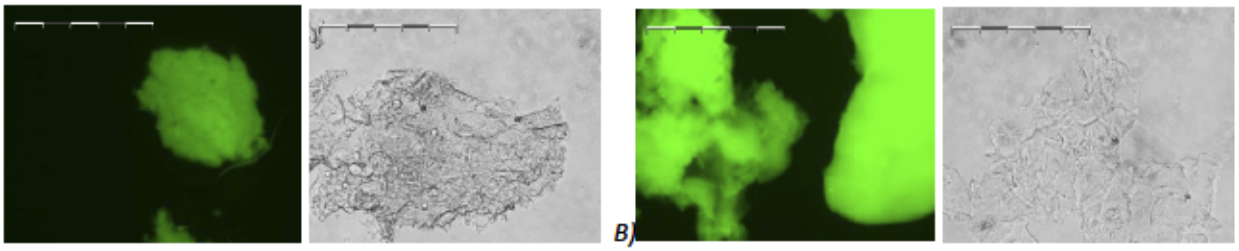
A)  B)

Fig. 90. Microscopy of thermo-aggregated glycated prion protein.

The photo in each of the pairs on the left is fluorescence microscopy with thioflavin T staining, on the right is optical microscopy of the preparation of the same sample without staining. Used prion protein, glycated with a reducing agent.

Sort by protein: A - before thermal incubation, B - after 60 minutes of thermal incubation. Scale bar 100 microns.

In the samples of glycated protein, both with and without reducing reagent, only amorphous aggregates were found, which after thermoaggregation are greatly enlarged and compacted. At the same time, the density of aggregates of glycated PrP was lower than that of unmodified protein, therefore thioflavin T penetrated into their depth. The fluorescence intensity of these aggregates after heat treatment also increased, but not as much as that of the non-glycated protein.

Conclusion: during glycation, the prion protein predominantly remains in monomeric form.

Conclusion: During heat treatment, the glycated prion protein aggregates.

Coaggregation of glycated prion protein and β-casein

After determination the main properties of glycated prion protein aggregation, it was decided to jointly aggregate it and β-casein, both unmodified and glycated, in order to understand whether a protein with antiaggregatory activity with respect to native PrP can disassemble the glycated protein.

The presence and properties of protein aggregates began to be investigated by dynamic light scattering.

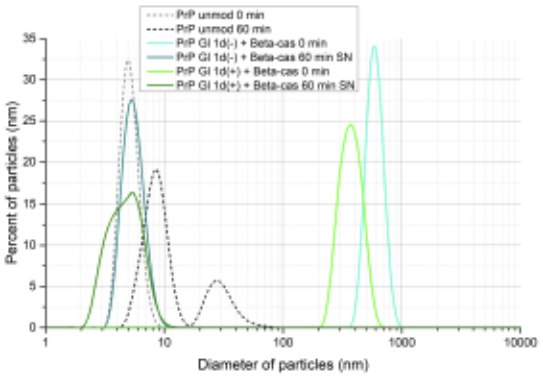
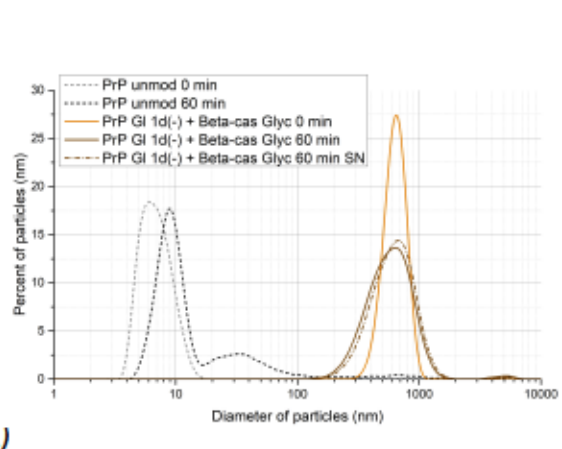


Fig. 91. Distribution of aggregates of glycated prion protein, co-incubated with unmodified β-casein by particle size. Detection by dynamic light scattering.

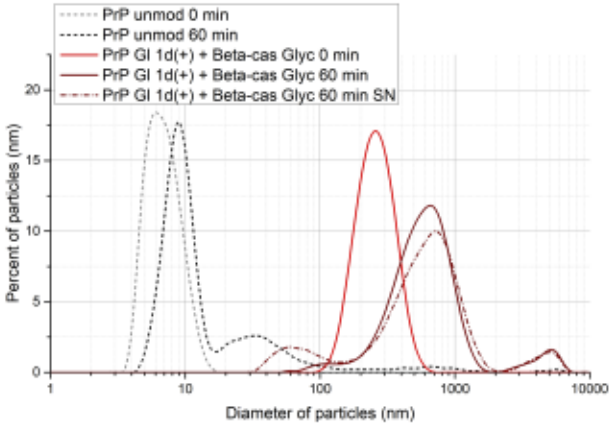
The distribution of the number of particles. The samples contained PrP glyc. 1d (-) - prion protein, glycated for 1 day without reducing agent, or PrP glyc 1d (+) - prion protein, glycated for 1 day in the presence of sodium cyanoborohydride.

Mixing of glycated prion protein and native β-casein leads to instantaneous turbidity of the sample and the formation of aggregates with a diameter of 600 (without reducing agent) and 360 (with reducing agent) nm. During thermal incubation, they form huge aggregates unmeasurable by the DLS method, however, the protein fraction continues to be present in the supernatant, reaching even the monomeric form.

The situation with coaggregation of both glycated proteins was different.



A)



B)

Fig. 92. The distribution of aggregates of glycated prion protein and β-casein in particle size. Detection by dynamic light scattering.

The distribution of the volume of particles.

Grouping pictures by protein in samples: A - prion protein, glycated for 1 day without reducing agent. B - prion protein, glycated for 1 day in the presence of sodium borohydride. In all cases, β-casein glycated for 2 days without reducing agent was used.

Mixing of glycated prion protein and β-casein leads to instant aggregation with the formation of large aggregates too, but in the presence of a reducing agent, particles are formed smaller than they were with unmodified β-casein (250 nm). In addition, after thermal incubation, unlike samples with unmodified β-casein, the monomeric form does not remain even in the supernatant - proteins completely aggregate, and the solution contains only fairly large particles with a diameter of ~ 700 nm (prion protein, glycated without reducing agent) or with an additional fraction with a diameter of ~ 90 nm (aggregation of prion protein, glycated with a reducing agent).

The turbidity of samples of isolated glycated prion protein even after thermoaggregation is small. In the presence of unmodified β-casein, turbidity is significantly increased due to the large number of aggregates that cannot be precipitated even by ultracentrifugation. However, after thermoaggregation, all these aggregates stick together and precipitate, which leads to a sharp decrease in the turbidity of the supernatant.

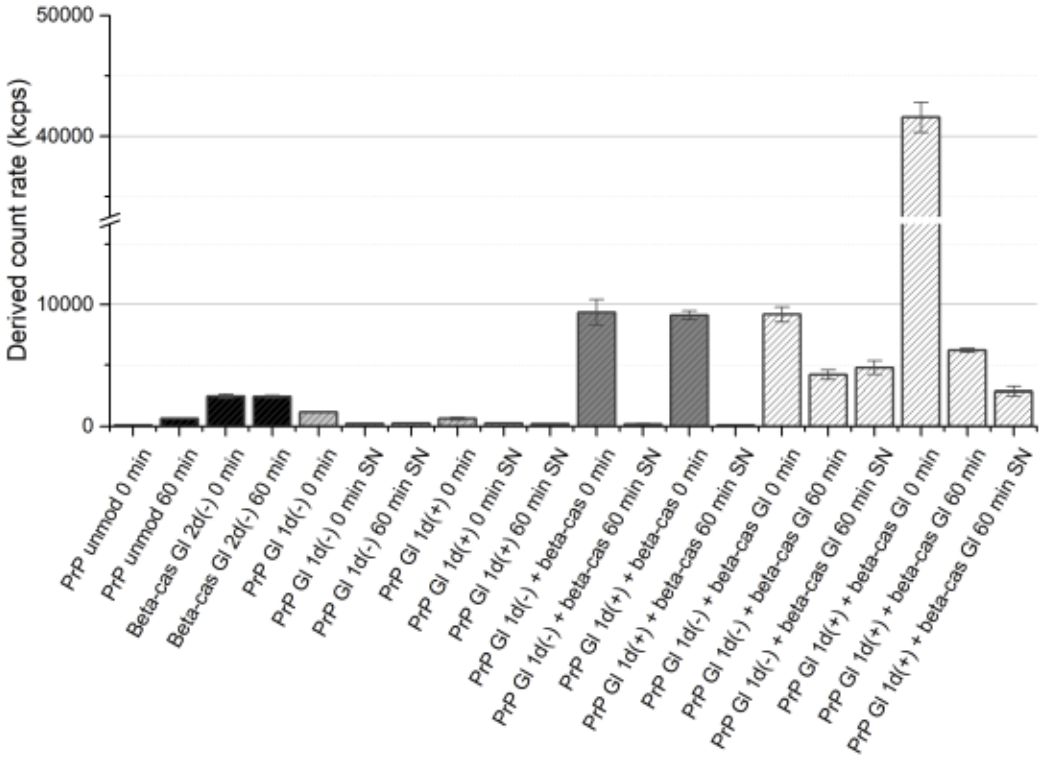
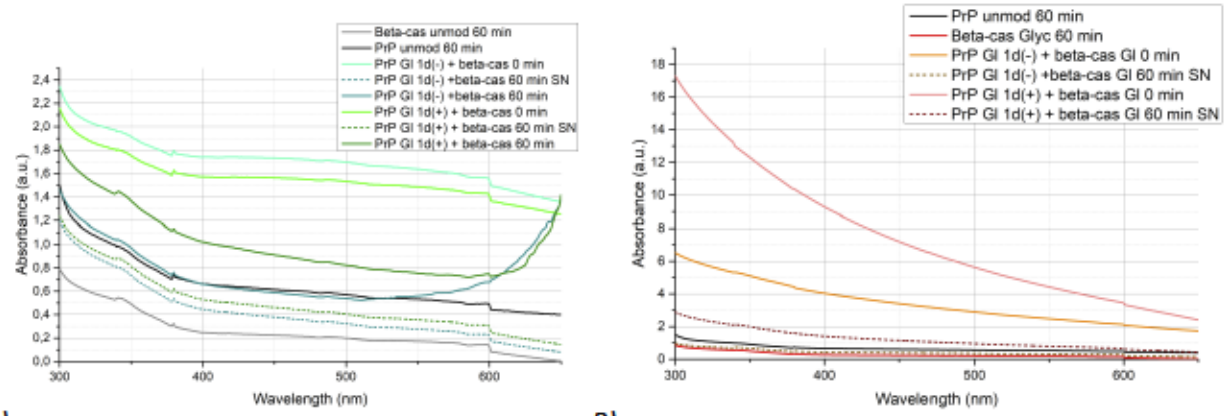


Fig. 93. Absorption of prion protein and β-casein suspension; measurement by dynamic light scattering. Notation used: 0 - before thermal incubation, 60 - after thermal incubation. SN - Result obtained from the supernatant of the sample. The black bars are the control unmodified prion protein and β-casein; light gray - samples of isolated glycated prion protein; dark gray bars - glycated prion protein samples with unmodified β-casein; white with shading columns - samples of glycated prion protein and β-casein.

In case of PrP with glycated β-casein co-incubation, the initial turbidity turns out to be even higher, and thermal incubation leads to its decrease, but not to its disappearance. So it can be argued that already at the first contact, glycated PrP and caseins coaggregate, and heat treatment significantly speeds up this process.

As in the case of isolated glycated β-casein, the turbidity of samples with protein coaggregation was analyzed and the absorption spectrum was removed in the range from 300 to 650 nm in order to capture the two main turbidity measurements of 320 and 600 nm.



A) *B)*

Fig. 94. Absorption spectra of prion protein samples and its mixtures with unmodified and glycated β-casein before and after thermal incubation.

A - samples with isolated glycated prion protein; B - samples with a mixture of glycated prion protein and unmodified β-casein; B - samples with a mixture of glycated prion protein and β-casein. The label "+" means the presence of 35 mM $NaBH_3CN$ during glycation, "-" - its absence. Incubation 1 h at 94°C.

The highest turbidity was again observed in samples of co-incubated glycated prion protein and β-casein. Isolated glycated β-casein, on the contrary, has the lowest turbidity. Unexpectedly, an increase in optical density was observed in the long-wavelength region in samples of glycated prion protein and unmodified β-casein after thermal incubation. This absorption disappears after centrifugation, so perhaps this is due to the fraction of the aggregates that can be removed from the solution by centrifugation.

In connection with the separation of the protein into precipitated and soluble fractions, it was decided to estimate the distribution of protein in these fractions using the Bradford method. It was shown that isolated glycated prion protein by itself practically does not aggregate, but after thermal incubation only ~ 1/3 of the protein remains in solution. The presence of unmodified β-casein leads to the involvement of ~ 1/3 of the total protein in the aggregation before thermoaggregation. Glycated β-casein carries along ~ 33% of protein into aggregates. At the same time, the amount of precipitated protein after thermoaggregation in all combinations is almost the same, regardless of its composition.

Also, the distribution of protein by fractions of aggregates was attempted to be evaluated in denaturing electrophoresis.

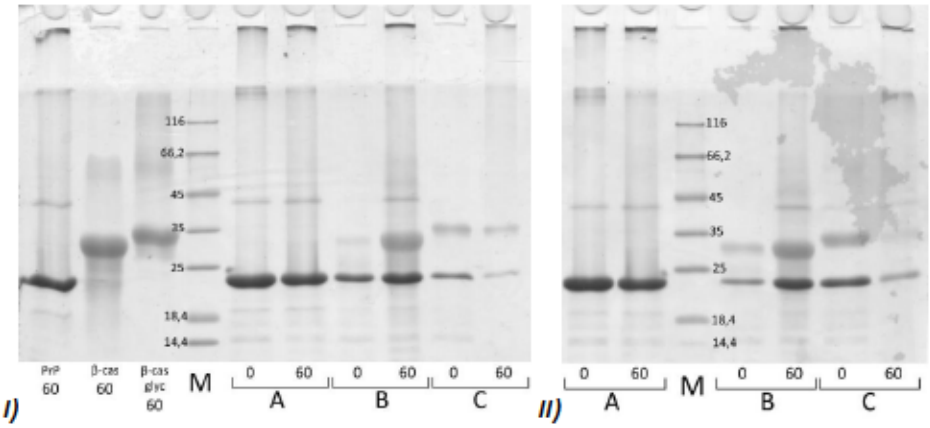


Fig. 95. Analysis of size of glycated prion protein aggregates after modification and coaggregation with β-casein. SDS electrophoresis.

The composition of the samples: A - glycated prion protein; B - glycated PrP and unmodified β-casein; C - glycated PrP and β-casein.

I - glycation of prion protein without reducing agent; II - prion protein is glycated in the presence of 35 mM NaBH3CN. Glycated β-casein in all samples is modified for 2 days without reducing agent. Prion protein was glycated for 1 day.

In the isolated glycated prion protein, at least 5 fractions were observed before thermoaggregation, considering the monomer and the part of the protein that did not enter the gel, 2 of which (intermediate in molecular weight, able to enter the concentrating gel, but retained at the entrance to the separating gel) are depleted during thermal incubation. Also noticeable is the decrease in the amount of monomer of the prion protein after heating.

The presence of β-casein completely prevented the formation of glycated prion protein aggregates without heat treatment; however, after incubation, the proteins formed a very dispersed fraction, from which the band at ~ 50 kDa was slightly separated.

In both previous cases, the presence or absence of sodium cyanoborohydride did not seriously affect the glycation of PrP, but there were still differences between the coaggregation of glycated differently prion protein and also glycated β-casein. When a modified protein with a reducing agent was involved in the process, the amount of

monomers visible on the gel turned out to be greater than in a sample with a protein glycated without a reducing agent. In addition, in the same sample, a band of intermediate-molecular aggregates passed through the concentrating gel, but stuck on the border of the separator. In the sample with protein without reducing agent, this band is extremely pale.

The presence of amyloid structures was checked by fluorimetry with thioflavin T staining, but taking into account the data of subsequent fluorescence microscopy.

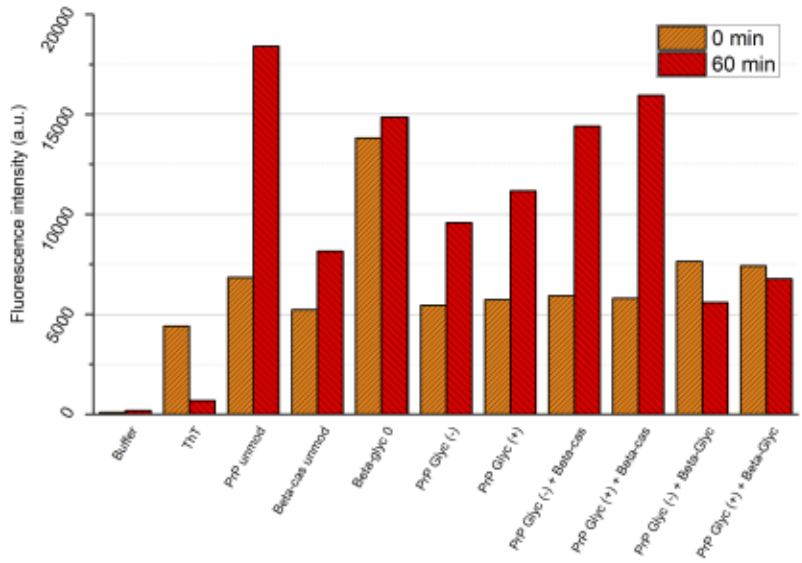


Fig. 96. Fluorescence intensity of glycated prion protein and β-casein, both unmodified and glycated, before and after thermoaggregation. Thioflavin T staining.
In each pair, the column to the left and lighter is the fluorescence intensity prior to thermal incubation, to the right after 1 h of heat treatment. The label "+" means the presence of 35 mM NaBH3CN during glycation, "-" - its absence. Heat incubation for 1 hour at 94°C.

Although the fluorescence of thioflavin T, bound to the glycated prion protein, increases after thermal incubation, it is lower than that of the unmodified prion protein and even lower than the fluorescence of the dye in the sample with glycated β-casein. At the same time, co-incubated glycated prion protein and unmodified β-casein triggered thioflavin T for fluorescence, which is slightly inferior to the fluorescence of the dye that bound the non-glycated thermo-aggregated prion. The fluorescence of the dye in samples of mixtures of glycated prion protein and β-casein was not so high, but

it seems that the measurement of fluorescence in them was made difficult by the rapid adhesion and precipitation of protein particles.

In samples of glycated prion protein and unmodified β-casein, fluorescent microscopy was dominated by spherical aggregates, as separate ones, and as part of large conglomerates. The large aggregates, in turn, especially after thermal incubation, consisted of stuck small balls, the fluorescence of these conglomerates was bright.

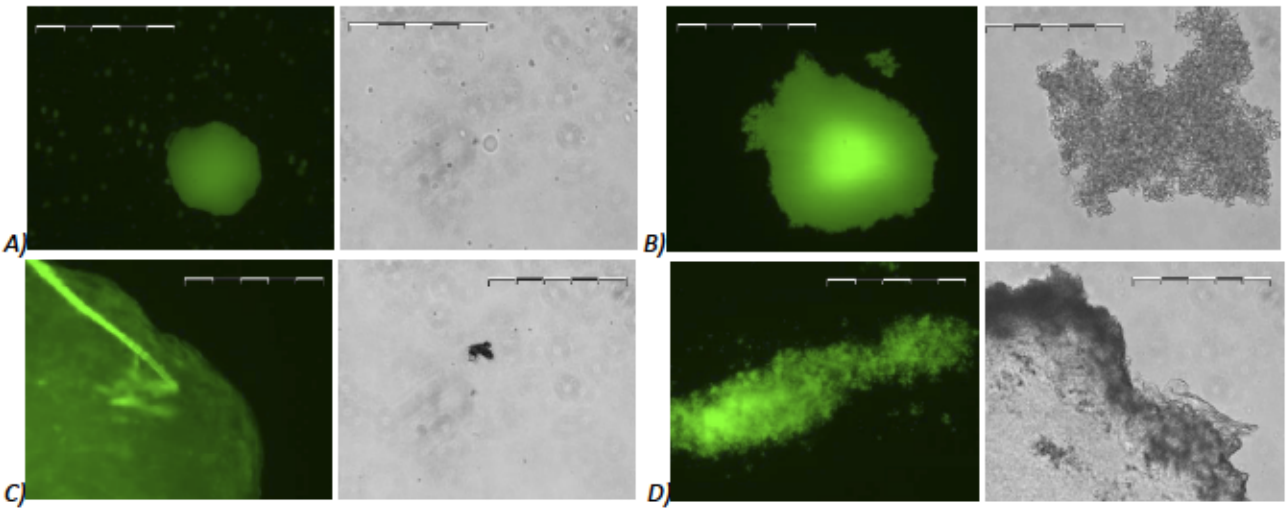


Fig. 98. Fluorescence microscopy of prion protein and β-casein with varying degrees of modification. Thioflavin T coloration.

The photo in pairs on the left is fluorescence microscopy with thioflavin T staining, on the right is optical microscopy of the preparation of the same sample without staining.

Sort by protein: A, B - prion protein, glycated without reducing agent, and unmodified β-casein; C, D - prion protein, glycated with 35 mM $NaBH_3CN$, and β-casein, glycated for 2 days without a reducing agent.

Sort by incubation time: A, B - before heat treatment, B, D - after 60 minutes of heat treatment.

Scale bar 100 microns.

Regardless of the reducing agent presence, during glycation in samples with both modified proteins 2 types of aggregates were found: large amorphous structures and stuck short threads. These filaments were similar to spiral-shaped aggregates except that they were thick, although they had the property of being formed only in the presence of a dye. The large amorphous aggregates found were as dense as in the case of coaggregation of non-glycated PrP and glycated β-casein. Despite the fact that the total fluorescence intensity of thioflavin T measured by fluorimetry in them was

weaker than in samples with unmodified and glycated PrP, the fluorescence brightness of individual aggregates was very high. Probably due to the deposition of aggregates, their fluorescence was difficult to measure. The amyloid nature of these aggregates is possible due to the presence of PrP in them.

Conclusion: The interaction of glycated prion protein and β-casein leads to the formation of the protein monomeric fraction, but does not exclude the formation of aggregates enriched with amyloid structures.

Conclusion: glycated prion protein changes the interaction of glycated β-casein with thioflavin T, suppressing the formation of spiral aggregates.

Part 6. Effect of anti-amyloid ligands on the aggregation of glycated prion protein

The interaction of glycated prion protein and anti-amyloid ligands

The final stage of the work was the analysis of effective anti-amyloid ligands curcumin and 3,4-dimethoxycinamic acid effect on the coaggregation of glycated proteins.

The change in the properties of glycated prion protein aggregates in the presence of ligands began to be investigated with the method of dynamic light scattering.

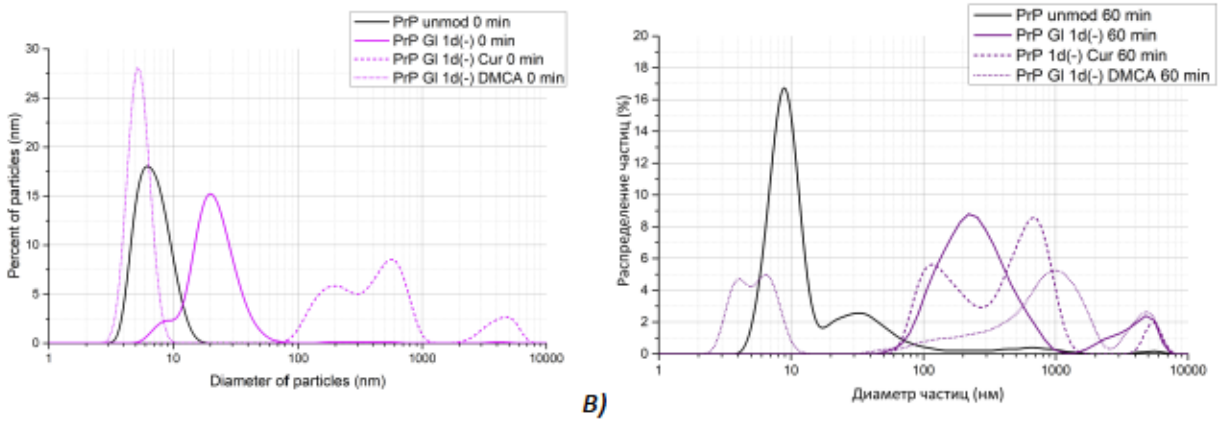
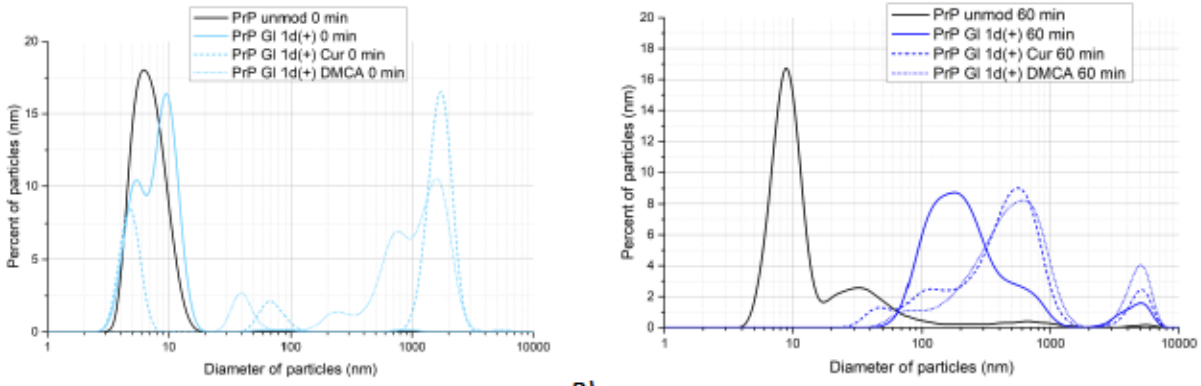


Fig. 99. The distribution of aggregates of glycated prion protein particle size. Detection by dynamic light scattering.

The distribution of the volume of particles. The label Cur indicates the presence in the sample of curcumin, DMCA - 3,4-dimethoxycinnamic acid. Tht PrP, glycated 24 h without reducing agent, was used.

A – samples before heat-treatment, B – samples after heat-treatment. Incubation 1 h at 94°C.

Curcumin stabilized the formation by glycated without reducing agent prion protein of aggregates with moderate size (2 peaks are distinguished at 150 and 700 nm), which, after thermal incubation, remained almost unchanged. 3,4-DMCA significantly reduced protein aggregation, as a result, prior to thermal incubation, it was predominantly in monomeric form, and even after warming up, part of the protein did not aggregate. So 3,4-DMCA shows a higher inhibiting effect against thermoaggregation of glycated PrP.



A) *B)*

Fig. 100. The distribution of aggregates of glycated prion protein particle size. Detection by dynamic light scattering.

The distribution of the volume of particles. The label Cur indicates the presence in the sample of curcumin, DMCA - 3,4-dimethoxycinnamic acid. A prion protein glycated for 1 day with 35 mM $NaBH_3CN$ was used.
A - samples before thermoaggregation; B - after thermoaggregation. Incubation 1 h at 94°C.

Unexpectedly, the aggregation of glycated in the presence of sodium cyanoborohydride PrP was activated by ligands: the particle diameter of the isolated protein after thermal incubation was 180–200 nm, while with ligands their diameter was 500 nm.

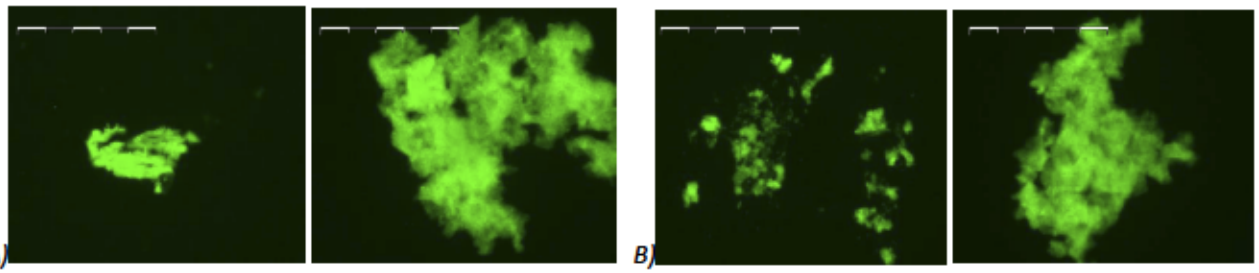
The results, obtained by DLS, correlate with the protein distribution between the supernatant and the precipitate after thermal incubation. When aggregating PrP, glycated without reducing agent, curcumin and 3,4-DMCA increase the part of protein in the soluble fraction by ~ 10%. The interaction with a prion protein, glycated with a

reducing agent, leads to the opposite effect - the amount of protein in the supernatant decreased by 5-10% of the total.

In the case of PrP glycated with a reducing agent, the ligand efficiency has changed - curcumin suppresses protein aggregation better in at least samples before heat treatment (after it and it is useless), the effect of cinnamic acid is almost absent both before and after heat treatment.

Also, the effect of ligands on the thermoaggregation of glycated PrP was evaluated by the results of SDS electrophoresis. However, the significant effect of ligands on PrP aggregation was not detected by this method.

The morphology of the largest aggregates was studied by fluorescence microscopy with thioflavin T staining.



A) *B)*

Fig. 101. Fluorescence microscopy of thermoaggregation of glycated prion protein. Thioflavin T coloring. Samples with prion protein glycated without reducing agent are presented.
Sorting by protein state: on the left - before thermal incubation, on the right - after 60 minutes of thermal incubation.
And - samples with curcumin, B - samples with 3,4-DMKK. Scale bar 100 microns.

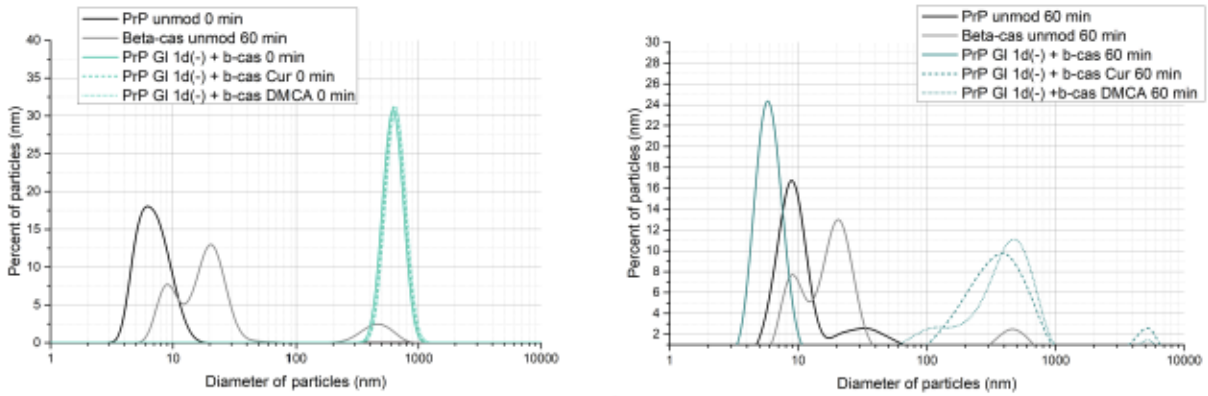
The presence of ligands leads to loosening of the aggregates of glycated prion protein: instead of dense large aggregates, film conglomerates are formed. The fluorescence intensity of individual aggregates decreases slightly. The presence or absence of a reducing agent during PrP glycation does not affect the morphology of protein aggregates in the presence of ligands.

Conclusion: curcumin more effectively suppresses amyloidization, and 3,4-DMCA more effectively suppresses the aggregation of glycated prion protein.

Conclusion: 3,4-DMCA more effectively suppresses aggregation of prion protein, glycated without reducing agent, curcumin - on the contrary, more effectively suppresses aggregation of PrP modified with reducing agent.

Effect of anti-amyloid ligands on coaggregation of glycated prion protein and β-casein

The process of two proteins coaggregation and the effect of anti-amyloid ligands, curcumin and 3,4-dimethoxycinnamic acid, on this process began to be studied by changing the size of a part of the aggregates with a diameter of up to 1 micron. Larger aggregates were usually precipitated during incubation, so the DLS method was mainly used for supernatant analysis.



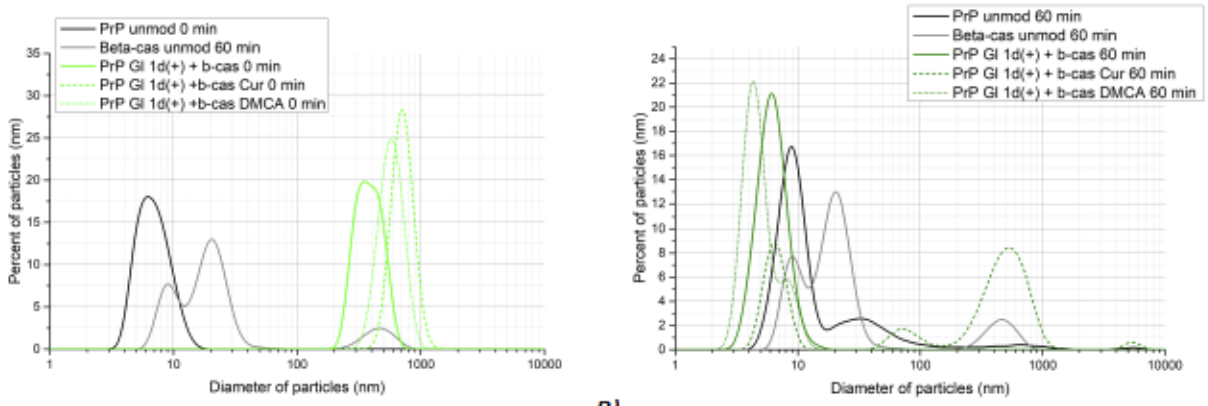
A) *B)*

Fig. 102. Distribution of aggregates of glycated prion protein, co-incubated with unmodified β-casein in particle size. Detection by dynamic light scattering.

The distribution of the volume of particles. The label Cur indicates the presence in the sample of curcumin, DMCA - 3,4-dimethoxycinnamic acid.

Unmodified β-casein and prion protein glycated for 1 day without reducing agent were used. A - samples before thermoaggregation, B - samples after thermoaggregation. Incubation 1 h at 94°C.

Before thermoaggregation of β-casein and prion protein, glycated without a reducing agent, ligands do not have a significant effect, but after thermal incubation, they seem to have a negative effect. In samples without ligands, a fraction of monomeric proteins is formed, while curcumin (that wasn't observed for DMCA) stabilize certain aggregates with a diameter 500 nm.



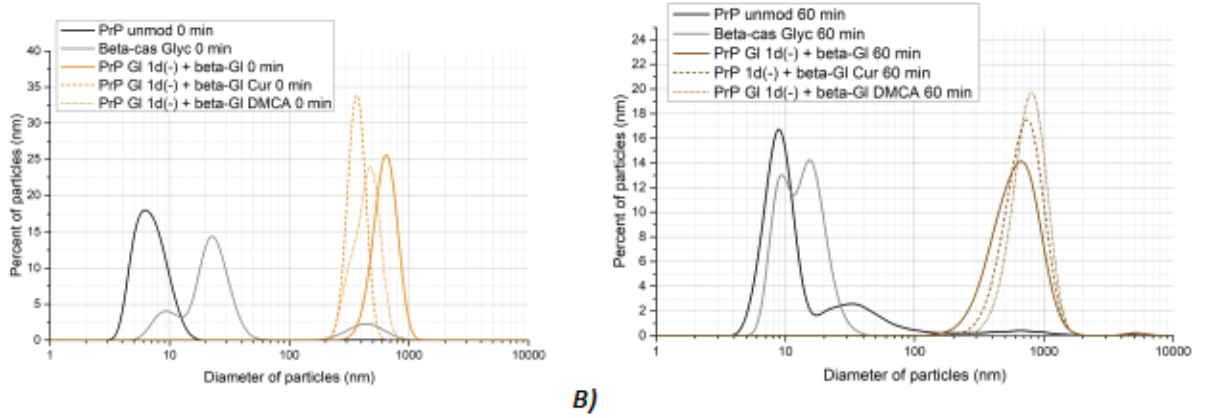
A) *B)*

Fig. 103. Distribution of aggregates of glycated prion protein, co-incubated with unmodified β-casein in particle size. Detection by dynamic light scattering.

The distribution of the volume of particles. The label Cur indicates the presence in the sample of curcumin, DMCC - 3,4-dimethoxycinnamic acid.

Grouping pictures by protein in samples: A - unmodified β-casein and prion protein, glycated for 1 day in the presence of a reducing agent before thermoaggregation; B - unmodified β-casein and prion protein, glycated for 1 day in the presence of a reducing agent after thermoaggregation. Incubation 1 h at 94°C.

Aggregation of glycated under reducing conditions prion protein with unmodified β-casein and anti-amyloid ligands prior to heat treatment also did not reveal the effect of low molecular weight substances, and analysis of samples after thermoaggregation revealed some degree of stabilization of large aggregates by curcumin and disassembly of oligomers to monomeric protein by 3,4-DMCA.



A) *B)*

Fig. 104. Distribution of aggregates of glycated prion protein and β-casein in particle size. Detection by dynamic light scattering.

The distribution of the volume of particles. The label Cur means the presence of curcumin in the sample, DMCA - 3,4-dimethoxycinnamic acid. Used prion protein, glycated for 1 day without reducing agent and beta casein, glycated for 2 days without reducing agent.
A - samples before thermoaggregation, B - after thermoaggregation. Incubation 1 h at 94°C.

Curcumin and 3,4-DMCA addition to glycated prion protein and β-casein (PrP modified without a reducing agent) before heat treatment led to a slight decrease in the size of aggregates from 600 nm in isolated proteins to 450 nm in the presence of DMCA and 350 nm in the presence of curcumin.

The principal effect of anti-amyloid ligands on thermally induced co-aggregation of glycated prion protein and β-casein was not observed; one could even say that in the presence of ligands, the aggregates became larger: their hydrodynamic diameter increased from 500 to 700 nm.

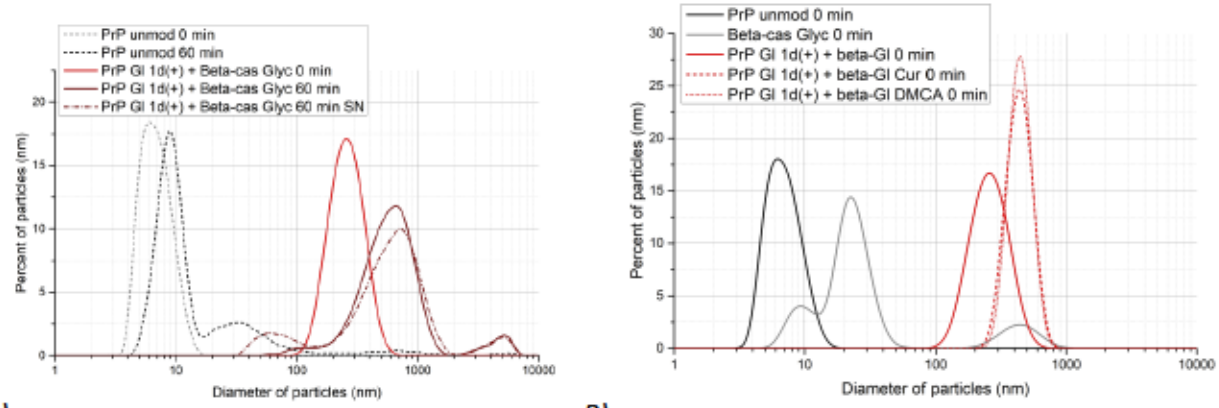


Fig. 105. Distribution of aggregates of glycated prion protein and β-casein in particle size. Detection by dynamic light scattering.
The distribution of the volume of particles. The label Cur indicates the presence in the sample of curcumin, DMCA - 3,4-dimethoxycinnamic acid. A prion protein glycated for 1 day with 35 mM $NaBH_3CN$ and beta-casein glycated for 2 days without reducing agent were used.
A - samples before thermoaggregation, B - after thermoaggregation. Incubation 1 h at 94°C.

The presence of anti-amyloid ligands before heating resulted in a negative effect on coaggregation of glycated β-casein and prion protein modified with a reducing agent: the size of the major population of the aggregates increased to ~ 440 nm, while the isolated protein mixture formed particles with a diameter of 240 nm. A similar analysis

of the samples after heat treatment showed a positive effect of ligands: the particle size in the sample with proteins only grew to 700 nm, while with ligands it decreased to 240 nm.

On average, according to DLS analysis, it can be argued that 3,4-DMCA slightly more effectively suppresses aggregation of glycated prion protein and β-casein than curcumin, allowing the protein to sometimes maintain a monomeric form or even provoking disassembly of a significant part of the aggregates, but the overall antiaggregation effect of ligands is small.

Additionally, the results of DLS were used to estimate the turbidity of the solutions both before and after heat treatment.

The presence of native or glycated β-casein leads to increasing of sample turbidity, probably caused by the process of large aggregates formation detected by DLS. Thermal incubation leads to adhesion of these aggregates into large particles, unlike these aggregates that settle in the solution, therefore, after thermal incubation, the number of large particles in the supernatant is greatly reduced. After thermal incubation, a significant level of absorption of the supernatant is retained by samples containing glycated β-casein and prion protein glycated under non-reducing conditions. The effect of ligands on the degree of aggregates precipitation is insignificant. In samples of unmodified β-casein and glycated PrP, the number of absorbing aggregates in the presence of ligands decreases, both before and after heat treatment. In samples with both glycated proteins, a weak antiaggregation effect was observed for both ligands against PrP, glycated without a reducing agent. In the case of a prion protein modified with sodium cyanoborohydride, curcumin and 3,4-DMCA either did not affect or activated aggregation.

Then an assessment of the distribution of proteins between soluble and precipitated fractions by protein concentration in the supernatant was made. The measurement was carried out according to the Bradford method in 96-well plates. As a result, it was

shown that, unlike isolated glycated prion protein, samples with β-casein, even unmodified and before thermal incubation, lead to rapid aggregation and a significant decrease in the protein content in the soluble fraction. Antiamyloid ligands somewhat improve the situation, but the effect remains within 5-10%. The presence of glycated β-casein leads to even greater involvement of the protein in the aggregation, the ligands also retain a slight effect of aggregation suppressing (about 5%). After heat treatment, the effect of ligands on the degree of protein deposition is significantly weakened.

Curcumin has shown the ability to keep proteins from precipitating during coaggregation of unmodified β-casein and glycated with a reducing agent prion protein before thermo-incubation, this ligand showed little effect for co-aggregation of glycated β-casein and prion protein in non-reducing conditions. In general, it can be said that 3,4-DMCA is more effective in suppressing the aggregation of the glycated prion protein without a reducing agent, while curcumin, on the contrary, suppresses the aggregation of PrP modified with sodium cyanoborohydride better.

Analysis of samples for the presence of stable detergent aggregates by SDS-electrophoresis showed that the formation of large aggregates that are not part of the gel and are stable when boiled with SDS, either does not depend on the presence of ligands, or even becomes more active.

Then we switched from the properties of the aggregates to the character of aggregation. Its analysis started with thioflavin T fluorimetry.

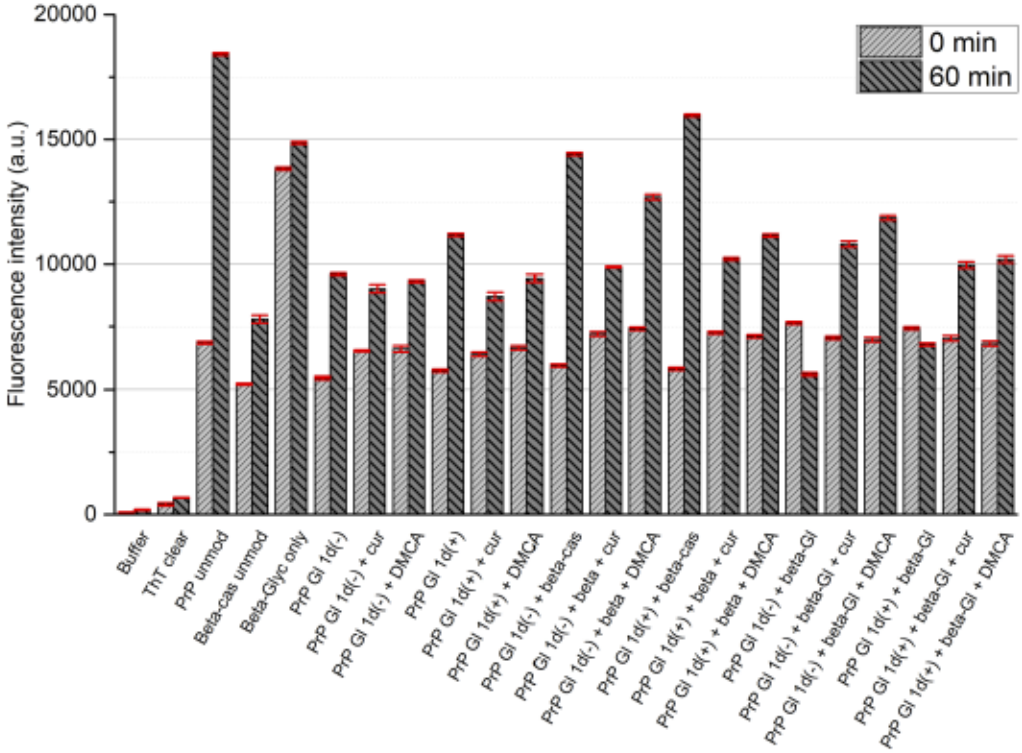
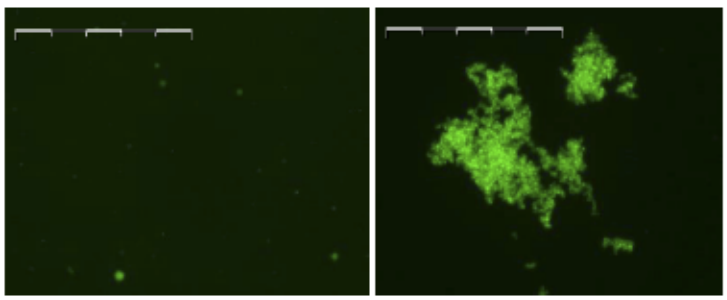


Fig. 106. Fluorescence intensity of glycated prion protein and β-casein, both unmodified and glycated, before and after thermoaggregation. Thioflavin T staining.

Block 1 - control preparations of unmodified prion protein and β-casein; Block 2 - samples of isolated glycated proteins; Block 3 - coaggregation of glycated PrP and unmodified β-casein; Block 4 - samples of coaggregation of glycated PrP and glycated β-casein.

On average, it can be argued that curcumin more effectively inhibits the growth of fluorescence of glycated prion protein samples. However, it was noted that the antiamyloid effect of ligands becomes significant only in the presence of unmodified β-casein, in the case of isolated prion protein, the effect is practically absent, and when both glycated proteins interact, on the contrary, the effect is activating.

Naturally, a more detailed analysis of samples by fluorescence microscopy with thioflavin T was performed.

A) 

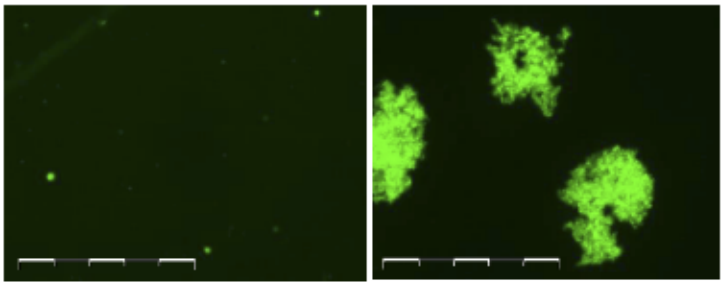
B) 

Fig. 107. Fluorescence microscopy of thermo-aggregated glycated prion protein and unmodified β-casein. Thioflavin T coloring.

In the presented samples prion protein, glycated without reducing agent, was used. B-casein in all samples was unmodified. Sorting by protein state: on the left - before thermal incubation, on the right - after 60 minutes of thermal incubation.

A - samples with curcumin, B - samples with 3,4-DMCA. Scale bar 100 microns.

Before thermoaggregation in samples with glycated PrP, unmodified β-casein and ligands, a large number of small spherical aggregates are observed. The presence of ligands during thermal incubation leads to loosening of large particles of glycated prion protein and unmodified β-casein, spherical aggregates are clearly distinguished in formed conglomerates, however, thioflavin T fluorescence in these aggregates does not become dimmer in these aggregates. Thermal aggregation with PrP, glycated with a reducing agent, is more active, the effect of ligands in this case is weaker, but the morphology of the aggregates remains the same.

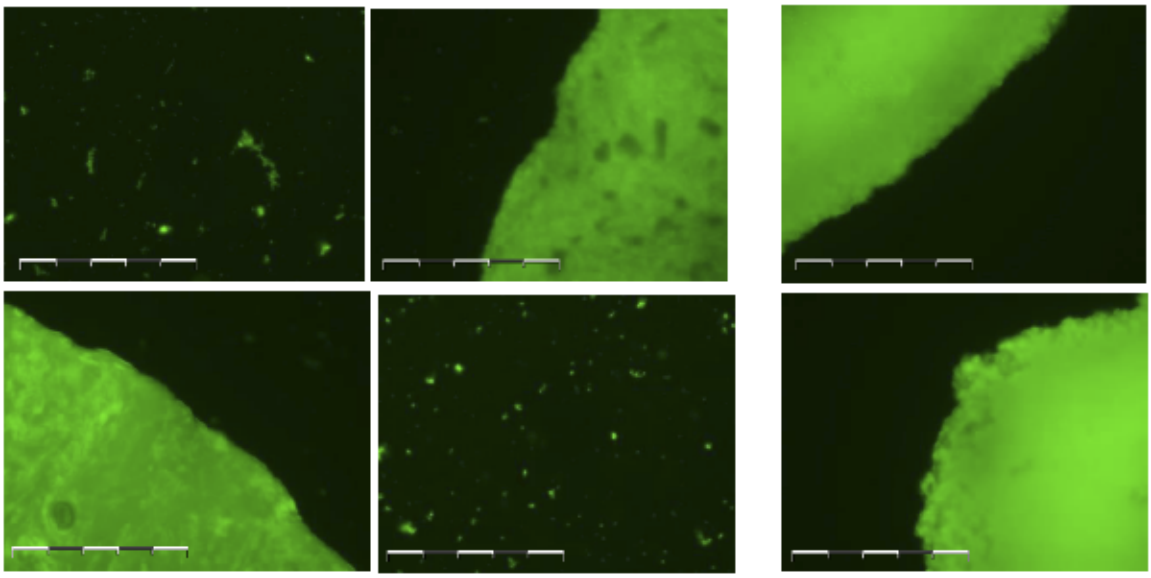
A) B) 

Fig. 108. Fluorescence microscopy of thermo-aggregated glycated prion protein and β-casein. Thioflavin T coloring.

Prion protein, glycated with a reducing agent, was used. B-casein in all samples was glycated for 2 days

without reducing agent. Sorting by protein state: on the left - before thermal incubation, on the right - after 60 minutes of thermal incubation.
A - samples with curcumin, B - samples with 3,4-DMKK. Scale bar 100 microns.

In samples with both glycated proteins and ligands prior to thermal incubation, small knotted fibrillar aggregates, resembling an intermediate shape between sticking needles from samples without ligands and sticking together conglomerates of spherical aggregates, formed from the interaction of glycated prion protein and unmodified β-casein, were found. After thermal incubation in the presence of ligands, the fraction of small aggregates disappears completely, but very large protein particles with high fluorescence don't change their structure, and their ThT fluorescence, according to required exposure time (decreases from 1000 ms without ligands to 100-200 ms with them), has increased. There was no principal difference in the morphology of the aggregates between the PrP samples glycated in the presence or without a reducing agent.

Conclusion: curcumin more effectively suppresses amyloidization, 3,4-DMCA more effectively suppresses the aggregation of glycated prion protein and unmodified β-casein.

Conclusion: 3,4-DMCA more effectively suppresses the aggregation of β-casein and prion protein, glycated with a reducing agent, curcumin - on the contrary, more effectively suppresses the aggregation of β-casein and PrP, modified without a reducing agent.

Conclusion: anti-amyloid ligands are ineffective against the co-aggregation of glycated prion protein and β-casein.

Results discussion

Coaggregation of amyloidogenic and dietary proteins

Effect of anti-amyloid ligands on prion protein aggregation

Isolated prion protein successfully passed amyloid conversion and aggregation, forming large dense aggregates. The aggregates contained a large number of amyloid

structures, and their density prevented the diffusion of thioflavin T dye into the interior. Curcumin and 3,4-dimethoxycinnamic acid showed a significant effect as inhibitors of amyloidization and aggregation of prion protein. Curcumin suppressed PrP amyloidization better; therefore, the percent of amyloid structures in its presence decreased significantly. 3,4-DMCA was more effective in suppressing PrP aggregation, therefore there were fewer aggregates in its presence and they became looser than under the influence of curcumin. Pentamidine isethionate had no significant effect on the studied processes, neither the shape, nor the density of the aggregates, nor the proportion of amyloid structures in them changed significantly. Resveratrol slightly suppressed amyloidization and PrP aggregation, but very slightly.

Effect of chaperonins on amyloid aggregation of prion protein

Activation of PrP aggregation in the presence of the chaperonin complex was found, as well as an increase in fluorescence of the amyloid-sensitive dye thioflavin T. Thus, it can be assumed that the presence of the GroEL/GroES complex not only does not suppress, but on the contrary, enhances the amyloidization of the prion protein. Probably, the chaperonins do not provide exactly the correctly folded protein, but only accelerate the folding process along one of the most energy-efficient ways, including amyloidization.

Effect of anti-amyloid ligands on co-aggregation of prion and milk proteins

The most actively aggregate mixtures are sheep prion protein with β-lactoglobulin, κ-casein and serum albumin. In the case of β-lactoglobulin and albumin, even at the minimum incubation time, large aggregates were formed, due to which the solution quickly became cloudy. Apparently, the process requires only a small push. In these cases, the aggregates are formed large, but amorphous and slightly reduced fluorescence intensity (Fig. 33 and 36). In addition, coaggregation of prion protein and bovine serum albumin led the formation of huge gel-like aggregates capable of

occupying the entire volume of the solution, with a very high fluorescence of thioflavin T. Thus, aggregates of both amyloid and nonspecific nature were obtained. It can be assumed that for β-lactoglobulin and α-lactalbumin, the prion protein is a good activator of nonspecific aggregation, probably due to its unstructured N-terminal domain.

For aggregation with κ-casein, visually aggregates forms during longer time, however, this is most likely due to a different structure of the aggregates: they are noticeably smaller in size, but more dense and have a higher fluorescence intensity (see Fig. 35). It can be assumed that the joint aggregation of prion protein and κ-casein leads to greater specificity and effectiveness of amyloid conversion than co-aggregation of PrP with β-lactoglobulin or serum albumin.

In the presence of β-casein, large aggregates do not form at all, and the fluorescence of thioflavin T also does not increase. B-casein itself is very reluctant to be involved in aggregation. It seems that in this case, β-casein exhibits chaperone-like properties known behind it and prevents amyloidization and PrP aggregation.

During incubation with anti-amyloid ligands – curcumin and 3,4-dimethoxycinnamic acid, a noticeable decrease in the rate of aggregation of isolated prion protein was observed, which correlates with the anti-amyloid properties of these low-molecular substances. The presence of curcumin has small effect on the formation of PrP aggregates with the rest of the proteins, which suggests that the ligand does not interact with those parts of proteins that are involved in coaggregation. In the presence of curcumin and 3,4-DMCA, as well as β-casein, it was possible to almost completely suppress the amyloid conversion of PrP. At the same time, in the presence of curcumin, the aggregates did form, but they did not have any structure, and the fluorescence intensity of thioflavin T in them was very low. In the presence of 3,4-DMCA, the aggregates became extremely small and rare, and the intensity of thioflavin fluorescence in them was also very low. Thus, the combination of curcumin and β-

casein made it possible to significantly suppress, and 3,4-DMCA and β-casein - to almost completely prevent amyoidization and aggregation of prion protein. The effect of β-casein, as a protein with chaperonin-like activity, with the anti-amyloid effect of ligands is summarized.

The effect of glycation on β-casein properties

Effect of glycation on casein anti-aggregation activity

In contrast to experiments with native β-casein, glycated protein showed a significant change in its properties. The modification reached approximately 50% of all groups available for glycation, which led to a change in the structure of the protein. This was shown both by circular dichroism and electrophoretic methods: protein mobility in the gel decreased even under denaturing conditions. These changes are expected to have an impact on the chaperone-like activity of the protein. In the presence of native β-casein, refolding of enzymes (by the example of GAPDH) was possible, while glycated β-casein almost completely suppressed the independent restoration of enzyme activity. It was also found that the interaction of other proteins with glycated β-casein in the event of violations in their own native structure leads to their rapid aggregation and precipitation, which indicate a complete denaturation of the enzyme. Naturally, this prevents the manifestation and enzymatic activity, in particular, GAPDH. It was assumed that the glycated β-casein, like the native, can interact with the hydrophobic regions of incorrectly folded proteins, but it is no longer able to create conditions for keeping them from aggregation due to modification and, moreover, becomes a kind of initiator of protein molecules sticking together. perhaps spatially organizing them around you. Moreover, it has been shown that glycated β-casein significantly inhibits the restoration of enzymatic activity of enzymes with the help of GroEL/GroES chaperonins. Apparently, the interaction of glycated β-casein and GAPDH is so strong that even true chaperonin cannot prevent or reverse it.

Specific aggregation of glycated β-casein with thioflavin T

In connection with such significant change in the properties of β-casein as a consequence of the modification, an analysis of its own propensity for aggregation was also carried out. Thus, a small fraction of the amorphous structures of the isolated protein was found. Being analyzed by these methods, excluding the presence of thioflavin T, these aggregates did not have confirmed amyloid properties; at the same time, the secondary structure of the protein in them was more ordered than the unmodified protein.

The interaction of glycated β-casein and thioflavin T was clearly specific, and therefore was studied separately. In the beginning, it was discovered that a sample of glycated β-casein after the addition of thioflavin T was turbid, and turbidity was increasing with time. This result was not expected, the analysis of dynamic light scattering also did not allow to establish definitely the causes of turbidity. As a result, samples with turbidity were studied by fluorescence microscopy. Specific helical aggregates were found, causing fluorescence of the thioflavin T dye. Based on their ordered structure, it can be assumed that these aggregates are not the product of random assembly, but of organized misfolding. In this case, by varying the protein and the glycating agent, it was shown that such aggregates are formed only from β-casein, glycated by glucose, with thioflavin T: congo red colorant did'tnot give them, detected structures with it were unorganized and unspun; isolated thioflavin T did not form anything like this. Glycation of β-casein by methylglyoxal led to the formation of spherical aggregates with high fluorescence intensity. Moreover, in the test with β-lactoglobulin, glycated with methyl glyoxal, straight rigid needle-like aggregates were observed, also painted with thioflavin T with small fluorescence nuclei at the edges, but no twisting of these "needles" into the helix was detected. This specificity can be explained by the formation of a special structure with the participation of glycated β-casein as the main framework element, and necessity ofinteraction between glycation products, obtained

by glucose modification, and thioflavin T, may be due to stacking interactions between cyclic structures in the molecules of the components. The presence of β-casein in the samples was confirmed by immunochemical staining.

Spiral aggregates are able to form without long-time incubation, although they take some time to form — they are not immediately detected after the dye addition, but after 5 minutes of storage at room temperature, they are detected in a noticeable amount, the longer storage time, the number of aggregates is increasing. If the sample was stored for 24 hours, the spiral aggregates accumulated that they formed a characteristic sediment, since during further storage the aggregates stuck together into large particles, visible in solution with the naked eye. Such sticking, however, deformed the structure of the spirals.

Thioflavin T in the composition of spiral aggregates demonstrates an increased brightness of fluorescence, but not so high as in classical amyloid structures. According to the results of microscopy, spirals can be both right-and left-handed, which suggests a certain symmetry in the main assembly element. At the same time, the structures never showed a change in the direction of rotation in an already assembled spiral, i.e. the structure of the aggregate is set by the initial seed and preserved as the aggregate grows, and the subsequent "links" fit into it according to the structure of the protein molecule that first folded incorrectly and created the "seed".

Based on the above observations, there were suspicions about the reliability of the results of fluorescent analysis of protein preparations with thioflavin T as a whole, since the dye itself significantly influenced the result of the experiment. Based on the fluorescence of thioflavin T in the composition of specific aggregates formed by several glycated proteins, it can be assumed that the interaction of thioflavin T with glycation products leads to an increase in its fluorescence intensity, in which case thioflavin T can be used as a variant for the detection of glycation products.

Coaggregation of prion protein and glycated β-casein

After confirmation of changes in properties of β-casein after glycation, the changes of its interaction with the prion protein were studied. Already in the process of samples preparation, it was noticed that the solution became turbid immediately after addition of both participants of the process to the sample, although isolated proteins, even under exactly the same conditions, remained stable. On fluorescence microscopy, very large helic-like aggregates were found together with classical huge dense amorphous aggregates, i.e. the presence of prion protein contributed to the assembly of β-casein-specific structures. The size of these spiral structures exceeded that one of aggregates with glycated β-casein only on approximately 2 times. Apparently, the prion protein activates the aggregation of glycated β-casein, without changing the path of this aggregation, so it is likely works as an assistant in the assembly of ordered structures. Dynamic light scattering assay gave significant results: the interaction of prion protein and glycated β-casein quickly formed a very homogeneous major fraction of microparticles with a hydrodynamic diameter of the last ~ 250 nm. Being heated, the resulting suspension includes two fractions: particles with a characteristic aggregated prion protein with a diameter of 90 nm and large spiral-specific aggregates for glycated β-casein with a length of 50–200 μm and a diameter of 2–3 μm. In addition, electron microscopy showed that sticky spherical aggregates of small diameter are formed in a mixture of prion protein and glycated β-casein. In other papers (El Moustaine, Perrier, Smeller, Lange, & Torrent, 2008), these aggregates were characterized as 20-nm spherical particles enriched with β-structures, but not amyloid. Similar in shape and size of the particles were noticed by other researchers, who indicated that such particles are formed during the amyloid conversion of the N-terminal unstructured PrP domain. Amorphous aggregates during thermal incubation under these conditions became very dense, in some cases the core region did not even penetrate thioflavin T, but a certain fibrousness was seen in their peripheral region (Fig. 63B).

Thus, the prion protein was shown to provoke the formation by β-casein not only amorphous, but also helical structures. In addition, it was shown that the level of thioflavin T fluorescence in samples containing both proteins is even higher than in a sample with an isolated prion protein in the same concentration. This is probably the result of the summation of the fluorescence of thioflavin T, bound to the amyloid structures of the prion protein and the part of the dye whose fluorescence is caused by the interaction with β-casein glycation products.

To study the effect of prion protein on the aggregation of glycated β-casein additionally, an attempt to study the formation of both fractions of specific aggregates was made.

So, microscopy of samples in which glycated β-casein and prion protein were mixed immediately prior to analysis, fibrillar structures were observed, subsequently sticking together into amorphous aggregates. It is possible that these structures are precursors of giant amorphous conglomerates found in samples with a mixture of proteins.

The collection of a large number of prion protein and glycated β-casein coaggregation samples made possible to make an assumption about the mechanism of assembly of spiral structures. This process is clearly slower than the formation of giant conglomerates. Apparently, the assembly begins with a certain nucleus, from which the threads that contain protein and fluorescent thioflavin T are already beginning to grow. These fibrils are already beginning to interact with each other, gradually folding into 1-2 thicker threads, which, in turn, twist around some axis. Probably at this stage the direction of rotation of the future helix is determined. Over time, the entire star-like aggregate begins to deform and curl. At the same time, it seems that the twisting axes can be several, and they are independent of each other, therefore branchy aggregates can be formed, each branch of which is a spiral structure. The core, which at one time served as the basis for assembling the threads, is also distributed among

the ready-made structures in the process of "spiralization", therefore the amorphous core does not remain in the branchy aggregates.

Effect of anti-amyloid ligands on co-aggregation of prion protein and glycated β-casein

In the presence of curcumin and 3,4-dimethoxycinnamic acid, amorphous aggregates became looser, and small spherical particles began to be released from the total mass. Large aggregates became similar to those observed in coaggregation samples of prion protein and β-lactoglobulin. The number of amyloid structures inside these spherical aggregates grows during heat treatment, but not as much as in large homogeneous conglomerates. According to greater efficiency of thioflavin T fluorescence suppression, curcumin can enter inside these aggregates more easily, and therefore inhibits their amyloidization more actively than 3,4-dimethoxycinnamic acid. At the same time, 3,4-DMCA better promotes the loosening of aggregates and suppresses the formation of really large particles.

Spiral aggregates in the presence of antiamyloid ligands are deformed: they become much shorter, distort, their ordered structure is disturbed, and thioflavin T fluorescence intensity in their composition suppressed. 3,4-DMCA in their relation is more effective, since in its presence the spiral-shaped aggregates are absent altogether, and in the presence of curcumin they, although in a smaller number, are deformed, but are present.

The effect of glycation on the properties of prion protein

Aggregation of glycated prion protein

Similarly with β-casein, it was decided to study the effect of glycation on the aggregation of the prion protein. Isolated glycated prion protein was shown to be subject to amyloid conversion to a lesser extent than unmodified. The aggregates formed by them are less dense, the level of non-aggregated protein is higher. Observations are consistent with experimental data obtained by other researchers.

At the same time, glycation did not prevent protein aggregation in principle. On the contrary, it facilitated non-specific amorphous aggregation. Unspecific shape aggregates were observed in these experiments, but the size of the shapeless aggregates exceeded the size of the particles formed by the unmodified prion protein. Amyloidization of the protein in these structures was significantly less active than pure recombinant PrP^C. This assumption is confirmed by fluorescence microscopy and fluorescence measurement with thioflavin T too. At the same time, when measuring the absorption spectra of samples to estimate the turbidity of samples at 320 and 600 nm, a slight increase in optical density was observed in the long-wavelength region of the visible spectrum. The reasons for this phenomenon have not yet been clarified.

Coaggregation of glycated prion protein and β-casein before and after glycation

The interaction of glycated prion protein with other polypeptides has also changed in comparison with the native protein. Addition of other components, even native β-casein, for which the ability to suppress thermoaggregation of unmodified prion protein has already been shown, into a solution with glycated prion protein led to the rapid formation of a stable fraction of aggregates with a hydrodynamic diameter of 800 nm, which did not precipitate during ultracentrifugation and remained after thermoincubation. Apparently, these same aggregates could be detected by fluorescence microscopy (Fig. 98A, B), moreover, the fluorescence of thioflavin T in these aggregates increased significantly after thermal incubation, which may be evidence in favor of amyloidization of the protein inside spherical particles. In addition, samples of a mixture of β-casein and glycated prion protein had an atypically high absorption in the long-wavelength region of the visible spectrum (above 600 nm). To a much lesser extent, this was typical of isolated glycated prion protein samples. This feature was not peculiar to similar aggregates formed by prion protein and β-casein, if both proteins were subjected to glycation. Instead, the latter combination of

proteins, among other things, also formed large amorphous aggregates with high hydrophobicity, with properties very similar to amorphous aggregates resulting from the interaction of the unmodified prion protein with glycated β-casein. Probably, in the process of amorphous aggregation, it is glycated β-casein that has the greatest effect. At the same time, the formation of spiral structures was not noticed: even in a mixture of both glycated proteins, the maximum that could be detected was deformed clumps of stuck short needle-like aggregates. Thus, after glycation, the prion protein no longer contributes to the specific aggregation of β-casein in the presence of thioflavin T, although, judging by the presence of needle-like formations, it does not completely prevent this interaction.

Table. 4. Comparison the results of β-casein and prion protein interaction depending on their glycation.

	No compopents	**PrP unmodified**	**PrP glycated without reducing agent**	**PrP glycated with $NaBH_3CN$**
No components	-	Aggregation, amyloid structures are presented	Aggregates are loosened, the number of amyloid structures is reduced	Aggregates are loosened, the number of amyloid structures is reduced
B-casein	Absence of aggregation	Significant suppression of aggregation and amyloidization	The aggregates are less dense, consist of small spherical particles, amyloid structures are presented	The aggregates are less dense, consist of small spherical particles, amyloid structures are presented
B-casein glycated	Aggregation itself is insignificant. In the presence of thioflavin T, helical aggregates are present.	Huge very dense aggregates, amyloid structures are present. The number of spiral units increased.	Huge very dense aggregates, the number of amyloid structures is reduced. There are lumps of stuck needle aggregates	Huge very dense aggregates, the number of amyloid structures is reduced. There are lumps of stuck needle aggregates

Thus, the glycation of the prion protein made it difficult for it to form amyloid structures, while at the same time facilitating non-specific protein aggregation, which became more difficult to influence. The presence of β-casein, even unmodified, against the aggregation of this protein is also ineffective.

Effect of anti-amyloid ligands on co-aggregation of prion protein and food proteins

The effect of anti-amyloid ligands on the co-aggregation of unmodified proteins

Experiments with co-aggregation of prion protein with β- and κ-caseins, β-lactoglobulin and alpha-lactalbumin in the presence of curcumin have shown that this anti-amyloid ligand is able to suppress aggregation of all with rather broad specificity, suppresses the amyloidization of many proteins. Curcumin is able to reduce the number of amyloid structures inside large aggregates, however, aggregation is not very effective in suppressing aggregation, possibly due to the fact that the ligand molecule is rather hydrophobic and cannot "loosen" such zones in aggregates, especially if they are of a more disordered nature.

3,4-DMCA demostrated greater efficiency in suppressing the amyloidization of the prion protein, the presence of other amyloidogenic proteins reduced its effect. At the same time, it is more effective than curcumin suppressed protein aggregation, but worse destroyed dense mature aggregates containing many amyloid structures, probably due to higher hydrophilicity and inability to enter into such dense aggregates. New potential ligands, pentamidine and resveratrol, showed no ability to inhibit protein aggregation. In the case of pentamidine, it was not possible to reveal any general pattern of the effect of this ligand on aggregation. Being coaggregation of prion protein with caseins, the protein aggregates in the presence of pentamidine became smaller, but denser than without ligand, while the aggregates of the remaining proteins in its presence became, on the contrary, looser. At the same time, the fluorescence of thioflavin T in them did not significantly decrease. In the presence of resveratrol, a rather interesting picture was observed: almost all the studied combinations of proteins formed aggregates of similar morphology, size, stability in the presence of detergents and thioflavin T fluorescence, regardless of the protein entering into co-aggregation

with PrP. That is, for example, in a sample of PrP with bovine serum albumin, amyloidization and aggregation were suppressed, and in the sample of prion protein with β-casein, on the contrary, they increased. Apparently, resveratrol directs the entire aggregation along a single path, possibly playing the role of a "mediator" between PrP and other proteins, thus eliminating the mutual influence on the aggregation mechanism.

The effect of protein glycation on the effectiveness of anti-amyloid ligands

The interaction of curcumin and 3,4-DMCA with glycated β-casein revealed a weak antiaggregation effect: antiamyloid ligands showed the ability to suppress the formation of ordered helical aggregates with slightly greater efficiency for 3,4-DMCA. Those aggregates that could still form were strongly deformed (in the case of 3,4-DMCA, they were practically absent), and the fluorescence of thioflavin T in them was reduced. The slightly more pronounced effect of DMCA may be due to the fact that β-casein with thioflavin T formed rather loose aggregates, therefore before the ligand there were fewer physical obstacles to reach the binding site. In addition, judging by the effect seen, it can be assumed that the glycation of β-casein did not block the binding of the protein to the ligands.

Due to changes in the characteristics of prion protein aggregation after glycation (amyloidization is less active, nonspecific aggregation is facilitated), the effectiveness of low molecular weight aggregation inhibitors has changed. The overall efficiency of the ligands has greatly decreased. The fact that the glycation of the prion protein contributes to a change in the priority pathway for the aggregation of the prion protein to a nonspecific one is an additional argument in favor of greater specificity of 3,4-DMCA, rather than curcumin, specifically to the unmodified prion protein - its glycation led to a significant decrease in specificity, and and ligand efficiency. At the same time, cinnamic acid retained more than curcumin, the effectiveness in suppressing protein aggregation - it was shown that 3,4-dimethoxycinnamic acid

suppresses protein aggregation better, up to complete disassembly of part of the aggregates to monomer. Curcumin is more effective in suppressing mature amyloid aggregation, therefore it is better to suppress aggregation in heat-treated samples. This is probably due to the greater hydrophilicity of cinnamic acid, which, on the one hand, increases its ability to suppress the formation of aggregates, but does not allow, in contrast to curcumin, to penetrate into already formed amyloid structures enriched in hydrophobic regions. The glycation of the prion protein in the presence of a reducing agent led to the fact that the efficiency of both ligands in suppressing even the aggregation of such a protein tends to zero.

Table. 5Effect of curcumin on amyloidization and aggregation of glycated prion protein and β-casein.

	No compopents	**PrP unmodified**	**PrP glycated without reducing agent**	**PrP glycated with $NaBH_3CN$**
No components	-	Aggregates are loosened, the number of amyloid structures is reduced	Aggregates are loosened, the number of amyloid structures is reduced	Aggregates are loosened, the number of amyloid structures is reduced
B-casein	Absence of aggregation	Significant suppression of aggregation and amyloidization	The aggregates are less dense, consist of small spherical particles, amyloid structures are present	The aggregates are less dense, consist of small spherical particles, amyloid structures are present
B-casein glycated	Aggregation itself is negligible. In the presence of thioflavin T, helical aggregates are present.	Large aggregates loosened. The number of amyloid structures has not changed. Spiral aggregates are very small and deformed.	Huge dense units. Activation of amyloidization	Huge dense units. Activation of amyloidization

The presence of unmodified β-casein did not lead to an increase in the effectiveness of anti-amyloid ligands in relation to the aggregation of glycated PrP. Both ligands were able to suppress the adhesion of small spherical protein aggregates, leading to loosening of the formed conglomerates, but they proved to be useless against amyloidization of the protein inside these aggregates. The number of detected amyloid structures did not significantly decrease, a large number of spherical particles with

diameters of about 700–800 nm were found in the preparations, and those painted with thioflavin T. However, it cannot be said that even large aggregates were very dense — thioflavin T freely penetrated and stained internal areas of aggregates, which caused a bright fluorescence of the dye in preparations after thermoaggregation. 3,4-DMCA was able to somewhat suppress aggregation, but it was also not available to suppress amyloidization.

It is possible that in this case the interactions of amino acids inherent in non-specific aggregation of proteins prevent the penetration of anti-amyloid ligands inside, without interfering with their internal structures to undergo amyloid conversion, which leads to the almost complete disappearance of the effect of curcumin and 3,4-dimethoxycinnamic acid.

Table. 6. Effect of 3,4-dimethoxycinnamic acid on amyloidization and aggregation of glycated prion protein and β-casein.

	No compopents	**PrP unmodified**	**PrP glycated without reducing agent**	**PrP glycated with $NaBH_3CN$**
No components	-	Aggregates are loosened, the number of amyloid structures is reduced	Aggregates are loosened, the number of amyloid structures is reduced	Aggregates are loosened, the number of amyloid structures is reduced
B-casein	Absence of aggregation	Almost full suppression of amyloidisatin and aggregation	Aggregates are less dense, consist of small spherical particles, the number of amyloid structures is reduced	Aggregates are less dense, consist of small spherical particles, the number of amyloid structures is reduced
B-casein glycated	Aggregation itself is negligible. In the presence of thioflavin T, helical aggregates are almost deformed	Large aggregates loosened. The number of amyloid structures has not changed. Spiral aggregates are very small and deformed.	Huge very dense aggregates, the number of amyloid structures is reduced. There are lumps of stuck needle aggregates	Huge very dense aggregates, the number of amyloid structures is reduced. There are lumps of stuck needle aggregates

Both antiamyloid ligands somewhat suppress aggregation of glycated prion protein and β-casein, destroying large aggregates, but they have almost no effect on small particles,

amyloidization in their presence, on the contrary, is only aggravated. The fluorescence of thioflavin T, both in samples as a whole and in individual aggregates caught in the lens of a fluorescent microscope, grew noticeably in the presence of ligands. In particular, without ligands, proteins form aggregates similar to short, tangled filaments, and in the presence of curcumin and 3,4-DMCA, small spherical aggregates are formed, which tend to stick together and have bright fluorescence.

Probably, the glycation of proteins led to the fact that the binding sites of proteins and ligands were no longer available, and the residual interactions between proteins and ligands led only to a spatial convergence of protein molecules, which only activated aggregation.

Conclusions

1. **3,4-dimethoxycinnamic acid, as well as native β-casein, were established to partially suppress amyloid transformation and non-specific aggregation of prion protein.**

2. **The combined effect of native β-casein and curcumin or 3,4-dimethoxycinnamic acid can be used to almost completely suppress aggregation and amyloid transformation of the prion protein.**

3. **β-casein glycation was shown to reduce the content of disordered structures in it and leads to the disappearance of its chaperone-like activity with respect to the prion protein.**

4. **The formation specific helicoidal fluorescent structures by glycated β-casein in the presence of thioflavin T was been found, while glycated β-casein is also involved in almost instantaneous aggregation with the native prion protein, forming fluorescent star structures in the presence of thioflavin T.**

5. **The detected increase in the fluorescence of thioflavin T, when it interacts with glycated prion and β-casein, which do not contain β-amyloid structures, as well as the appearance of unusual spiral structures from these proteins in the presence of thioflavin T shows that using this dye to identify amyloid structures can give false positive results in the presence of glycated proteins.**

6. **Prion protein glycation was established to greatly reduce the effectivity of suppressing its amyloid conversion and aggregation by curcumin and 3,4-dimethoxycinnamic acid.**

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Titre : Recherche de facteurs affectant la transformation amyloïde et l'agrégation des protéines.

Mots clés : amyloïde, protéine prion, bêta-caséine, glycation, thioflavine T, agrégation

Résumé : L'objectif de ce travail était l'identification de nouveaux facteurs influençant la capacité des protéines à s'agréger ou ayant un impact sur l'agrégation d'autres protéines. Nous avons étudié l'influence des ligands anti-amyloïdes, interactions protéine-protéine et la glycation sur l'agrégation des protéine prion. La protéine prion a activée l'agrégation amyloïde dans d'autres protéines habituellement stables. La glycation de la protéine prion entraîne une diminution de son amyloïdogénicité. En même temps, cette modification facilite légèrement l'agrégation amorphe de la protéine prion. Dans ce cas, l'effet antiagrégant total de la curcumine et de l'acide 3,4-diméthoxycinnamique a été affaibli, car après la modification, l'agrégation s'est faite davantage par la moyen non spécifique.

La présence de bêta-caséine avec une activité semblable à celle du chaperon supprime significative tout type d'agrégation des protéines prions, et combinaison de bêta-caséine et de ligands anti-amyloïdes est une méthode efficace de suppression complète de l'amylose de la protéine prion. La bêta-caséine glyquée a montré sa capacité à s'agréger par elle-même et à former des agrégats ordonnés fluorescent spécifiques avec un colorant thioflavine T. La présence de protéine prion a grandement facilité la formation de ces agrégats, au contraire les ligands anti-amyloïdes l'ont supprimé. L'interaction de la protéine prion glyquée et de la bêta-caséine conduit à la formation des agrégats sphériques, que sont enrichis en structures amyloïdes pendant le traitement thermique. Dans ce cas, l'effet antiamyloïde de la curcumine et de l'acide 3,4-diméthoxycinamique disparaît presque complètement.

Title : Research of factors affecting amyloid transformation and protein aggregation

Keywords : amyloid, prion protein, beta-casein, glycation, thioflavin T, agregation

Abstract : The main aim of this work was a search of new factors influencing the ability of proteins for aggregation themselves or impact the aggregation of other proteins. We investigated the influence of anti-amyloid ligands, protein-protein interactions and glycation on prion protein aggregation. The prion protein activated amyloid aggregation in other usually stable proteins. The glycation of the prion protein causes a decrease in its amyloidogenicity. At the same time, this modification slightly facilitates the amorphous aggregation of the prion protein. Also, the total anti-aggregating effect of curcumin and 3,4-dimethoxycinnamic acid in this case was weakened, since after the modification the aggregation was done more by non-specific means.

The presence of beta-casein with chaperone-like activity significantly suppresses all types of prion protein aggregation, and a combination of beta-casein and anti-amyloid ligands was decided to be an effective method of total prion protein amyloidosis suppression. The glycated beta-casein showed its ability to aggregate by itself and to form specific fluorescent ordered aggregates with a thioflavin T dye. The presence of prion protein greatly facilitated the formation of these aggregates, unlike the anti-ligands, which inhibits it. The interaction of glycated prion protein and beta-casein leads to the formation of spherical aggregates, which are being enriched by amyloid structures during heat treatment. In this case, the antiamyloid effect of curcumin and 3,4-dimethoxycinamic acid disappears almost completely.