UNIVERSITÉ DE NANTES
FACULTÉ DES SCIENCES ET DES TECHNIQUES

ÉCOLE DOCTORALE VENAM

Année 2010

N° attribué par la bibliothèque

Application & comparison of commercial pectins and Iranian keylime pectin stabilizers in "doogh" (drinkable yogurt) making and the study of intra & intermolecular properties of the best pectin type stabilizer

THÈSE DE DOCTORAT

Discipline : Science des Aliments
Spécialité : Biotechnologie

Présentée
et soutenue publiquement par

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Le 7 Decembre 2010, devant le jury ci-dessous

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ABBREVIATIONS LIST

A, arabinan; **AEC**, anion exchange chromatography; **ADD**, acidified dairy drink; **AFM**, atomic force microscopy; **AG-I**, arabinogalactanI; **AG-II** arabinogalactan II; **Ara**, arabinofuranose; **B-series**, base treated HG; **BP-series**, p-PME treated B-series; **Ca^{2+}**, calcium ion; **CSP**, Ca^{2+} sensitive pectin; **DA**, degree of acetylation; **DB**; degree of blockiness; **DB_{abs}**, absolute degree of blockiness; **DM**, degree of methylation; **DMI**, Dairy Management Inc^{TM}; **(DMi-DMf)/DMi**, (initial degree of methylation-final degree of methylation)/initial degree of methylation; **dp**, degree of polymerization; **EEA**, European economic area; **endo-PG**, endo-polygalacturonase; **ESI-IT-MS**, ElectroSprayIonisation Ion-Trapp Mass Spectrometry; **exo-PG**, exo-polygalacturonas; **EU**, European Union; **FAO**, food and agriculture organization of the United Nation; **f-PME**, fungal pectin methylesterase; **FRP**, factor de reponse parameter; **Gal**, Galactopyranose; **GalA,** Galacturonic acid; **GLC**, gass/liquid chromatography; **h**, hour; **HCSP**, high- Ca^{2+} sensitive pectin (p#4); **HG**, homogalacturonan; **HIC**, Hydrophobic Interaction Chromatography; **HM**, high methoxyl; **HPAEC**, performance anion exchange chromatography; **HPLC**, high pressure liquid chromatography; **HP-SEC**, high-performance size-exclusion chromatography; **INS**, international numbering system; **ISIRI**, institute of standards and industrial research of Iran; **kDa**, kilo Dalton; **LM**, low methoxyl; **M**, molar; **MALDI-TOF-MS**, matrix-assisted laser adsorption/ionization time of flight mass spectrometry; **MCSP**, medium- Ca^{2+} sensitive pectin (p#3); **Mg^{2+}**, magnesium ion; **MHR**, modified hairy region; **mM**, millimolar; **MS**, mass spectrometry; **MSNF**, milk solid non-fat; **MT**, metric ton; **M_w**, molecular weight; **NCSP**, non-Ca^{2+} sensitive pectin (p#2); **nm**, nanometer; **NMR**, nuclear magnetic resonance; **PG**, polygalacturonase; **PL**, pectin lyase; **PME**, pectin methylesterase; **p-PME**, plant-pectin methylesterase; **P-series**, p-PME treated HG; **RG**, rhamnogalacturonan; **RG-I**, rhamnogalacturonan-I; **RG-II**, rhamnogalacturonan-II; **Rha**, rhamnose; **SEC**, size exclusion chromatography; **SDS**, sodium dodecyl sulfate; **USDA**, United State department of agriculture; **v/v**, volume/volume; **w/v**, weight/volume; **w/w**, weight/weight; **XGA**, Xylogalacturonan; **Xyl**, xylopyranose; **µm**, micrometer; **κ-casein**, kappa casein; **α-casein**, alpha casein; ***β*- casein**, beta casein; **γCa^{2+}**, calcium reactivity; **$\gamma_{exp.}$ /$\gamma_{theo.}$**, experimental calcium reactivity/theoretical calcium reactivity.

INTRODUCTION

Pectin is a heterogeneous branched polysaccharide, which is mainly extracted from citrus peels (lime and lemon) and apple fruits (Albersheim *et al.,* 1996). 80-90% of the citrus dry weight is galacturonic acid (GalA). The majority of GalA is found in homogalacturonan (HG) region, an unbranched polymer of methylated and/or non-methylated α-(1-4) linked GalA residues (Ridley *et al.*, 2001). Functional property of pectin is related to the degree of methylation (DM) of GalAs and their distribution pattern within HG regions (Willats *et al*., 2001). High methoxyl pectin is widely used in acidified dairy drink (ADD) as a stabilizer ingredient to avoid flocculation of casein in acidic condition and subsequent macroscopic whey separation. The stabilization mechanism is proposed to adsorb pectin onto the micellar surface of casein by blocks of non-methylated GalAs along the HG while the uncharged parts extend to the solution to cause repulsive interaction between pectin-covered micelles (Laurent and Boulenguer, 2003). The blockwise distribution of methyl esters depends on the demethylation mechanism either by enzyme or alkaline/acidic process. Chemical or acidic fungal pectin methylesterase (PME) result in random distribution of carboxylic acid groups while alkaline PMEs from higher plants lead to a blockwise distribution (Thibault and Rinaudo, 1986; Grasdalen *et al*., 1996; Limberg *et al*., 2000a, 2000b; Ralet and Thibault, 2002; Verlent *et al*., 2004). The length of the demethylated stretches has been studied by limited *endo*-PG, *exo*-PG and pectin lyase digestion and subsequent analysis of the degradation products with high performance anion exchange chromatography (HPAEC), mass spectrometry (MS), capillary electrophoresis and mathematical modeling by different authors (Dass *et al*., 1998, 1999; Guillotin *et al*., 2005; Williams *et al*., 2010). It has been said by Glahn and Rolin (1996) that a calcium sensitive HM pectin (pectin that forms gel particles when calcium is added, which implies a high blockwise pattern) is capable of stabilizing ADDs while non-calcium sensitive pectin with the same DM cannot. However, Laurent and Boulenguer (2003) showed that non-calcium sensitive pectin can be quite good for this application, especially when milk solid non-fat (MSNF) is not too high. Thus, great efforts have been made over the past ten years for development of methods for isolation of pectin degrading enzymes with specific mode of action, introduction of modeling

tools/substrates better suited to find out the length of demethylated stretches and to describe different aspects over stabilization process by pectin.

In the present work, we tried to use pectin as stabilizer and texture enhancer in the Iranian acidified dairy drink (ADD) called "Doogh" (consumption ≥ 1500 000 tons/year), which is prone to protein aggregation and whey separation during time. In this regard, some industrial citrus pectins (with different calcium sensitivity), used in this type of products, have been characterized. Pectin was extracted from the peels of "*Citrus aurantifolia*" under different conditions, seeking for characteristics close to the industrial ones. The different pectin types were applied in "Doogh" and compared for their stabilization properties. In order to find out more information about the role of demethylated stretches and calcium sensitivity on stabilizing process in this kind of products, several homogalacturonans with different molecular structure have been produced and characterized. Various experimental approaches have been carried out that can be grouped into 4 categories as:

- Preparation of model homogalacturonans by alkali or/and plant-*PME*
- Isolation and characterization of pectin lyase
- Application/characterization of *endo*-PG and pectin lyase on the model homogalacturonans
- Pectin extraction from lime peels, characterization/comparison of extracted pectins and industrial pectins used in ADD, application of pectins in ADD and comparison of textural properties of the final products.

This manuscript consists of different chapters as follows:

- Bibliography
- Methods, results and discussion
- General discussion, conclusion and perspectives

The first chapter consists of the reports of previous investigations about pectin (composition, extraction, and properties) and ADD (general properties, challenges). The second chapter includes the description of the methods used and the presentation and discussion of the results obtained, being categorized in 4 chapters (Isolation and characterization of model substrates (HG), Purification and mode of action of pectin lyase, Appraisal of degree of blockiness of model HG using *endo*-PG

& pectin lyase (PL) and characterization of commercial pectins and extracted pectin from lime peels and application in ADD. The last chapter gives an overall overview of the thesis and suggestions/perspectives explored out of the results.

In order to present this work, 3 articles and 1 poster have been offered as follows:

A. T. Nasseri, J.-F. Thibault & M.-C. Ralet. 2008. Citrus pectin: structure and application in acidified dairy drink. *In*: Teixeira da Silva, J. A., Kagawa University, Japan. *Global Science Book*, *Tree and Forestry Science and Biotechnology, Citrus I,* 2, 60-70.

M. A. K. Williams, A. Cucheval, A. T. Nasseri & M.-C. Ralet. 2010. Extracting *intra*molecular sequence information from *inter*molecular distributions: highly non-random methylester substitution patterns in homogalacturonans generated by pectinmethylesterase. *Biomacromolecules*, 11 (6), 1667-1675.

A. T. Nasseri, M.-J. Crépeau, J.–F. Thibault, N. Moazami & M.-C. Ralet. Commercial pectin and extracted pectin of *Citrus aurantifolia* in Iranian acidified dairy drink. The manuscript is in preparation for *Food Hydrocolloids.*

A. T. Nasseri, M.-C. Ralet, M.-J. Crépeau, E. Bonnin, J. Vigouroux, N. Moazami & J.-F. Thibault. 2009. Application and comparison of commercial pectins and *Citrus aurantifolia* extracted pectin as stabilizer in Iranian drinkable yogurt "Doogh" and study of intra and inter molecular properties of best pectin type stabilizers. *Total Food, sustainability of the agri-food chain.* Norwich Bioscineces Conference Center, Norwich, Norwich, UK, 22-24 April.

BIBLIOGRAPHY

1. Pectin localization and chemistry

Pectin was discovered in the 19th century by a French scientist named Braconnot (Braconnot, 1825a; Braconnot, 1825b). He found this "acid" in many plants so he continued his investigation on this molecule and its gelling properties. He named this molecule "pectic acid" from "pektos", which means coagulum in Greek. Pectin is found in almost all higher plants, namely in the primary cell walls and in the middle lamellae where it represents around 40% by weight on a dry matter basis (Brett and Waldron, 1996). In citrus fruits, it can be found in membranes, juice vesicules and core in different quantities depending on the fruit variety and maturity stage (May, 1990). Pectin has a lubricating and cementing function in the cell walls (Sakai *et al.*, 1993).

1.1. Localization

Pectins are common to the cell walls of higher plants and contribute to many cell wall functions. Cell walls determine the size and shape of cells and, consequently, the integrity and rigidity of plant tissues (Schols and Voragen, 2002). In some species, the interface regions between cells, the middle lamella and the cell corners, are rich in relatively unesterified pectins, which may function in cell-cell adhesion and play an important structural role in tissue integrity (McCann and Roberts, 1996). In addition, pectins play role in ion transport and water retention. They determine the pore size of cell walls and they are involved in defense mechanisms against infections by plant pathogens, wounding, and stress. The specific functions of pectins are strongly influenced by the amount and nature of the pectic molecules present. The structure of pectins is further influenced by enzymatic and chemical modification reactions during the growth of plants, during the ripening and storage of fruits and as a result of the processing of fruits and vegetables. The structure of pectic molecules therefore depends on many parameters and is subjected to considerable change. In addition to all these functions in living tissues, pectins are also of commercial interest (Schols and Voragen, 2002).

1.2. Chemical structure

Pectin is a complex polysaccharide composed of a α-1,4-linked D-galacturonic acid (GalA) linked linear chain (so-called homogalacturonan or smooth region) with varying proportions of the acid groups esterified with methanol. They are interrupted by the insertion of α-(1,2)-linked L-rhamnosyl and α-1,4-linked D-galacturonosyl residues in adjacent or alternate positions with side chains of arabinans, arabinogalactans and galactans, which are linked to rhamnogalacturonan segments within the pectin molecule (branched rhamnogalacturonans or hairy regions). Other structural elements of pectin include xylogalacturonan and apiogalacturonan (Voragen *et al.,* 1995; Schols and Voragen, 2002) (Fig. 1). The proportion of 'smooth' to 'hairy' regions can vary greatly depending on the type of tissue or its developmental stage (Schols and Voragen, 2002).

1.2.1. Homogalacturonan

Homogalacturonan (HG) is the simplest and most abundant pectic structural domain. It consists of a linear backbone of (1→4)-linked α-D-GalA residues (Hotchkiss *et al*., 1996; Zhan *et al*., 1998). The possible presence of single rhamnose (Rha) residues within HG regions was convincingly argued against by Zhan *et al*. (1998). The minimum estimated length of this domain is ~ 100 GalA residues (Thibault *et al*., 1993). For citrus, chemical as well as enzymatic approaches led to the isolation of HG domains of narrow molar mass distribution with a degree of polymerization (dp) of 100-120 GalA residues (Thibault *et al.,* 1993; Hellín *et al*., 2005; Yapo *et al.,* 2007a). Hellín *et al*. (2005) claimed that acid hydrolysis can not underestimate this length since the average weight of HGs isolated by enzymatic degradation of RG-I were in good agreement with values observed for HGs isolated after mild acid hydrolysis of lime pectin. However, the recent estimation of GalA dp in HG region by Round *et al*. (2010) was ~320, a three or even four-fold increase on previous estimates. In this research, the sodium carbonate pectin extract from unripe tomato pericarp has been observed to consist of linear and branched homogalacturonans (HG) and aggregated complexes containing rhamnogalacturonan-I (RG-I), irreducible aggregates of HG and HG polysaccharide stretches. Significant amounts of individual HG polysaccharide stretches either not linked to the RG-I, or linked through bonds, are broken during the extraction from the cell wall. During acid hydrolysis, the neutral sugars and thus the RG-I polymers

were degraded into their constituent parts, whilst the individual HG domains, and a major fraction of the complexes, retain molecular weights similar to their initial values. After 72 h of hydrolysis only trace amounts of arabinose, galactose and rhamnose remain and the observable pectin structures consist of individual HG stretches and HG aggregates. The lack of change in size of individual polymers in the event of almost total loss of arabinose, galactose and rhamnose demonstrated that these polymers consist of HG only, and therefore measurements of the size of these polymers in the freshly extracted pectin prior to hydrolysis represent a new minimum size distribution for HG polymers.

GalA residues are commonly partly methyl-esterified at C-6 (Voragen *et al.,* 1995) and, in some plant species, partly acetyl-esterified at *O*-2 or *O*-3 (Ralet *et al.,* 2005, 2008a). Both the degree of methyl-esterification (DM) (i.e. the number of methyl-esterified GalA residues for 100 total GalA residues) and degree of acetylation (DA) (i.e. the number of acetyl-esterified GalA residues for 100 total GalA residues) have a profound impact on functional properties. Lime pectin is particularly rich in HG, which account for ~ 80-90% of raw pectin mass (Ralet and Thibault, 1994; Hellín *et al*., 2005; Yapo *et al*., 2007a). Native citrus pectin, and its constitutive HG domains, is highly methyl-esterified and lowly acetyl-esterified (Ralet and Thibault, 1994; Ros *et al.,* 1996, 1998). The distribution of methyl esters is very complex at an intramolecular level (within one molecule) and at an intermolecular level (over various pectin molecules within a mixture) (Daas *et al*., 1998, 1999). Within a single molecule, the distribution of ester groups may vary from a rather random distribution, in which all esters are 'spread out' over the polymer, to a more blockwise distribution in which long stretches of nonesterified galacturonic acid residues are interspersed with segments that are almost completely methyl-esterified (Voragen *et al.,* 1995; Daas *et al.*, 2001a,b). It has been found that the distribution of methyl esters over the HG backbone varies much more than it can be covered by terms such as regular, random or blockwise (Daas *et al.*, 2001b). The degree and distribution of esterification vary according to the plant species, age and location in the plant cell wall and is implicated in controlling functional properties of pectin (Round *et al*., 2010).

1.2.2. Xylogalacturonan

Xylogalacturonan (XGA) consists of a HG backbone with mostly single-unit substituents of β-D-xylopyranose (Xyl) linked at *O*-3 of GalA residues. In lemon albedo pectin, the presence of very limited amounts of XGA has been reported (Ros *et al.,* 1998).

1.2.3. Rhamnogalacturonan I backbone and neutral sugars side-chains

The rhamnogalacturonan I (RG-I) backbone consists of [→2)-α-L-Rha*p*-(1→4)-α- D-Gal*p*A-(1→] repeats (McNeill *et al*., 1984; Albersheim *et al*., 1996) covalently linked to HG domains (Round *et al*., 1997, 2001, 2010). RG-I domains isolated from several plant species, including citrus, were shown to be highly acetylated at *O*-2 and/or *O*-3 of GalA units (Komavilas and Mort, 1989; Schols and Voragen, 1994; Ros *et al.,* 1996; Ralet *et al.,* 2005, 2008a). So far, no evidence has been published that GalA units in RG-I domains are methyl-esterified. The Rha residues of RG-I backbone are substituted, mainly at *O*-4, with α-L-arabinofuranose (Ara)- and β-D-galactopyranose (Gal)-containing side-chains (Voragen *et al.,* 1995). Lemon pectins were shown to contain arabinan structures with a central core of (1→5)-linked Ara*f* residues carrying essentially single Ara*f* substituents at C-3 (Ralet and Thibault 1994; Ros *et al.,* 1996). Both types of galactans: (1→4)-linked type I (arabino)-galactans and (1→3) and (1→6)-linked type II arabinogalactans were also detected (Ralet and Thibault, 1994; Ros *et al.,* 1996) as well as single D-Gal*p*-(1→4)] substitutions (Ros *et al.,* 1996, 1998). Rhamnogalacturonans I was recently recovered from citrus peels pectins after extensive degradation by homogalacturonan-degrading enzymes (Yapo *et al.,* 2007a). Further use of side-chains-degrading enzymes allowed the recovery of a high molar mass RG-I backbone (Yapo *et al.,* 2007a).

1.2.4. Rhamnogalacturonan II

Rhamnogalacturonan II (RG-II) is a highly conserved structure in the plant kingdom. It is a low molar mass (5-10 kDa) highly complex macromolecule with a short HG-like backbone substituted by four different side-chains encompassing several unusual sugar residues (Schols and Voragen, 2002; Matsunaga *et al*., 2004). It is composed of 12 different monomers, including rare glycosyl residues (Ridley *et al*., 2001). This structural element, although present in very limited amounts, plays a key role as it is strongly believed to be involved in the cross-linking of two pectin molecules

within the cell wall through a borate di-ester (Ishii *et al*., 1999; Ishii and Matsunaga, 2001). RG-II has been also isolated from citrus peels pectin by homogalacturonan-degrading enzymes (Yapo *et al*., 2007a). The structure of RG-II can be identified by mild acidic conditions coupled to the analysis of the resulting fragments by mass spectrometry since enzymes which are able to efficiently cleave RG-II have not been identified to date (Séveno *et al*., 2009).

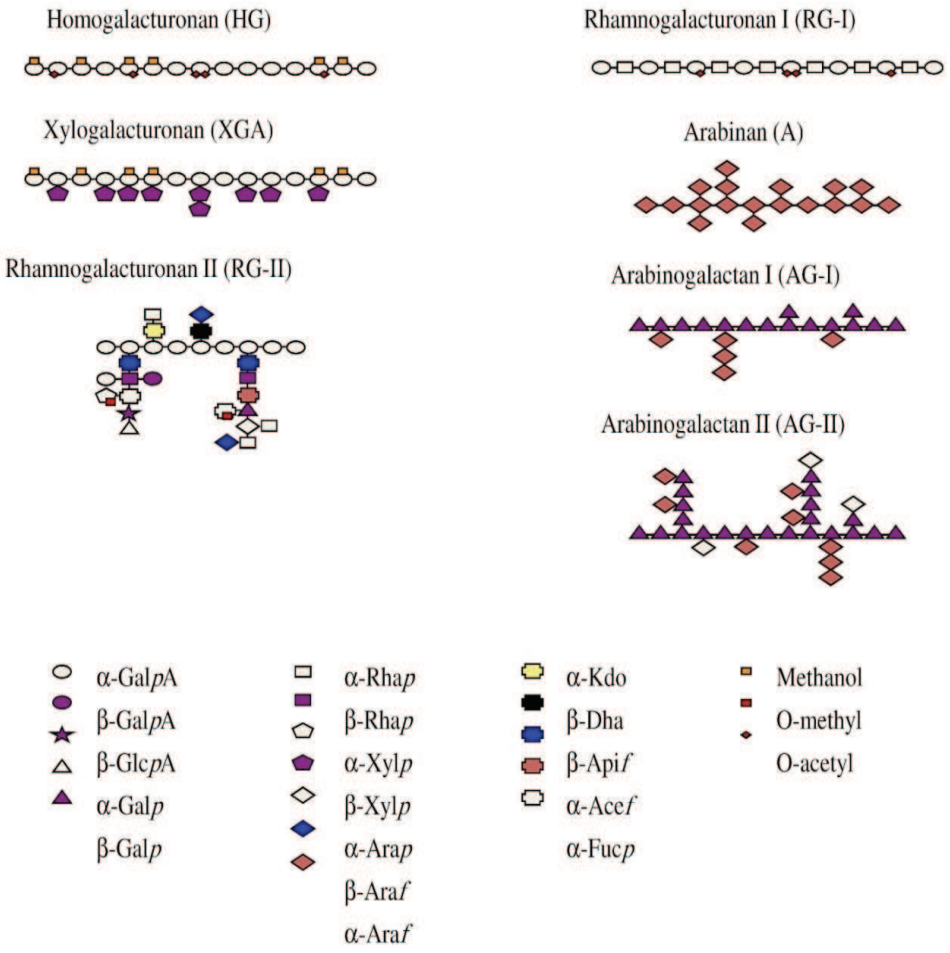


Fig. 1. Different structural elements present in pectins (Tanhatan Nasseri *et al*., 2008).

1.3. Macromolecular models

Although the structure of the different pectic domains is now quite well known, the way they are connected one with another to form a macromolecular structure is still a matter of debate. Two pectic models are considered nowadays: the "smooth and hairy regions" model (Schols and Voragen, 1996) (Fig. 2) and the "RG-I backbone" model (Vincken *et al*., 2003) (Fig. 3). In the first one, "smooth regions" (HG) alternate with "hairy regions" (XGA and RG-I encompassing neutral sugars side chains) to form the pectin molecule backbone, RG-II being an integral part of some HG domains. In the second one, HG, XGA, arabinan and galactan would occur as side-chains of RG-I backbone, forming a "*molecular brush*" (Vincken *et al*., 2003), RG-II being again an integral part of some HG domains.

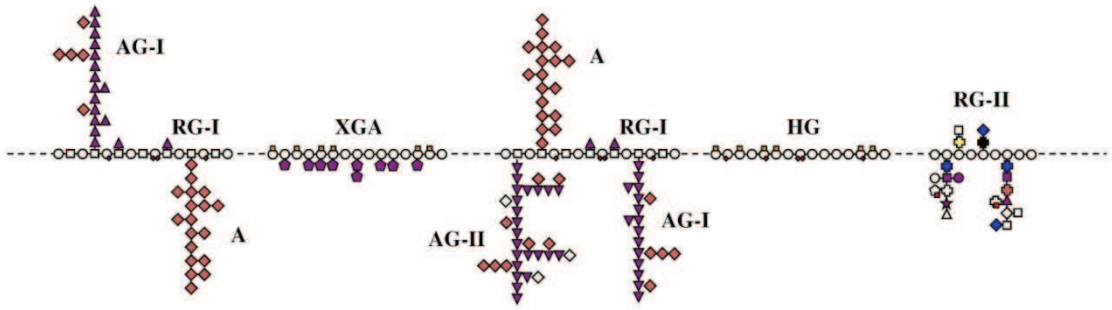


Fig. 2. Pectin schematic representation, "smooth and hairy regions" model (Schols and Voragen, 1996). The pectic backbone consists of alternating homogalacturonan (HG) (smooth regions), xylogalacturonan and rhamnogalacturonan-I (RG-I). Rhamnogalacturonan-II (RG-II) is considered as an integral part of HG domains. Arabinan (A) and arabinogalactan I and II (AG-I, AG-II) constitute the "hairy" part of the macromolecule (Tanhatan Nasseri *et al*., 2008).

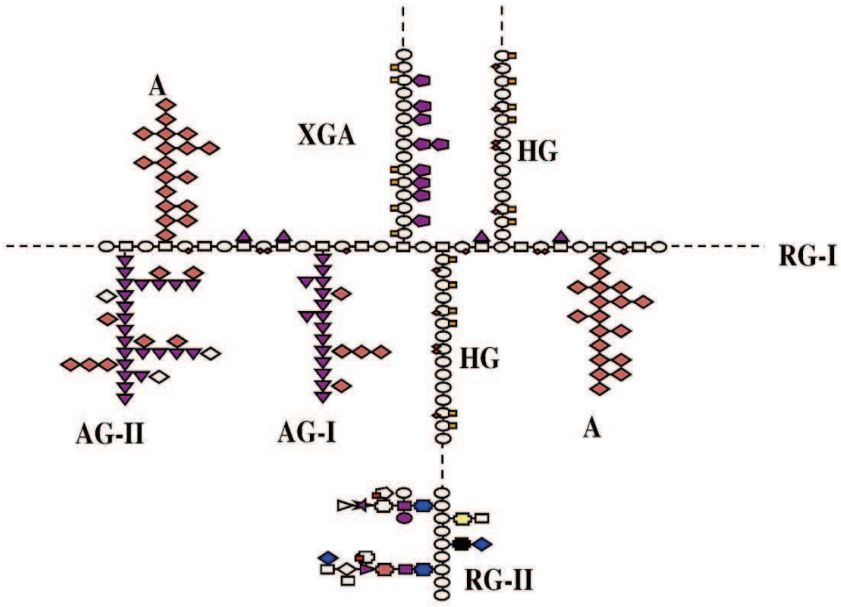


Fig. 3. Pectin schematic representation. In this "RG-I backbone" model (Vincken *et al.,* 2003). The RG-I backbone is decorated, not only with arabinan and arabinogalactan I and II side chains, but also with homogalacturonan (HG) and xylogalacturonan (XGA) domains. Rhamnogalacturonan-II (RG-II) is considered as an integral part of HG domains (Tanhatan Nasseri *et al*., 2008).

In order to get more insight in the inter linkage between the various structural elements; apple pectin modified hairy regions (MHR) were degraded by controlled acid hydrolysis by Coenen *et al*. (2007). From the degradation products, oligomeric fragments were selected which could represent interconnection points, and these oligomers were characterized. HPAEC at pH 5 in combination with MALDI-TOF-MS revealed that the position of the RGI block was at the reducing end of the oligomer and the HG/XGA block was at the non-reducing end. Blocks of RGI were α-(1 → 2) linked to blocks of HG/XGA, as indicated by enzymatic degradation with Exo-PG and NMR spectroscopy. These results partly correspond with the model of Voragen *et al*. (1996), where the backbone consists out of consecutive HG and RGI structural elements but the model where HG is positioned as a RGI side chain (Vincken *et al*., 2003) could not be excluded irrevocably since only some part of the connecting points present in the pectin were identified and not all unknown oligomers could be identified so far.

1.4. Chemical stability

Pectin is stable at pH 3-4, even at high temperatures. At lower pH and temperature, esters split and the neutral sugar side chains are hydrolyzed (Voragen *et al.,* 1995). Linkages between galacturonic acid residues are more stable than linkages with neutral sugars. Arabinosylfuranose linkages are particular sensitive to low pH. Ester linkages present in the pectin can be cleaved rather easily in alkaline conditions and low temperatures (0.05 M NaOH; 0-4°C; 2-6 h) without influencing the molecular weight significantly. Release of methyl esters at low temperatures already starts at pH 8 and will accelerate rapidly with increasing pH. No distinct difference has been reported about the stability of methyl esters and acetyl esters. In competition with the saponification reaction, a ß-eliminative depolymerisation reaction may occur, even at a pH slightly higher than 7 at room temperature. At elevated temperatures, this degradation takes even place under slightly acidic conditions (pH 5), resulting in a dramatic shift in the molecular weight. ß-elimination affects only a glycosidic linkage on the non-reducing side of a methyl-esterified galacturonic acid residue. This depolymerization reaction can easily be monitored spectrophotometrically at 235 nm, based on the formation of an unsaturated bond at the non-reducing end of the degradation products (Schols and Voragen, 2002).

2. Pectin extraction

Citrus peels and apple pomace can be used for pectin manufacturing. Apple pomace contains 15-20% pectin and citrus peels contain 30-35% pectin. Historically, the major part of the pectin industry developed from utilization of apples (Rolin, 2002; Mesbahi *et al.,* 2005), but nowadays, dried citrus peel is the largest source. Among the citrus, lime and lemon are preferred and orange and grapefruit are less often used. Indeed, lime and lemon yield pectin of higher specific viscosity and lower Ca^{2+} sensitivity than orange and grapefruit (Rolin, 2002). Pectin has been manufactured from citrus peel for more than 50 years. Pectin manufacture involves leaching, acid extraction, precipitation, purification and standardization (Fig. 4).

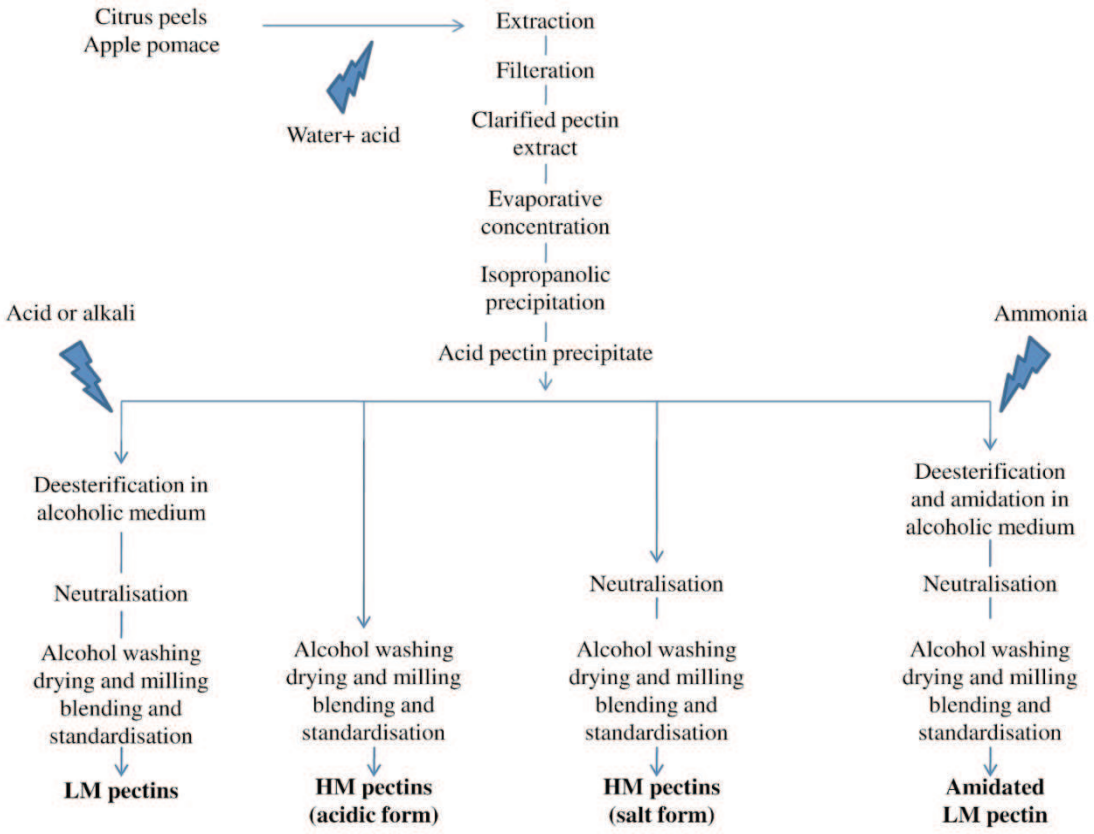


Fig. 4. Industrial production of pectin (Ralet and Thibault, 2003).

Most pectin extraction in industry is done in Europe using lime peels imported from Mexico and South America. Before industrial extraction, the raw materials are generally subjected to a pre-treatment (blanching, washing, drying, *etc*) to inactivate enzymes that otherwise would rapidly degrade the pectin molecules and to increase the product stability during transportation. Washing in water in order to leach out sugars is necessary prior to drying with the aim of minimizing caramelisation. Washing may even be preferred when the raw material can be used without drying, because the leachable material will have to be separated from the pectin at a later stage to reach a pure product. Citrus peel is extensively leached with water. This leach water has the potential for large pollution problems.

Pectin is extracted from leached citrus peels or apple pomace by water acidified with nitric acid. The extraction conditions (pH, temperature, time) must be optimised in order to provide good yields and desired gelling properties through typical conditions of pH 1.5-3, temperature 60-100°C and time 0.5-6 h. The solid/liquid ratio for citrus should be 1/35 in order to lead to efficient separation of phases. Acid

extraction has at least two demerits: (i) it may degrade pectin structure, and (ii) it does not meet environmental safety due to acid usage. Acid-extracted pectin often contains a mixture of both Ca^{2+} sensitive (CSP) and non-Ca^{2+} sensitive (NCSP) molecules. Separation, from extracted juice, of theses two types of pectin by selective precipitation has been described by Glahn (1995a, b). Selective precipitation was achieved by a solution of water and alcohol that contained a dissolved polyvalent cation such as Ca^{2+} (usually nitrate salt), which causes the CSP to gel. It is also possible to separate CSP and NCSP directly *in-situ* by fractional extraction at differential pH. At pH around 3, NCSP is separated and at pH 2 (typical of normal pH for pectin extraction), extracted pectin turns out to be CSP. Extraction technology is being continually studied and extraction processes using steam injection under pressure, microwave heating, enzymatic and microbial tools have been proposed (Fishman *et al.,* 2000, 2006; Panouillé *et al.,* 2006). It has been shown that flash-extracted pectin from orange albedo by microwave heating under pressure exhibited increased molar mass and intrinsic viscosity compared to pectin extracted by conventional heating techniques (Fishman *et al.,* 2000). Moreover, gel forming properties of orange and lime pectin prepared by rapid microwave heating were better than those of commercial citrus pectin (Fishman *et al*., 2003). An enzyme-hydrolytic technology would be environmentally safer and potentially more effective in terms of pectin yields (Panouillé *et al.,* 2006; Zykwinka *et al.*, 2009). Analysis of the scientific and patent literature shows that a number of research centres have been conducting studies to develop a biotechnological method for pectin extraction but these works are of exploratory nature only and their results are still far from industrial application (Ptichkina *et al*., 2008).

Centrifugation and filtration of the viscous (pectin concentration should be 0.6-1%) aqueous pectin at this stage is a bottleneck of pectin manufacturing. Some filter aids like diatomaceous earth with the help of rotary dry vacuum are often used (Rolin *et al*., 1998; Joye and Luzio, 2000). Extracts are rapidly brought to pH 3-4 and temperature is lowered to avoid any demethylation/depolymerization of pectin. The clarified extract is concentrated to 3-4% of pectin. For recovery of pectin, aluminium precipitation has replaced alcoholic precipitation as an environmental friendly process. The precipitate is filtered and then washed to remove any contaminants like acid, sugar, polyphenol, pigment and heavy metals.

Suspension in alcohol is the most used for DM modifications. Acidic or alkali treatment of alcohol solution at 50°C can deesterify pectin to the desired DM (DM of 55-75% for HM pectin and 20-45% for LM pectins). Weak bases such as sodium carbonate or ammonia are used to minimize ß-elimination reactions. Enzyme-hydrolytic technology would be environmentally safer and potentially more effective in terms of pectin yields (Panouillé *et al.,* 2006).

Drying and milling are the final steps. During processing, the pectin may undergo an ion-exchange step to remove Ca^{2+} ions and make a sodium form of pectin for easy use in food applications (Joye and Luzio 2000). Standardization is a current industrial practice to produce pectin samples with consistent properties. Diversity of natural raw material, climatic conditions to which the plants have been exposed, ripeness and peel pre-treatment processes may lead to large differences in pectin properties and functionalities. HM-pectin is therefore diluted and standardized with sucrose, dextrose, glucose or lactose to a given gelling power defined as degree sag. The standard of 150° sag means that 1g of pectin is able to gel 150g of sucrose under defined conditions of pH and temperature. Due to their large application range, LM-pectins are not necessarily standardized. For pharmaceutical purposes, pectin without admixed sugar is also available from the major manufacturers. So, when using pectin, it is obviously important to choose a type, which has been standardized in a way that corresponds reasonably to the intended use.

Pectin extraction is an intensive energy utilizing process that requires sophisticated operations and control. Tropical developing countries may have a locally owned pectin manufacturing operation, but it is typically hard pressed to compete with imported pectin unless the native operation is given governmental protection. Typically pectin operations are co-located with large-scale juice operations that run at least 300000 MT of fruit per year (Bates *et al.,* 2001).

3. Physico-chemical characteristics of extracted pectin

Both the nutritional value and physicochemical properties of pectin are important in the potential development. The physical characteristics of the pectin, as well as hydration, ion-exchange and adsorption capacities are important for their optimal utilization in foods (Thibault *et al*., 1993).

3.1. Solubility

Pectin is soluble in water but not in organic solvents. By having carboxylic acid groups, pectin is a polyelectorolyte and a weak organic acid. When pectin is added to water, carboxylic acid groups dissociate and the pectin molecules become negatively charged. Solubility is increased by all factors diminishing possibilities of intermolecular association. These factors can be of sterical (presence of substituents) or chemical (charges) nature. Mostly, solubilization is proceeded by a slow swelling (Thibault and Ralet, 2001). At a pH of ~ 3.6 (depending on pectin type), around half of the carboxyl groups of pectin are dissociated. Repulsion between the charged groups contributes to solubility and decreases the tendency for gelation. The solubility of pectin in aqueous media depends on multiple parameters, the main ones being the counterion nature, the ionic strength and the pH (Rinaudo, 1996).

3.2. Macromolecular features

The macromolecular characteristics of pectin mainly include its molar mass and conformation. These are determinants of pectin industrial applications as the strength of pectin gels is positively correlated with apparent molar mass (Rolin, 2002). Pectin molar mass varies with plant source, raw material stage of ripening and extraction conditions. It should be emphasized that a large number of factors, such as charge density, neutral sugars content and solvent quality can affect pectin aggregation and molecular state in solution. It was also shown that pectin molecules of similar molar masses may exhibit different hydrodynamic properties due to differences in DM, branching and neutral sugars content which make their solution behaviour even more complex (Rolin *et al.,* 1998). Molar mass determination is still a challenge due to problems of heterogeneity and aggregation in addition to the usual broad molar mass distribution. Developments in high-performance size-exclusion chromatography, coupled with laser light scattering and/or viscometric detection, led to an improvement in pectin characterization, although aggregation and poly-dispersity can disturb light-scattering data (Ralet *et al*., 2002). Pectin conformation is a matter of complexity. As the individual sugar rings are essentially rigid, the overall conformation of the chain is primarily determined by the bridge angle and the relative orientations of the component sugars as defined by the rotational angles. The linkages between monomers in HG are axial-axial, giving the polymer an intrinsic stiffness due to the severe conformational constraints imposed by this type of linkage

(Burton and Brant, 1983). A rather extended conformation with a persistence length of 4.5 to 13 nm was determined on isolated HG domains or HG-rich citrus pectin (Cros *et al.,* 1996; Morris *et al.,* 2008; Ralet *et al.,* 2008b). Some contradictory conclusions have been reported concerning the influence of DM on the conformation of the pectin macromolecules. Molecular modelling, nuclear magnetic resonance and small angle neutron scattering have shown that methoxyl groups have no significant influence on the flexibility of the linkages between GalA residues (Cros *et al.,* 1992, 1996). However, other studies have shown a general decrease in the hydrodynamic volume of the pectin molecules and an increase in chain stiffness with decreasing DM, with both steric and electrostatic interactions playing an important role in conformational changes (Morris *et al.,* 2000). Using another experimental approach combining intrinsic viscosity, sedimentation coefficient and weight-average molar mass determinations, the same authors recently concluded that all citrus pectin molecules tested were of similar conformation, whatever their DM (Morris *et al.,* 2008). Isolated RG-I domains or RG-I-rich pectin appear very flexible (Hourdet and Muller, 1991; Axelos and Thibault, 1991; Ralet *et al.,* 2008b). A complex set of different factors may influence the conformation of the pectin molecules and may partly explain some of the discrepancies found in literature about this subject.

The average molecular weight of pectins from various sources is typically in the order of $10^4 \sim 10^5$ g/mol. High values of intrinsic viscosity for a small molecular weight, compared with those of amylose indicate that the pectin molecule has a rather extended conformation in solution. Branched pectin may be expected to lower molecular size in solution. The complexity of structure means that no simple relationship between molecular size (in solution) and molecular weight exists for pectins (Perez *et al*., 2000).

3.3. Viscosity

Due to the high hydrophilicity of pectic substances, they generally exhibit higher hydration properties than other fibers. Aqueous solutions of 1 to 2% (w/v) of pectin already have a relative high viscosity (Rolin, 2002). Dilute pectin solutions are Newtonian but at a moderate concentration, they exhibit the non-Newtonian, pseudoplastic behavior characteristics. Viscosity is proportional to molar mass and is also influenced by degree of esterification, ionic strength, pH, and temperature (Sakai *et a*l., 1993). Factors that increase the tendency to gel or gel strength, decrease

solubility, and increase viscosity, and vice versa. Monovalent cation salts of pectins are highly ionized in solution, and the distribution of ionic charges along the molecule tends to keep it in an extended form by reason of columbic repulsion (Paoletti *et al*., 1986). Furthermore, this same columbic repulsion between the carboxylate groups prevents aggregation of the polymer chains. (The number of negative charges is, of course, determined by DM.) In addition, each polysaccharide chain, and especially each carboxylate group, will be highly hydrated. Solutions of monovalent salts of pectins exhibit stable viscosity because each polymer chain is hydrated, extended, and independent of each other.

The viscosity of pure aqueous solutions of pectin is inversely correlated with pH (Gilsenan *et al.,* 2000). As the pH is lowered, ionization of the carboxylate groups is suppressed, results in a reduction in hydration of the carboxylic acid groups, the molecules no longer repel each other over their space entire length, and as a result, they can associate and form a gel (Izydorczyk *et al*., 2005). Apparent pK-values (pH at 50% dissociation) vary with the DM of the pectin (Plaschina *et al*., 1978). Pectins with increasingly greater degrees of methylation will generate aggregation at somewhat higher pH, because they have fewer carboxylate groups at any given pH.

In absence of external salt or at low ionic strength, pectin chains exist in an extended conformation because of the strong intramolecular electrostatic repulsion and the solutions show the highest viscosity. Addition of salts of monovalent cations to pectin solutions suppresses the electrostatic repulsion, hence reduces viscosity (Izydorczyk *et al*., 2005). The presence of salts of di- or tri-valent metals suppresses the solubility of pectin, and this can, depending upon the conditions, either reduce or increase the viscosity, or gel the solution. The viscosity of an aqueous solution of pectin together with Ca^{2+} is usually positively correlated with pH within the interval of $2.5 < pH < 4.5$, which is in contrast to the behavior of a pure pectin solution. Interactions with divalent metal ions are also possible when high methylester pectins with a blockwise distribution of non-esterified carboxylic acid groups are used (Daas *et al*., 1999). Blocky HM pectin may form pseudoplastic, semi gelled solutions (Rolin, 1994, 2002). If Ca^{2+} salt is added, Ca^{2+} ions may form bridges between molecules (LM-pectin mechanism) and this increases the viscosity or causes gelation (L□fgren *et al*., 2005). Protons compete with Ca^{2+} especially at low pH. In the absence of Ca^{2+}, the

viscosity is inversely related to pH. That's why pectin of very low DM can gel at pH values below 2.5 (Löfgren *et al.,* 2005).

4. Gelation properties

Once pectin gets solubilised, they may gel through specific inter-chain associations. Pectin molecules also form random coil configuration due to the linear characteristic of the molecules and interactions between pectin molecules through hydrogen bonds. Pectin molecules tend to associate to form stiff rods or segmented rods in solution through noncovalent interactions (Rinaudo, 1996). It seems highly plausible that non-homogalacturonan part kinks the molecules from aligning, preventing a gel from turning into a precipitate (Rolin *et al*., 1998). Pectins are used in food industry mostly as gelling agents rather than thickening additives (Corredig *et al*., 2000).

The properties of pectin gels are strongly influenced by several factors. Extrinsic factors such as pH, ionic strength, co-solute concentrations and temperature influence the strength, texture and general viscoelastic properties of the pectin gels (Rao and Lopes da Silva, 2006). Intrinsic variables of the pectin macromolecules, such as molar mass, sequence of sugars along the chain, DM and distribution of the methyl groups along the chain, greatly impact on gelling properties of this polymer.

Among them, DM plays the most critical role (Rolin *et al.,* 1998; Cardoso *et al.,* 2003). The distribution pattern of free and esterified carboxyl groups also has a profound effect on gelling properties (Ralet *et al.,* 2001).

HM pectins (≥ DM% 50) forms gels under acidic conditions in the presence of a cosolute wherease LM pectin (< DM% 50) forms gel by a different mechanism in the presence of calcium ions (Rolin and De Vries, 1990; May, 1990).

4.1. HM- Pectin

HM-pectin is used in the confectionary industry for making fruit jellies, in fruit juices and fruit drink concentrates as a stabilizer and/or to provide mouth-feel, and also in fermented and directly acidified dairy drinks (Ralet *et al.,* 2002). Jam manufacture is the main user of industrially-extracted pectin. They use HM pectin to form gel with sugar and acid –so-called low water-activity gels or sugar-acid-pectin

gels. Such a gel is considered as a three-dimensional network of pectin molecules in which the solvent (water) encompassing the co-solutes (sugar 55-75% w/w), and acid (pH 2.5-3.5) is immobilized, resulting in a system resisting deformation and showing a stress/strain relationship for small deformations.

The high sugar concentration creates conditions of low water activity, which in turn promote chain-chain rather than chain-solvent interactions. The fact that sucrose can be replaced by other polyols, that less acid is necessary for pectin with higher DM and that a completely methoxylated pectin will gel without any acid are considered as proofs of these functions (Voragen *et al.,* 1995). Generally, raising the concentration of sugar increases gel strength, gelling temperature and gelling rate. The minimum concentration of sugar or polyol required for gelation depends on its ability to sufficiently stabilize hydrophobic interaction (Rao and Lopes da Silva, 2006).

Acidic conditions are required to reduce the dissociation of the carboxylic acid groups reducing the negative charge density of pectin molecules. By reducing negative charge density, repulsion of pectin molecules is reduced, and once attracting forces grow stronger than repelling forces, junction zones are formed (Fig. 5) (Lopes da Silva and Rao, 1995).

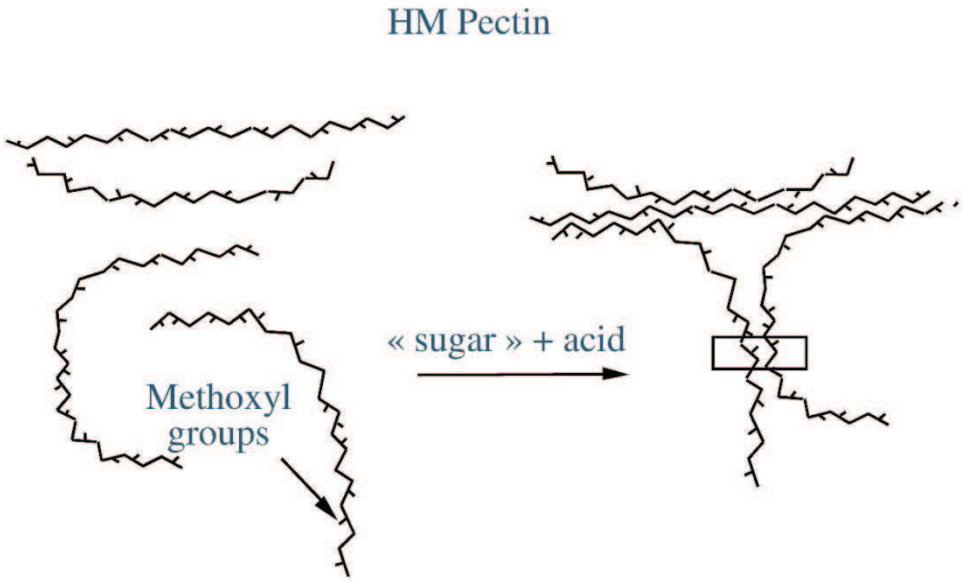


Fig. 5. Schematic representation of the junction zones in HM pectin gels.

Generally, the upper limit of pH for HM-pectin gelation is raised if, either the DM, total solid concentration or pectin concentration, increases. In the pH range where gelation of HM-pectin usually occurs (~2.0-3.7), gel strength and gelling temperature increase by pH reduction, when other conditions such as total solids content and ionic strength remain unaltered. This is due to the enhancement of macromolecular interaction resulting from the reduction of the pectin charges. Gels formed under these conditions are stabilized by aggregated helices supported by hydrogen bonds and grouping of methyl-ester groups through hydrophobic interactions within a cage of water molecules (Rao and Lopes da Silva, 2006).

Many factors influence the conditions of gel formation and strength, among which DM is a key factor. In general, increasing the DM leads to a faster gelation and higher gelling temperature. Moreover, at constant pH and co-solute concentrations, the final gel strength increases with increasing DM. As the DM of HM-pectin decreases, a lower pH is required for gelation, although the apparent pk_a increases due the decrease of DM (Rao and Lopes da Silva, 2006). DM correlates with the gel setting rate and gel texture under otherwise similar conditions, which means that very highly esterified pectin gel quicker at higher temperature. It will also form more elastic and brittle gel texture (Herbstreith and Fox KG Corporate Group, 2003).

HM pectin is classified into 'rapid set', 'medium rapid set' and 'slow set' types. Rapid set typically has a DM around 72 and will solidify jam at about 75 °C under normal conditions, while slow-set has a DM around 62 and causes gelation at about 60°C under similar conditions. High DM means high gelling temperature. In products that are deposited above setting temperature, slowly a network is formed stabilized by junction zones. If products are deposited below setting temperature in general a softer gel is formed with a rough gel structure. This process is also named pre-gelation. When the gel batch is cooled below the gelling temperature, gelation occurs after a delay, which is short with pectin of high DM and longer with pectin of lower DM. HM pectin gelation exhibits hysteresis in the sense that the melting temperature is higher than the setting temperature. So, it is impossible to re-melt the gel without causing destruction, and gels with HM pectin are accordingly often characterized as 'thermo-irreversible'.

The distribution pattern of free and esterified carboxyl groups has a profound effect on gelling properties (Ralet *et al.,* 2001). A random distribution of methyl esters gives a low Ca^{2+} sensitivity while a blockwise distribution generates a local charge concentration, which may hold Ca^{2+} ions in place in gel structure. HM pectin with blockwise distribution of carboxyl groups can follow egg- box mechanism with estimated 3-13 (Luzio and Cameron, 2008) or 10-20 units of GalA (Kohn and Luknar, 1977) as local charge concentrations. Calcium sensitive pectin (CSP) can gel in the presence of Ca^{2+} ions without sugars and are therefore useful for low-fat or sugarless acidic food stuff formulations (Joye and Luzio, 2000). Blocky HM pectin gels at higher temperature than pectin with the same (average) DM in a more uniform distribution. When used with calcium-rich fruit material, blocky pectin may create heterogeneous gels, in which the surface that forms upon cutting with a sharp knife contains visible grains. In some cases, the breaking strength of gels may increase with the amount of Ca^{2+}, but in other cases the heterogeneity becomes so pronounced that gels have poor breaking strength and increased tendency to syneresis. Blocky HM pectin may form pseudoplastic, semigelled solutions (Rolin, 1994).

In the presence of monovalent salts, such as NaCl, less Ca^{2+} is needed for the pectin to pass sol-gel transition points. More Ca^{2+} ions are indeed able to establish junction zones while the charge of the polymer is increasingly screened by monovalent ions. The combine effect of pH and sugars leads to promotion of gelation at lower Ca^{2+} levels. pH and co-solute effects on chain-on-solvent interactions counterbalance the decrease in the number of carboxyl groups available for Ca^{2+} binding and chain-chain interactions are promoted (Voragen *et al.,* 1995). The affinity of pectin for Ca^{2+} increases with decreasing average DM and increasing length of unsubstituted galacturonan stretches. The affinity of HM-pectin for Ca^{2+} is generally not high enough to lead to sufficient chain association for gelation to occur (Rao and Lopes da Silva, 2006).

4.2. LM-Pectin

Ionic-mediated gelation through divalent cations, of which the most relevant is Ca^{2+}, is the classical mechanism of LM-pectin gelation. The mechanism of Ca^{2+} binding to the ionized carboxyl groups on the pectin chains is similar to the egg-box proposed for alginate (Grant *et al*., 1973). Dimerization of polygalacturonic chains

occurs in the presence of divalent ions, each chain have a two-fold symmetry and forms a series of electronically negative cavities that divalent cation can fit in known as “egg-box” binding (Rao and Lopes da Silva, 2006) (Fig. 6).

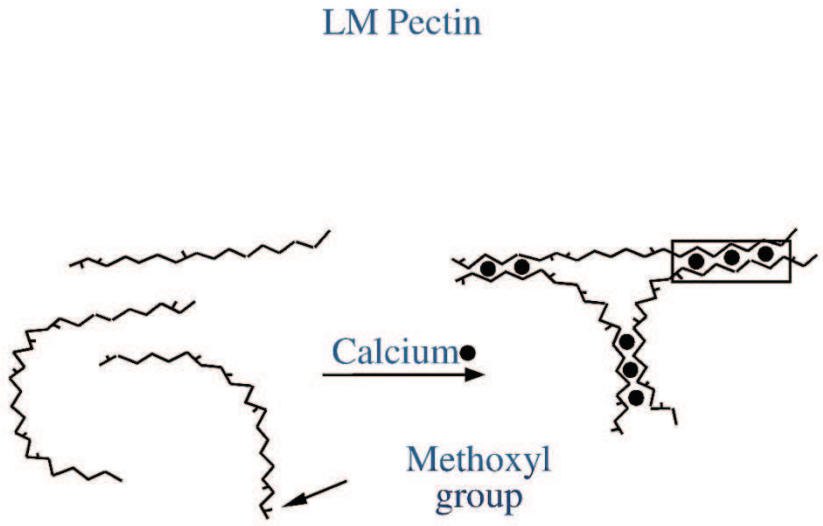


Fig. 6. Schematic representation of the “egg-box” model in LM pectin gels.

Because of the electrostatic nature of the bonds, LM-pectin is very sensitive to intrinsic parameters that can modify the environment of the carboxyl groups such as the nature, distribution and amounts of substituents along the galacturonic backbone.

In the case of LM pectin, the setting temperature is inversely correlated to the DM. Gel forming ability increases with decreasing DM and LM-pectin with a blockwise distribution of free carboxyl groups are very sensitive to low Ca^{2+} levels (Kohn *et al.,* 1983; Thibault and Rinaudo, 1986; Ralet *et al.,* 2001).

An appropriate level of Ca^{2+} is required for gelling LM pectin, and the gelling temperature as well as the firmness of gels is both positively correlated with the concentration of Ca^{2+}. In spite of its similarity to Ca^{2+}, Mg^{2+} has much less impact on pectin rheology, and it is unable to gel LM pectin systems (Malovikova *et al.,* 1994). LM pectin may gel in the absence of dissolved solids, apart from the pectin itself, but gels with less than about 20% soluble solids are not very resilient and have a high tendency to syneresis (Rolin, 2002). With LM-pectin, as the soluble solids increase, Ca^{2+} requirement decreases and the Ca^{2+} bandwidth becomes narrower (Ralet *et al.* 2002). For LM blockwise pectin at low level of Ca^{2+}, the gel structure will be less elastic, rather pasty with lower breaking strength compared to a gel prepared with an

optimum level of Ca^{2+} (Herbstreith and Fox KG Corporate Group, 2003). Overdose of Ca^{2+} will lead to pectin precipitation, also called pre-gelling, which is reversible only to limited extent, even when the gel is once more heated above its setting temperature and cooled down without destruction (Herbstreith and Fox KG Corporate Group, 2003).

pH is a factor that is not so critical in the development of LM-pectin gels (2.0-6.0). However, it has a significant role in the final properties of the gels. A decrease in pH leads to different kinetics of gel formation and to a decrease in shear modulus, due to the decrease in the number of ionized carboxyl groups necessary for ionic complexation and gel formation. At a low pH, more Ca^{2+} is needed to induce gelation than at neutral pH. Compared to a salt-free solution, less Ca^{2+} is necessary to form gels when the ionic strength increases. However, gels in salt-free solution are formed more rapidly but the final modulus is lower (Rao and Lopes da Silva, 2006). When pH is below 3.5, there is a predominance of non-dissociated acid groups, which leads to more hydrogen binding in the gel network. This gives rise to a more rigid, non-shear-reversible gel network. When pH is above 3.5, there is a predominance of ionized acid groups, which favours Ca^{2+} cross-linking, leading to the formation of a more spreadable, shear reversible gel network.

The traditional application of LM-pectin is in jams with soluble solid below 55% (low-calorie jams, jelly preserves and conserves), which is the limit for the use of HM-pectin. The heat reversibility of LM-pectin gels may be utilized in bakery jams and jellies for glazing purpose. LM-pectin also finds application in the production of fruit preparations for yogurt and fruit/milk desserts (Ralet *et al.*, 2002).

Amidated LM-pectin can gel under the same conditions as the LM-pectin does. It has a lower gelling temperature than non-amidated pectin of the same DM. Amidated LM-pectin accounts for the major part of today's use of LM-pectin. Compared to non-amidated LM-pectin, gels are less prone to syneresis and their texture is easier to control due to functionally saturating with respect to Ca^{2+} ions in most applications (Rolin, 2002). Amidated pectin needs less Ca^{2+} to gel and is less prone to precipitation at high Ca^{2+} levels suggesting that its gelation cannot be fully explained by the “egg-box model”. Indeed, blocks of amide groups along the chain promote association through hydrogen bonding (Racapé *et al.*, 1989; Rolin, 2002; Alonso-Mougan *et al.,* 2002).

Amidated LM pectin is widely used for jam with reduced sugar content because, compared to conventional LM pectin, it yields gels with lower tendency to syneresis. Further, at typical conditions of use, the texture is not affected by moderate variations in the presence of Ca^{2+}.

5. Acid Dairy Drink (ADD) and pectin functionality as stabilizer

Acid Dairy Drinks (ADD) are worldwide products popular in many variations, e.g. fruit milk drinks, yoghurt drinks, soy milk, butter-milk, whey drinks, kefir, *etc*. These beverages can be described as an acidified protein liquid system with stability and viscosity similar to natural milk. Such drinks are usually composed of an acid dairy phase (fermented base) or a neutral base (milk, soy milk, *etc.*) with an acidic medium (fruit phase) (Laurent and Boulenguer, 2003). According to DMI Dairy Management Inc^{TM} (2007), drinkable yoghurt stands out as the fastest growing seller, not just in dairy, but in the food and beverage industry as a whole. China topped the list of producer with a whopping 49% year-over-year growth rate. Annual sales of drinkable yoghurt grew by 18.4% to $7.76 billion from mid-2005 to mid-2006 (DMI Dairy Management Inc^{TM} 2007). Broad opportunities for functional ingredients, such as Omega-3, phytosterols and probiotics in drinkable yoghurt have made the products an attractive area for innovation.

5.1. Stabilizing properties (pectin-protein interactions at interfaces)

A substantial portion of pectin is today used for stabilization of low-pH dairy drinks including fermented drinks and mixture of fruit juice and milk. The drinks may be heat-treated in order to increase their shelf life. Low viscosity and homogenous appearance are preferred characteristics. Casein, however, is prone to aggregation at low pH, particularly when subjected to heat treatment. Thus, in the absence of a stabilizer, high viscosity, whey exudation and sandy mouth feel are likely quality defects in these types of drinks (Rolin *et al.* 1998). In the late 1950s, it was proven that addition of HM-pectin to acidified milk drinks prevented the formation of sediment. Casein in milk, at its natural pH of approximately 6.6, is in the form of stable sub-micron particles, often called micelles (Ye, 2007). At this pH,

caseins are negatively charged. They are mutually repellent, which prevents precipitation. During acidification, electrostatic repulsion decreases and casein particles tend to aggregate. At the isoelectric point (pH 4.6), casein particles are uncharged and exhibit the weakest hydration. Below the isoelectric point, casein particles exhibit a net positive charge. The detailed changes of the micelles above the isoelectric point were summarized as follows by Laurent and Boulenguer (2003):

- *Between pH 5.8 and 5.5, the zeta potential decreases, conducting the micelles to form clusters (particle size change from 180 to 1300 nm at pH 5.5).*
- *Between pH 5.5 and 5, a re-organization of the different kinds of caseins occurs in the micelle by solubilization of α and β casein. Micelles are then associated in multi-strands and are no longer spherical.*
- *Below pH 5, Ca^{2+} is completely soluble, the aggregation of casein is irreversible and forms a three-dimensional network constituted by the cluster of aggregated strands*.

A mechanism of stabilization by pectin under acidic conditions was proposed in which the adsorption of the pectin on the casein micelle surface throughout carboxylic-rich zones of HG domains is involved. The uncharged pectin segments in between form entropy-rich loops that extend into the solution. These loops cause a repulsive interaction between the micelles at low pH in the same way as κ-casein chains do at milk pH (Rao and Lopes da Silva, 2006) (Fig. 7). The stabilization of casein with pectin is only effective in the pH interval 3.2-4.5. By hypothesis, the pectin is not sufficiently dissociated at a pH below 3.5, and then it either does not anchor efficiently or the repulsion between "hairs" in an interpenetration zone becomes too weak. At pH above 4.5, casein does not posses enough positively charged areas and there is no longer attraction between pectin and casein (Rolin *et al*., 1998). In more concentrated acidified milk systems, it was suggested that stability is associated with the existence of a network of pectin-coated casein micelles, but a large fraction of pectin does not interact directly with acidified milk gel (large casein micelles). It was shown that less than 20% of the pectin added directly interacts with casein particles (Syrbe *et al.,* 1998). The remaining 80% is involved in a network with casein-pectin complexes but plays no role in stabilizing the final product. This excess fraction seems however necessary to produce a stable

system (Janhøj *et al.,* 2008). Jensen *et al*. (2010) showed that the excess fraction of pectin is not a matter of importance in stabilized ADD.

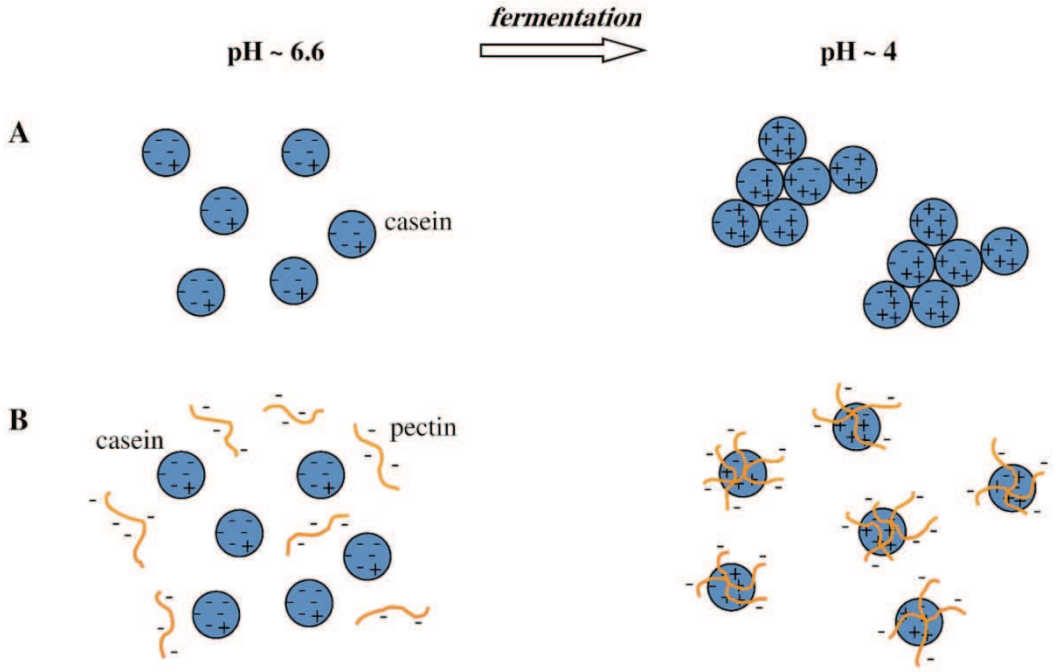


Fig. 7**.** Schematic representation of pectin stabilizing properties in acidified dairy drinks. (A) Casein in milk at its natural pH (~ 6.6) is in the form of stable sub-micron particles that are globally negatively charged. During fermentation, acidification occurs and casein particles become globally positively charged. Below pH 5, aggregation of the casein is irreversible. (B) At pH > 3.5, pectin is negatively charged due to the presence of carboxylic functions. At pH 6.6, both polymers are negatively charged and repel each other. At pH ~ 4, pectin electrostatically stick to the positively charged areas of casein particles producing a highly hydrated coating, which prevents casein aggregation. Such pectin-stabilized particles are often depicted as "fuzzy golf balls" (Tanhatan Nasseri *et al*., 2008).

Recently, it was shown that (i) stabilization in ADD might be caused by a combination of depletion interaction between pectin coated casein micelles and a pectin network, (ii) that 50-90% of all pectin is bound to casein and (iii) that stability is not affected by the remaining non-bound fraction (Tromp *et al.,* 2004; Boulenguer and Laurent, 2003).

Pectin dosage is a determinant factor in acidified milk drink stabilization. Too little pectin destabilizes the product compared to no pectin addition. If a series of milk drinks is prepared with different pectin additions, the viscosity first increases until a certain dosage is reached. A further increase in pectin dosage causes a sharp decline in viscosity. A point of minimum viscosity is then reached, beyond which further pectin addition causes a new increase in viscosity. The tendency for sediment formation grossly follows viscosity until the point of minimum viscosity. It is believed that the de-stabilization at low pectin dosages is because the adsorbing pectin molecules tend to wrap around casein particles when the surface is not crowded with pectin. Electrostatic repulsion is at a minimum because pectin has balanced the initial casein charge rather than increased numeric net charge. At full stabilization, only those pectin molecular areas, which interact strongly with casein, are anchored at the casein particles surface, whereas those areas, which are less strongly attached, are forced away from the surface (Rolin *et al.,* 1998). Viscosity overshoot and final viscosity reduction become particularly pronounced when the casein particle size is low. It is striking that, independently of casein particle size, the maximum of low shear viscosity occurs at about half the pectin concentration at which the viscosity minimum (and product stability) is observed. This could point to bridging by weakly adsorbed polymers, expected to be the most pronounced at about 50% surface coverage. In contrast to the results at low shear rates, the limiting high-shear viscosity increased steadily with pectin concentration. This was interpreted as an increase in particle volume by the absorbed pectin layer, matching the results on the increased sediment volume after centrifugation (Rolin *et al.,* 1998). Pectin addition also changes the flow behavior of ADD from shear-thining and thixotropic to quasi-newtonian. This means that the system undergoes a transition from a flocculated state with partial surface coverage into a free-flowing state, where surface coverage is completed and particle attraction minimized. This conclusion is corroborated by microscopy and particle size analysis (Rolin *et al.,* 1998). In industry, the pectin dosage required for optimum acidified milk stabilization depends on the formulation and production technology of the product. The stability of acidified milk drinks is evaluated on the basis of the viscosity, the amount of sediment after defined centrifugation and by microscopic examination of the protein particles (Herbstreith and Fox KG Corporate Group, 2003). Important parameters are:

- pH value of the drink (optimum range 3.9-4.1)
- protein content and particle size (1-2 μm~ smooth, 10-20 μm~ chalky, 40-60 μm~ grainy)
- fermentation conditions during yoghurt preparation (temperature, time, bacteria culture used to produce uniform-sized protein particles)
- conditions during direct acidification using juice or acid (direct acidification makes large protein particles)
- heat treatment during the production process (the more intense the heat treatment, the greater the risk of thermal agglomeration of protein particles).
- homogenization (10-20 MPa, to destroy caseinate gel)
- heat treatment of the finished products (it has adverse effect because of the weakest bound molecules dislodge from the complex)
- Addition of Ca^{2+} (more Ca^{2+} reacts with more pectin) (Ye, 2007; Boulenguer and Laurent, 2003; Herbstreith and Fox KG Corporate Group, 2003; Janhøj *et al.,* 2008; Lucey *et al*., 1999; Sedlmeyer *et al.,* 2004; Tholstrup Sejersen *et al.,* 2007; Syrbe *et al.,* 1998; Tromp *et al.,* 2004).

Dilution drastically hampers the stabilization of ADD. In fact, except for Ca^{2+} enriched drinks, water-dilution modifies the ionic strength, which induces a decrease in stability (Ye, 2007). A high Ca^{2+} concentration inhibits stabilization since it strongly enhances caseinate self-association (Syrbe *et al.,* 1998).

5.2. Type of pectin used for ADD stabilization

ADD are commonly stabilized by HM-Pectin (Ye, 2007; Laurent and Boulanguer, 2003; Herbstreith and Fox KG Corporate Group, 2003; Janhøj *et al.,* 2008; Lucey *et al*., 1999; Tholstrup Sejersen *et al.,* 2007; Syrbe *et al.,* 1998; Tromp *et al.,* 2004). LM-pectin with more numerous carboxyl groups should associate more strongly with the positively charged casein particles that HM-pectin does. However, LM-pectin exhibit lower stabilization properties than HM-pectin. Tighter binding of pectin molecules could be the reason, leading to a flatter configuration and less polymeric stabilization, but without detailed information on molar mass and carboxyl residues distribution, all this remains speculation (Syrbe *et al.,* 1998). HM-apple pectin is especially suited for this stabilization mechanism when a high viscosity

ADD is assumed. If a low-viscosity end-product is required, HM-citrus pectin is mainly used. Only HM-pectin within a very specific range of esterification (approx. 68-72%) is suited for the stabilization of ADD. For optimum stabilization, pectin requires a high molar mass and defined Ca^{2+} reactivity.

6. Regulation and market

The pectin industry is dominated by large multinational firms, which have undergone major changes in the past five years. The world leaders include CP Kelco (owned by I.M. Huber Corporation since 2005), Danisco sugar (Denmark), Cargill (acquired Citrico and Degussa Food Ingredients) and Herbstreith and Fox (Germany) (USDA Foreign Agricultural Service, 2007). Approximately 35000 tons of pectin are produced and used each year in the world (USDA Foreign Agricultural Service, 2007). CP Kelco has around 40% of the market (35-40% of the world market and 35-45% of European economic area (EEA) market) followed by Danisco (20-30% of the world market and 15-25% of EEA market), Degussa Food Ingredients (10-20% of the world market and 15-25% of the EEA market), Herbsteith and Fox (5-15% of the world market and 10-20 of EEA market), Cargill and Obipektin (10% of the world and EEA market, separately). The remaining 5% is held by small processors (USDA Foreign Agricultural Service, 2007). Pectin is an essential additive in many food applications such as beverages, protein drinks, yogurts, jams, jellies and desserts. In addition to these general applications, pectin may also hold applications as a prebiotic, potential source of soluble fiber and fat replacer in functional food and nutraceutical applications. *In vitro* tests have shown that pectin acts as prebiotic, preventing pathogens from binding to the intestine and increasing the growth of probiotic bacteria in the large intestine (Iisakka, 2003). In regard to fiber additives, Functional Food and Nutraceuticals estimates that besides oat bran, psyllium and soya fiber, pectin will fare the best in nutraceutical application based on combination of cost, quality, performance and versatility advantages. In addition, a physically modified version of pectin is in the market as fat replacer. Pectin also holds medical applications for colon specific drug delivery which may face a potential $1 billion market in medicine field (Kalorama Information, 2007). The market of pectin has been estimated to be growing at a rate of 3-5% annually since 2001. The average

price of HM and LM-pectins are approximately $ 16000 per ton since 2006 (USDA Foreign Agricultural Service, 2007). At the FAO/WHO joint Expert Committee on Food Additives and the EU, no numerical acceptable daily intake (ADI) has been set, as pectin is considered safe, although some national regulations may limit the amount of pectin added in some applications. In the US, pectin is GRAS-Generally Recognized as Safe. In most food it can be used according to good manufacturing practice in the levels needed for its application "*quantum satis*". In the International Numbering System (INS) pectin has the number 440. In Europe, it is differentiated into E440 (i) for non-amidated pectin and E440 (ii) for amidated one (USDA Foreign Agricultural Service, 2007).

7. HG-degrading enzymes

Studies have shown that DM has a very strong influence on pectin functionality, being a major basis for differentiation of pectin used in food industry (May, 1990). The distribution pattern of methyl esters over the HG pectin domains influences its functional properties (Willats *et al*., 2001; Guillotin *et al*., 2005; Luzio and Cameron, 2008). This distribution pattern within HG regions is related to the mechanism of demethylation. Alkali demethylation results in random pattern. Enzymatic tools potentially can be used to modify the amount and the pattern of methyl esterification. Plant-PME has been shown to produce ordered (blocky) distribution of methyl esters while fungal-PME produces random distributions (Grasdalen *et al*., 1996; Limberg *et al*., 2000a, b; Ralet and Thibault, 2002; Verlent *et al*., 2004). To characterize the DM pattern of methyl ester over the HG stretches, different enzymes can be use to digest HG and liberate different oligomers according to their cleavage sites (Limberg *et al*., 2000a, b).

The main chain of HG can be cleaved by endo/exo-polygalacturonases (PG) or pectin/pectate lyases (PL). The activity of these enzymes depends strongly on the DM and methylation pattern of pectin, which can be modified by pectin methylesterase (PME).

7.1. Pectin methylesterases

These enzymes are produced by many higher plants, fungi and bacteria (Pilnik and Voragen, 1991). A few PMEs were also found in yeast (Gainvors *et al.*, 1994; Nakagawa *et al.*, 2000). In many plants, several isoforms of PME are found. There are three generally recognised modes of action for PME (Grasdalen *et al.,* 1996):

• The 'multiple-chain' mechanism, in which the enzyme-pectin complex dissociates after the de-esterification of a single galacturonic acid residue, which results in random de-esterification of the pectin backbone.

• The 'single-chain' mechanism, where the enzyme de-esterifies all the residues in an esterified blocks of GalA.

• The 'multiple attack' mechanism in which the enzyme de-esterifies a fixed number of residues within an esterified block (known as the degree of multiple attack) with each enzyme substrate encounter.

Acidic PMEs, such as those found in fungi, tend to follow random patterns of de-esterification, while PMEs with alkaline pH optima, and such as found in plants and bacteria, results in blockwise deesterification of the pectin (Benen *et al*., 1999).

Catoire *et al.* (1998) examined the mode of action of three isoforms of PME isolated from mung bean and found that they exhibited different mechanisms. This group also demonstrated that the mechanism differed depending on whether the PME being characterised was soluble or in the cell wall-bound form. Dènes *et al.,* (2000) have determined the mode of action of apple fruit PME and have shown that this can vary depending on the pH of the assay. In addition, Limberg *et al.,* (2000b) have observed variations in the mode of action of PME from orange peel depending on the DM of the initial pectin. In general, plant and bacterial PMEs have pH optima between pH 6 and 9 whereas most fungal PMEs have pH optima between pH 4 and 6. Presence of di- or trivalent cations has been shown to influence the activity of microbial (Laurent *et al*., 2000) as well as plant PMEs (Schmohl *et al*., 2000). Calcium could affect the enzyme or the substrate, by modifying their conformation or by altering the enzyme-substrate interactions (Slavov *et al*., 2009).

7.2. Polygalacturonases

PGs can split glycosidic linkages adjacent to free carboxyl groups (Pilnik and Voragen, 1991) and are thereby considered as key enzymes for depolimerisation of pectin. They can be divided into exo and endo-PG. PGs are generally active between pH 3 and 7, with a more acidic pH optimum for the fungal enzymes (Kapoor *et al.,* 2000; Kobayashi *et al,* 2001).

7.2.1. Exo-PG

Enzymes that act from non-reducing end to remove *mono* and *di*-GalA are *exo*-PGs (Pařenicová *et al.,* 2000a). With respect to their mode of action, exo-PGs are much simpler enzymes than endo-PGs because only one product is released, either a GalA monomer or dimer. Typically, all bacterial exo-PGs analyzed so far only released dimers, whereas in fungi like *Aspergillus* and *Fusarium oxisporum* only the monomer-releasing activity was reported (Garcia-Maceira *et al.,* 2000). From *Clostridium thermosaccharolyticum* (van Rijssel *et al.,* 1993), an exoPG activity was purified as part of a complex with PME activity that had a low degree of trimer-releasing activity in addition to dimer release. An optimium of 0.4-0.5 mM Ca^{2+} is required for activity in exo-PG from most sources. EDTA and citrate can inactivate exo-PG. Exo-PG is independet of substrate size (Pressey and Reger, 1989).

7.2.2. Endo-PG

The optimal substrate for endo-PG is polygalacturonan with a low degree of esterification. The pectinase preparation studied by Kester and Visser (1990) contained two major endo-PG activities designated PGI and PGII classified into 5 additional categories (A-E). Using partially methyl-esterified substrates with various DM, it was established that except for PGA and PGB, the enzymes gradually became less active while DM increases. PGA and PGB were the most active on moderately esterified pectin (DM 22±5%) (Pařenicová *et al.,* 2000a). For PGD it was proposed that this enzyme is in fact an oligogalacturonase since with a limited number of subsites (4), it has a high digalacturonate-hydrolyzing capacity and extreme processive behavior on oligogalacturonides dp > 4 (Pařenicová *et al.*, 2000b). For PGC and PGE it was concluded that the natural substrate is most likely different from polygalacturonic acid while for the other polygalacturonans with a low degree of esterification is favored.

Endo-PGs are considered to be random-acting enzymes on polymer substrates. For some enzymes it turned out that after the first random encounter with the substrate, the enzyme remains bound to the substrate to further degrade this in a multiple-attack fashion. For the enzymes characterized in this respect, the multiple attack or processivity releases monomers from the reducing end, in contrast to exo-PGs that release monomers from the nonreducing end, and thus the enzymes appear to act in an exolytic way. Even for the truly random-acting enzymes, non-random hydrolysis occurs when the dp of the substrate is smaller than the number of subsites, as then the affinities of the individual subsites become important. As a result, the appearance/disappearance and amounts of individual oligogalacturonides during hydrolysis of polymer substrate is typical for each endo-PG and can be presented as a product progression profile.

The product progression of PGII is typical for random endo-acting enzymes and is therefore easier to understand than the one for PGI. Random hydrolysis of a polymer substrate will result in large products at the onset of the reaction. These larger products will then be hydrolyzed into smaller products until the smallest substrate that can efficiently be hydrolyzed is depleted. The product progression for PGII closely follows this theoretical profile. Since the trimer is only very slowly hydrolyzed, it will only be degraded after prolonged incubation. A similar profile was also recorded for A. *niger* PGB and PGE (Pařenicová *et al.,* 1998; 2000a) and for *F. moniliforme* PG (Bonnin *et al.,* 2001). The product progression of PGI is characterized by a rapid increase of the monomer from the onset of the reaction and, moreover, no substantial amounts of oligoGalA of dp > 5 were detected. This demonstrates that PGI is not a fully random-acting enzyme. Similar profiles to that described for PGI were found for PGA, PGC and PGD (Pařenicová *et al.,* 1998, 2000a; Benen *et al.,* 1999).

Endo-PG has no metal requirement. The effect of cations such as Na^{+} has been attributed to increase availability of the substrate by dispersion of the polygalacturonans in solution (Pressey and Avants, 1977). Cations like Ca^{2+}, which interact with polygalacturonans to form aggregates, are inhibitory at even low concentration. High molecular weight polymers are hydrolysed at faster rate than low molecular weight substrates by endo-PG.

7.3. Lyases

Pectin transeliminases or pectic lyases degrade the pectic substances by trans-elimination mechanism yielding unsaturated oligogalacturonates. Pectate lyases, acting on polygalacturonic acid, and pectin lyase, acting on pectin, are the two important transeliminases (Gummandi and Kumar, 2005).

7.3.1. Pectin lyases

These enzymes split glycosidic linkages between methyl-esterified GalA by a *trans*-elimination reaction that causes the formation of a double bond between carbons 4 and 5 of one of the galacturonic acids at the newly formed non-reducing end. They are only produced by microorganisms, predominantly fungi such as *Neurospora, Botrytis,* penicillia and aspergilli, in contrast to pectate lyases, which have predominantly been isolated from bacteria. Currently, only endo-acting pectin lyases are known. Their optimum pH is around 6 on almost fully methylated pectins. On pectin with lower DM and random distribution of methyl esters, lower optimum pH was observed (Voragen, 1972; Wijesundera *et al*., 1989; Yadav *et al*., 2009). Although pectin lyases do not require Ca^{2+} for activity, Ca^{2+} appeared to influence the activity of the enzymes (Voragen, 1972). Ca^{2+} compensates the charge of the carboxylates of the substrate and modifies the pH optimum (8.5) (Voragen, 1972). Ca^{2+} at 0.5-1.0 mM has been shown to increase activity with pectin as substrate (Wijesundera *et al*., 1989) that promotes optimal interaction of substrate with enzyme.

Three three-dimensional structures have been solved; two structures of pectin lyase A (PLA) (Mayans *et al*., 1997) and one of pectin lyase B (PLB) (Scavetta *et al*., 1999), all from *Aspergillus niger*. Pectin lyase consists of two types as PLI (8 subsites) and PLII (9 or 10 subsites). A family of six pectin lyase genes was identified in *A. niger* (Gysler *et al.,* 1990: Harmsen *et al.,* 1990; Kester and Visser, 1994).

The detection of unsaturated uronic acid ester from PL activity is generally performed by using two different methods: (i) detection of the olefinic bond of the unsaturated uronic ester by its UV absorption at 230-240 nm (Ceci and Lozano, 1998) and (ii) detection by transformation of the unsaturated uronic ester into a colored species possessing UV absorption at 550 nm. Thiobarbituric acid (TBA) is

claimed to be a colorimetric test specific for the quantification of the PL activity (Kato *et al*., 1997).

It has been demonstrated by Voragen (1972) that pectin lyase activity is highly dependent on the distribution of the methyl esters. Pectin lyase activity on blockwise deesterified pectin is stronger than random pattern since the effect of unesterified GalA is stronger as a stretch of ideal substrate is hardly available (van Alebeek *et al*., 2002, 2003).

Purified saturated and unsaturated oligoGalAs (van Alebeek *et al.*, 2000) were derivatized at the C-6 position and/or the C-l position. The purity and molecular weight were checked by MALDI-TOF MS (van Alebeek *et al.,* 2000). The mode of action of PL of A. *niger was* first studied using fully methyl esterified oligoGalA. The reactions were monitored by high-performance anion-exchange chromatography (HPAEC) using pulsed amperometric detection (PAD) showing all compounds and UV detection that reveals only the unsaturated products (between GalA-4 and GalA-5 with dp=4-8) which demonstrates that at least eight subsites are present on the enzyme. Enzymatic degradation proceeds more rapidly when the substrate contains blocks of esterified residues than when a random distribution of methyl esters occurs. Larger substrates are preferred by the enzyme (Baldwin and Pressey, 1989).

7.3.2. Pectate lyases

The pectate lyases cleave internal glycosidic bonds in polygalacturonate by β-elimination, releasing both saturated and unsaturated oligogalacturonates from the reducing end (Roy *et al*., 1999). They are commonly associated with pathogenic fungi and bacteria (Collmer and Keen, 1986; Gummandi and Kumar, 2005). They act preferentially on LM pectin rather than on pectic acid and need Ca^{2+} ions. They encompass *endo*- and *exo*- types.

The exo-acting forms of the enzyme can result in the production of di- or tri-oligomers while endo-acting forms generate boths (Lojkowska *et al*., 1995). Pectate lyases have been classified into five families, based on their relative amino acid sequences (Shevchik *et al.,* 1999). In a report by Soriano *et al.* (2000) a pectate lyase from *Bacillus* sp. is described that displays higher activity on both low- and high-DM pectin with a low activity on partially methylesterified pectins. The eight *E. chrysanthemi* 3937 pectate lyases form the largest group of lyases from one organism

that have been characterized in detail. Four closely related enzymes from *E. chrysanthemi* B16 were partially characterized by Preston *et al.* (1992). Some of these enzymes prefer partially methyl-esterified pectins, whereas others prefer polygalacturonic acid (Roy *et al.,* 1999). The optimum pH for endo-pectate lyase is 8 and 9.5. The optimum pH decreases as the molecular weight decreases (Nagel and Anderson, 1965). The enzyme has a requirement for Ca^{2+} of about 1 mM Ca^{2+} is thought to interact with the substrate to form more favorable substrate geometry for enzymatic attack.

8. Methodology in pectin research and methyl-esterification pattern determination

Various extraction and fractionating procedures have been developed for the purpose of designed changes to pectins during growth, ripening, storage or processing of plant materials (Selvendran and Ryden, 1990; Voragen *et al.,* 1995, 2001). In general, extracts obtained are fractionated to homogeneity using size exclusion and/or anion-exchange chromatography. For structure elucidation of such homogeneous fractions, sugar and glycosidic linkage composition and anomeric configuration of sugar residues are determined. But strategies for establishing the fine structure of pectin further include fragmentation with pure, well-defined and specific enzymes (or with less specific chemical reactions) and fractionation to homogeneity of the fragments in the digests (Fransen *et al.,* 2000; Huisman *et al.,* 2001). Indeed, these fragments often fit within the analytical ranges of advanced NMR and mass spectroscopic techniques that allow the establishment of the absolute structure, including the presence and distribution of substituents. By degrading purified pectins in galacturonan backbone, chemically by ß-elimination or enzymatically with *endo*-PG and *endo*-pectin lyase or *endo*-pectate lyase, it is possible to separate pure, degraded galacturonan fractions from high molecular weight pectin. Some of the strategies and procedures used in elucidation of oligosaccharide structures will be illustrated through the separation and identification of oligogalacturonic acids.

Detailed characterization of pectins having completely different functionalities has been given by Daas and co-workers (1998, 1999, 2001a). Rather than employing

the frequently used high performance anion-exchange chromatography (HPAEC) methods at pH 12 that result in an excellent separation of oligogalacturonic acids, Daas and co-workers used a HPAEC method at pH 5 (as previously described by Hotchkiss and Hicks, 1990; Hotchkiss *et al.,* 1996) to obtain additional information about the methyl ester groups present (Daas *et al.,* 1998). They were able to separate and to identify the various degradation products present in a polygalacturonase digest (PG from *Kluyveromyces fragiles)* with respect to dp and number of methyl esters present. Matrix-Assisted Laser Desorption/Ionisation Time of Right Mass Spectrometry (MALDI-TOF MS) was a very valuable tool for determining the number of methyl esters present on each oligomer (Daas *et al.,* 1998). In subsequent publications, Daas *et al.* (2001b) offered various new parameters to characterise the different pectins. From their work, Daas and co-workers introduced the concept of "degree of blockiness" (DB) in pectin. In this regard, degree of blockiness is calculated by measuring the amount of monomers, dimers and trimers of GalAs released from pectic molecules treated with *endo*-PG divided by the amount of free GalAs present in the sample. The DB increases when the GalA residues are distributed in a more blockwise way over the pectin molecule. At the same time, Körner *et al.* (1998, 1999) used MALDI-TOF MS and nanoelectrospray ionization ion trap mass spectrometry to locate methyl-esterified galacturonic acid residues in oligomers up to a degree of polymerization of 10 present in a complex mixture obtained after pectin digestion by *endo*-polygalacturonase and *endo*-pectin lyase with known mode of actions. The data sets obtained were used to draw conclusions about the structure of the parent pectins as well as on the mode of action of the enzymes (Körner *et al.,* 1998, 1999; Limberg *et al.,* 2000a, b).

9. Objectives of the thesis

In the present work, we were up to realize the impact of molecular structure of pectin on stabilizing process in special type of Iranian acidified dairy drink (Doogh). In this regard, several commercial pectins were characterized at molecular level. Pectins were extracted from two dried types of *Citrus aurantifolia* peels (oven-dried and air-dried peels) by different types of acidic methods with the aim of recovering pectin exhibiting high similarity to the commercial ones. *Citrus aurantifolia* is the

main lime production of Iran with more than 600 thousand tons per year (FAO, 2007). The extracted and commercial pectins were applied in Doogh and some intra-molecular properties characterized by simple tests to find out the stabilizing effects of them. Doogh is traditional Iranian fermented milk with a consumption of more than 1 million ton per year (FAO, 2007). In order to get a better understanding of the pectins stabilizing properties in Doogh, we focused on DM distributions over the pectin molecules. Since pectin is a complex molecule, to simplify the investigations in order to interpret the DM distribution patterns, different HGs were prepared. We probed into the HG structure with different DM and methyl distribution prepared by basic and p-PME action on different substrates. Endo-PG and pectin lyase were used to find out more information about DM distribution and simplify the comparision of pectin over HG stretches in order to better translate the pattern of methyl distribution for each case. In order to perfom the analyses, some original methods have been conducted to recognize and better classify the samples in accordance with the DM distribution over the pectin HG stretches. The first keystone was to generate adequate model substrate and the next was to purify a pectin lyase with a very specific mode of action to have an accurate interpretion of enzymatic digestion. The other one was to have different types of oligomers (saturated, unsaturated) defined as standards, for the recognition of enzymatic digests by HPAEC.

RESULTS

1. Isolation and characterization of model substrates (HG)

1.1. Introduction

As developed in the Bibliography chapter, pectin's functional properties and reactivity toward calcium and other cations is largely dependent on the amount of methylated GalAs and their distribution pattern within the HG stretches (Willats *et al*., 2006). Two general patterns of methyl ester distribution are recognized, random or ordered (blockwise) (Willats *et al*., 2001). Demethylation of pectin can be performed by chemical (alkaline) or fungal pectin methyl esterase (*f*-PME) to form a random distribution while plant pectin methyl esterases (*p*-PME) have been shown to lead to blockwise removal of esters (Denès *et al*., 2000).

Chemically similar pectins display differences in functional properties (Guillotin *et al*., 2005). The size of the demethylated blocks along HG backbone was indeed shown to affect the reactivity of pectin towards cations such as calcium (Limberg *et al*., 2000b; Ralet *et al*., 2001; Thibault and Rinaudo, 1986). The impact of structural change by PMEs on functional properties of pectin has been investigated by several biological, biochemical and physicochemical methods.

To simplify the chemical complexity of pectin in order to propose detailed estimation at molecular level, HGs have been successfully isolated by acidic means (Thibault *et al*., 1993). Acid-resistant totally demethylated HG with a length of 72-100 GalA units has been obtained.

In the present work, different HG models have been prepared by chemo-enzymatic methods from an originally fully demethylated HG. The aim of this work was to fully master DM and methyl esters distribution onto model substrates to be used for further enzymatic investigations.

1.2 Materials and methods

1.2.1 Preparation of model HGs

1.2.1.1 Preparation of unmethylated HG

Unmethylated HG has been prepared according to Thibault *et al*. (1993). Citrus pectin (Danisco, Lot 0080677, DM% 70.7) was first deesterified. Pectin (~ 25

g) was solubilized in 3.6 L of water under gentle magnetic strirring overnight at 4°C. 500 mL of cold 0.2N NaOH were added drop by drop to raise pH 11.8. After 20 min at 4°C, 0.2N NaOH was added to maintain pH at around 12. pH was checked regularly for 5 h. After 17 h at 4°C, 1N HCl was added drop by drop to reach pH 5. The slurry was then concentrated to 2 L. Precipitation of demethylated pectin was performed by adding 3 volumes of ethanol to the slurry. After 17h at 4°C, the suspension was filtered through Nylon (150µm). The precipitate was washed twice with ethanol 70%, then twice with ethanol 96% and finally twice with acetone before being dried in a ventilated oven at 40°C. The recovered deesterified pectin was dissolved in 2.5 L of water to have a concentration of ~ 10g/L. 0.25N HCl was added to the pectin solution to reach a final concentration of 0.1N HCl. Hydrolysis was performed at 80°C for 72h. After cooling, the acid-soluble and acid-insoluble fractions were separated by centrifugation of the mixture at 15000g for 20 min. The acid-insoluble fraction, corresponding to HG, was washed twice by 0.1M HCl, then distilled water, and resuspended in distilled water (unmethylated HG in H^+ form). An aliquot of the suspension was taken, in which 1N NaOH was added drop by drop under magnetic stirring to reach pH 6. The thereby obtained solution was freeze-dried to yield unmethylated HG (quoted HG0) in Na^+ form.

1.2.1.2 Preparation of highly methylated HG

Highly methylated HG was prepared according to Renard and Jarvis (1999). Tetrabutyl ammonium hydroxyde (TBA) was added to the rest of HG0 in H^+ form to reach pH7 and the resulting solution (HG-TBA) was extensively dialyzed against distilled water before freeze-drying. HG-TBA (40 g) was dissolved in dimethyl sulfoxide (900 mL) in a capped erlenmayer flask by overnight magnetic stirring at room temperature. Methyl iodide was then added (HG-TBA/CH_3I, 1/1, mol/mol) (3 times 2 mL at 30 min intervals). The solution was kept in the dark under continuous gentle stirring for 24 h. Residual CH_3I was removed through a nitrogen flux (1 h at room temperature). The solution was dialyzed twice against 0.2 M NaCl, then extensively against distilled water before freeze-drying.

1.2.1.3 Demethylation of highly methylated HG with NaOH

Aliquots (1g) of highly methylated HG were demethylesterified by adequate amounts of 0.2 M NaOH to yield samples of known DM. At the end of the reaction,

samples were freeze-dried. The samples thereby obtained are quoted **HG-Bx** (with B = basic deesterification and x=degree of methylesterification).

1.2.1.4 Demethylation of highly methylated HG with p-PME

Aliquots of highly methylated HG (1g) were deesterified by *p*-PME (Sigma 5400; 194 U/mg) to yield a series of *p*-PME-deesterified HG samples of varying DM. Highly methylaled HG aliquots were dissolved in phosphate buffer 100mM pH 7.6 for 30 min at 30°C. Adequate amounts of *p*-PME were added and solutions were kept under gentle stirring at 30°C for various times. At the end of the reaction, enzymes were inactivated: solutions were cooled in an ice bath and pH was brought to 5 with 2N phosphoric acid. Solutions were then extensively dialysed, pH was brought to 6 with NaOH and solutions were finally freeze-dried. Various samples were recovered; they are quoted **HG-Py** (with P=PME deesterification and y = degree of methylesterification.

1.2.1.5 Demethylation of selected NaOH-demethylated HG with p-PME

Aliquots of selected samples from HG-B series (1g) were deesterified by p-PME (Sigma 5400; 194 U/mg) to yield a series of HG-B-P samples of varying DM. The selected HG-B aliquots were dissolved in phosphate buffer 100mM, pH 7.6 for 30 min at 30°C. Adequate amounts of *p*-PME were added and solutions were kept under gentle stirring at 30°C for various times. At the end of the reaction, solutions were treated as described above. Various samples were recovered; they are quoted **HG-Bx-Pz** (with B=basic deesterified HG, P=PME deesterification and x and z = degree of methylesterification).

1.2.2 Analytical

1.2.2.1 Galacturonic acid

The galacturonic acid content was quantified according to the automated meta-hydroxybiphenyl method (Thibault, 1979).

It is based on the analysis of the degradation products of pectin obtained from hydrolysis with concentrated acid (sulfuric acid) at high temperature. These compounds react then with meta-hydroxydiphenyl and form color complex. The coloration obtained is proportional to the galacturonic acid concentration.

Galacturonic acid was quantified after saponification of the pectin samples (0.2M NaOH, 30 min, room temperature) and neutralization (0.2M HCl).

1.2.2.2. Neutral sugars

Individual neutral sugars were analysed as their alditol acetate derivatives (Blakeney *et al.*, 1983) by gas chromatography after hydrolysis in 2M trifluoroacetic acid at 121°C for 2.5 h (Ralet and Thibault, 2002). *Myo*-inositol was used as an internal standard. The separation was performed on a DB-225 fused-silica capillary column (30 mL× 0.32 mm i.d., J&W Scientific, Courtabœuf, France).

1.2.2.3 Methyl groups

Methanol released by alkaline de-esterification (1N NaOH) for 1h at 4˚C in the presence of $CuSO_4$, was quantified by HPLC on C_{18} Superspher eluted at room temperature with sulphuric acid solution at pH 3.5, at a flow rate of 0.7 mL/min (Levigne *et al.* 2002a). Isopropanol was used as internal standard. DM was calculated as the molar ratio of methanol to galacturonic acid.

DM was also determined by titrimetry. All samples were recovered in acidic form by passing through a strong H^+-exchanger (Rohm and Hass Amberlite IR 120) ($25cm^3$). Free carboxylic acid functions were quantified at the neutralization point by conductimetric titration with NaOH of known molarity and total carboxylic function were determined by colorimetry on the same solutions after saponification (Ralet and Thibault, 2002). DM was calculated as shown in eq. 1:

DM= 100 × (total carboxylic functions- free carboxylic functions)/ total carboxylic functions (1)

1.2.3 Macromolecular characteristics

HP-SEC was performed at room temperature on a system constituted of one Shodex OH SB-G pre-column followed by two columns in series (Shodex OH-Pack SB-804 HQ and OH-Pack SB-805 HQ, Shodex, Showa Denko KK, Miniato, Japan) eluted with 0.05 M $NaNO_3$ buffer containing 0.02% NaN_3 as preservative at a constant flow rate of 42 mL/h. A differential refractometer (RI) (ERC 7517A), a multiple-angle laser light-scattering device (MALLS) (Mini Dawn, Wyatt, Santa Barbara, CA) operating at three angles (41°, 90° and 138°) and a differential viscometer (T-50A, Viscotek) were used as detectors. Samples were solubilised in

0.05 M $NaNO_3$ buffer containing 0.02% NaN_3, left overnight with tail-over-head continuous mixing, heated at 40°C for 15 min, cooled and filtered (PVDF filter 13 mm diameter, 0.45 μm pore size) (Whatman Inc., Sandford, ME, USA) prior to analysis. Samples were injected automatically through a 50 μL loop. Data for molar mass determinations were analysed using Astra 1.4 software (Wyatt, Santa Barbara, CA) taking dn/dc 0.146. Unsmoothed M_w and M_n values were obtained from raw data. Intrinsic viscosity values were obtained using Tri SEC Software (Version 3.0, Viscotek, Houston, TX).

1.2.4 Calcium activity

HGs were dissolved in ultra-pure water (~7 mequiv/L) by overnight magnetic stirring at room temperature. Percolating the samples through a strong H^+-exchanger (Rohm and Hass Amberlite IR 120) allowed the recovery of HG samples in H^+ form at a concentration of ~1mequiv/L. Conductimetric measurements were carried out at 25±0.2˚C with a CDM83 conductimeter (Radiometer Analytical) equipped with a double platinum electrode CDC 241U (Radiometer Analytical). The cell constant was determined with 0.05 % (w/w) NaCl before each set of measurements. The titrations were performed with freshly prepared $Ca(OH)_2$ (10 mequiv/L). The calcium activity coefficient at the neutralization points (γ_{Ca2+}) was determined by means of a dual-wavelength spectrophotometric method (Φ=A530/A493 nm) using tetramethylmurexide as an activity probe for calcium ions (30μL, 10^{-3} M + 0.97 mL of HG solution in Ca^{2+} form) (Dronnet *et al.*, 1996; Ralet *et al.*, 2001). Values reported correspond to the ratio of the activity of the calcium ions in ideal $CaCl_2$ solutions at the same ionic concentration (eq. 2, 3).

$$Ca^{2+} \text{ Total (mol/L)} = N\ Ca(OH)_2 \times Vol\ Ca(OH)_2 \times 10^{-3} / (Vol\ Pectin + Vol\ Ca(OH)_2) \times 1000/2 \quad (2)$$

$$\gamma^{++} = \text{Free } [Ca^{2+}] / \text{ Total } [Ca^{2+}] \quad (3)$$

The theoritical values of calcium activity were calculated by the Manning theory (eq. 4, 5 and 6):

$$\gamma^{+} = \exp(-0.5z\xi) \quad \text{when } \xi<1/z \quad (4)$$

$$\gamma^{+} = \exp(-0.5/z\xi) \quad \text{when } \xi>1/z \quad (5)$$

$$\xi = 1.61 \times [1-(DM/100)] \quad (6)$$

ξ = effective charge density

z = charge of the counterion

1.2.5 Degree of blockiness

6 µl of poly-galacturonase (*endo*-PGII; 36 nkat/mL) (the enzyme purified as described by Sakamoto *et al*. (2003) from a liquid preparation of *Aspergillus niger*, Novozymes) was added to 3 mL of HG solution (~1mg/mL) in 50mM acetate buffer pH 4. Hydrolysis was performed at 40°C for 3 days, fresh enzyme (6 µl) being added at 24h and 48h. After 3 days, hydrolysates were filtered (0.45µm Millipore) and injected to HPAEC.

On alkaline conditions (pH 13) the hydroxyl groups over the HG stretches convert to anionic form and can be separated by anion exchange chromatography. A Dionex system equipped with a Carbopac PA1 column (2mm) with pulsed amperometric detection was used. The elution was carried out with Na-acetate and NaOH for 90 min. Flow rate of the mixture was constant at 0.25 mL/min and concentration of NaOH was kept constant at 100 mM. The linear gradient of Na-acetate in the mixture solution was as follows:

Table 1. HPAEC separation conditions for GalA oligomers

Time (min)	1M NaOAc%	0.5M NaOH %
0-20	25-50	20
20-40	50-60	20
40-60	60-70	20
60-65	70-80	20
65-66	80-25	20
66-90	25-25	20

Chroméléon Software (Dionex) was used for data acquisition and processing. Monomer, dimer and trimer of GalA were used as standards (Bonnin *et al.,* 2002).

Degree of blockiness was calculated by measuring the amount of monomers, dimers and trimers of GalAs released from pectic molecules treated with *endo*-PG divided by the amount of free GalAs present in the sample (Fig. 8) (eq. 7) (Daas *et al*., 1999, 2001a, b; Löfgren *et al*., 2005). Once the amount of released oligomers is expressed as a percentage of total GalA (eq. 8) it is called DB absolute (DB_{abs}), which can more illustrate the blockiness pattern of pectic molecules (Fig. 9) (Guillotin *et al*., 2005; Ström *et al*., 2007).

$$DB = \frac{[(1 \times 1^0) + (2 \times 2^0) + (3 \times 3^0)]\, M_w{}^{GalA}}{(1 - DM/100)\, m_{pectin}\, (m_{uronicacid}/ m_{pectin})} \times 100 \qquad (7)$$

$$DB_{abs} = \frac{[(1 \times 1^0) + (2 \times 2^0) + (3 \times 3^0)]\, M_w{}^{GalA}}{m_{pectin}\, (m_{uronicacid}/ m_{pectin})} \times 100 \qquad (8)$$

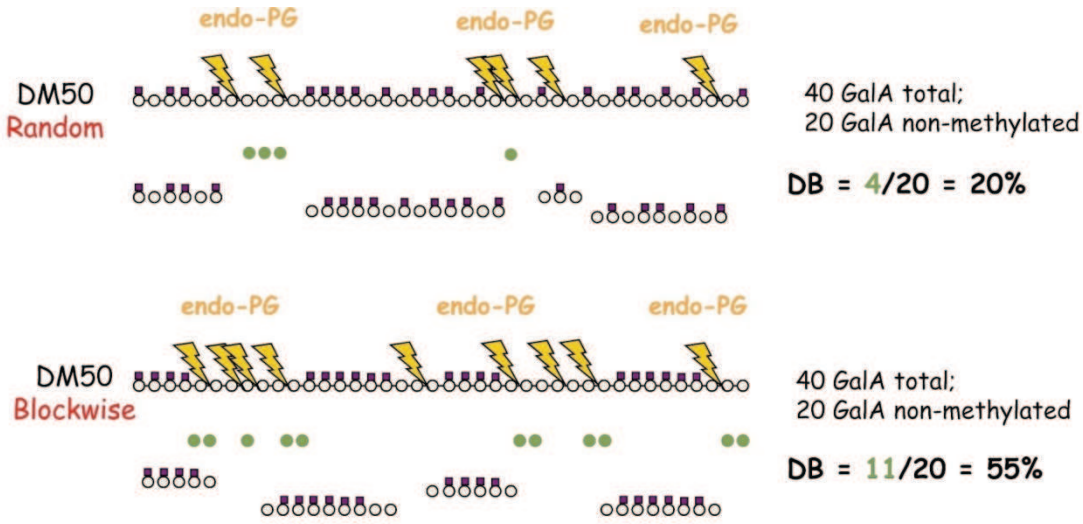


Fig. 8. Schematic representation of a random and a blocky pectin. The circles represent GalA residues while square symbols correspond to methyl groups.

Fig. 9. Schematic representation of a blocky pectin and its values of DM, DB and DB_{abs}. The filled symbols represent the methyl esterified GalA and the open circles represent the non-methylesterified GalA (Ström *et al*., 2007).

1.3. Results

1.3.1. Non-methylated HG isolation

Pectin was first deesterified before isolating HG in order to avoid differences in susceptibilities of acid hydrolysis of glycosidic bonds because of the ester groups. The deesterification was performed under cold alkaline conditions to minimize β-elimination of the GalA chains (Fig. 10).

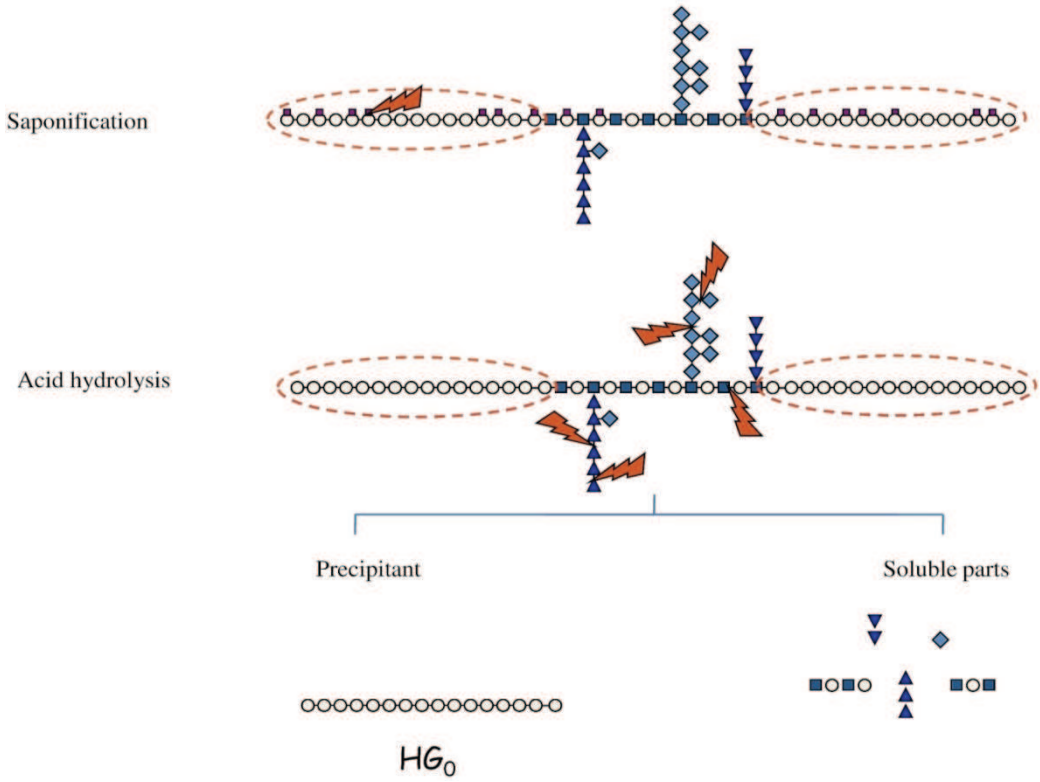


Fig. 10 . Isolation of HG0

The recovered HG0 contains > 99 sugar mol% of GalA with traces of Rha, Xyl and Gal. The molecular weight of HG0 is 17430 g/mol (+/- 10%) (~ 100 units of GalA) (Fig. 11) with a low polydispersity index (1.1). HG0 exhibits an intrinsic viscosity of 78 mL/g. Those results are in good agreement with values reported in the literature for homogalacturonan obtained from citrus pectin using the same procedure (Thibault *et al.*, 1993; Yapo *et al.*, 2007).

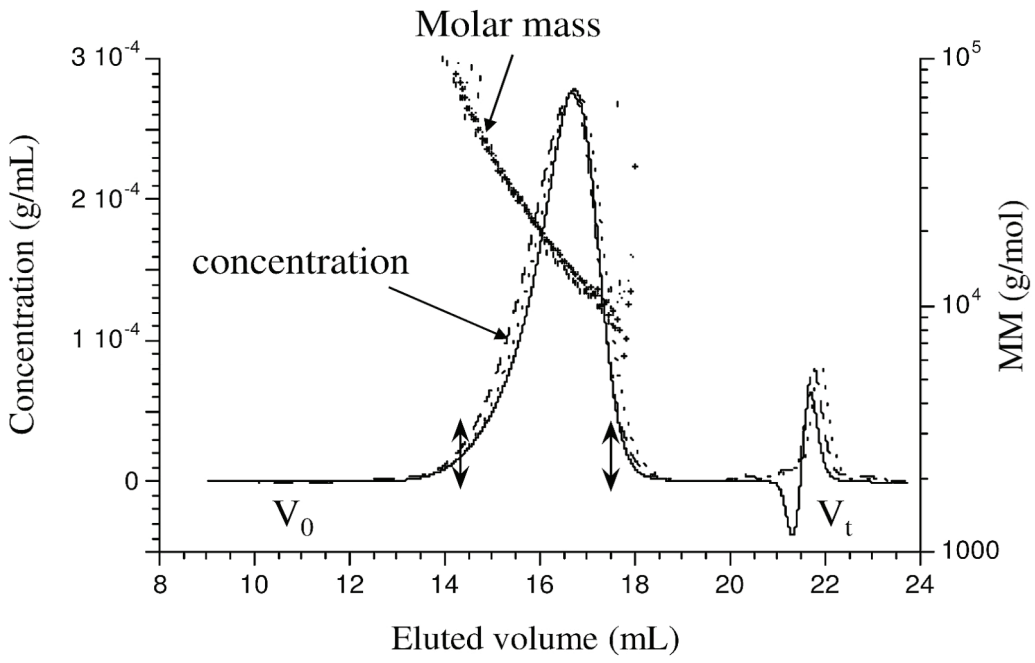


Fig. 11. HPSEC chromatogram of HG0

1.3.2. Chemical methylation of the isolated HG

Methylation of HG0 was carried out with some modifications of the procedure performed by Matricardi *et al*. (1995) and Renard and Jarvis (1999). A DM of 96 was reached. Such a high DM made HG96 hardly soluble in water at room temperature but visual solubilization was achieved by treating the sample for 15 min at 40°C. M_w of 23070 (+/- 10%) (~ 121 units) was estimated. It is noteworthy that due to the presence of remaining aggregates, this value is most probably overestimated. Indeed, an intrinsic viscosity (which is not sensible to the presence of aggregates) value of 80 mL/g was observed, very close to the value obtained for HG0 (78 mL/g). It seems that degradation of HG stretches did not occur, contrary to what was reported by Renard and Jarvis (1999).

1.3.3. Chemical and enzymatic demethylation of the highly methylated HG

HG96 has been modified by enzymatic and chemical methods to generate two series of model HGs. The alkali treated HGs are addressed as HG-Bx and the *p*-PME treated ones correspond to HG-Py. Chemical composition and some macromolecular features of generated HGs are shown in Table 2.

Table 2. Some chemical and physicochemical features of HGs (B- and P- series)

Sample	DM (mol%)	Enzymatic condition	*M*w (g/mol)	Intrinsic viscosity (mL/g)
B-series				
HG-B82	82	-	15030	65.6
HG-B69	69	-	15230	61.2
HG-B56	56	-	15610	57.2
HG-B40	40	-	15800	57.0
HG-B20	20	-	16750	53.9
P-series				
HG-P75	75	353 units/g of HG, 25 min, 30°C	19040	67.7
HG-P64	64	353 units/g of HG, 70 min, 30°C	18760	64.9
HG-P56	56	1765 units/g of HG, 40 min, 30°C	21520	63.6
HG-P36	36	1765 units/g of HG, 120 min, 30°C	19610	63.5
HG-P14	14	1765 units/g of HG, 480 min, 30°C	18810	64.9

DM, degree of methylation (mol %); *M*w, weight-average molar mass (g/mol)

HGs from B- and P-series exhibit homogenous molar mass distributions with no major degradation compared to the original HG, which is consistent with the results of Yapo and co-workers (2007) for HG isolated from extracted pectin of citrus peels by different chemical methods and the findings of Hellín and co-workers (2005) who isolated HGs from lime pectin by enzymatic degradation. The intrinsic viscosity of HGs is comparable to previous values reported for HGs of citrus pectin (Thibault *et al*., 1993; Yapo *et al*., 2007). Isolated HGs were characterized by a very low polydispersity index (1.0-1.1), revealing that they are very homogenous with respect to molar mass, which is in good agreement with the previous findings (Hellín *et al.*, 2005).

1.3.4. Enzymatic modification of selected NaOH demethylated HG

In order to find out the impact of p-PME on different HG substrates and to potentially generate other types of methylesterification patterns, selected NaOH-demethylated HG samples (either already available, either newly generated) were treated with p-PME. The newly generated HGs are addressed as HG-Bx-Pz.

The conditions used to generate these new substrates are presented in Table 3.

Table 3. HG-BP-series generated from HG-B77, HG-B76 and HG-B82 at different conditions of enzymatic digestion

Initial sample	Initial DM	Enzymatic condition	Final samples	Final DM
HG-B77	77	5.5 units/g of HG, 5 min, 30°C	HG-B77-P63	63
HG-B76	76	27 units/g of HG, 5 min, 30°C	HG-B76-P56	56
HG-B76	76	109 units/g of HG, 7 min, 30°C	HG-B76-P49a	49
HG-B76	76	109 units/g of HG, 15 min, 30°C	HG-B76-P49b	49
HG-B82	82	109 units/g of HG, 15 min, 30°C	HG-B82-P48	48

1.3.5. Methylester distribution of HG model samples

The two approaches chosen for exploring the distribution pattern of methyl esters over the different HG model samples were degree of blockiness and calcium activity determination (Table 4).

Table 4. Degree of blockiness and calcium activity of the different HG model samples

Sample	DM	(DMi-DMf)/DMi	DB	DB_{abs}	γCa^{2+}	γCa^{2+} exp/theo
B-series						
HG-B82	82	0.146	4	1	0.82	1.10
HG-B69	69	0.281	9	3	0.65	1.07
HG-B56	56	0.417	21	9	0.56	1.31
HG-B40	40	0.583	33	20	0.43	1.37
HG-B20	20	0.792	56	44	0.06	0.26
P-series						
HG-P75	75	0.219	61	15	0.23	0.34
HG-P64	64	0.333	81	29	0.14	0.27
HG-P56	56	0.417	73	32	0.12	0.28
HG-P36	36	0.625	71	46	0.08	0.27
HG-P14	14	0.854	66	57	0.06	0.27
BP-series						
HG-B77-P63	63	0.182	5	2	0.62	1.22
HG-B76-P56	56	0.263	20	9	0.56	1.31
HG-B76-P49a	49	0.355	35	18	0.38	1.03
HG-B76-P49b	49	0.355	43	18	0.38	1.03
HG-B82-P48	48	0.415	53	22	0.29	0.79

DM, degree of methylation (mol %); (DMi-DMf)/DMi, the difference between initial and final DM of the HG divided by DM of initial HG that new HG originated from; DB, degree of blockiness (mol %); DB_{abs}, absolute degree of blockiness (mol %)

As shown in Table 4, DB values are low for high DM samples originating from the B-series (HG-B56 to HG-B82). DB values then increase, all the more DM is low (HG-B40 and HG-B20). Although NaOH is known to induce demethylation in

a random fashion, long sequences of non-methylesterified GalA residues able to be degraded by endo-PG are statistically generated when DM decreases below a value of 35-40 (Ralet and Thibault, 2002). DB values of samples from the P-series are extremely high compared to corresponding samples from the B-series, those values remaining roughly similar whatever the DM. Finally, samples from the BP-series show intermediate values.

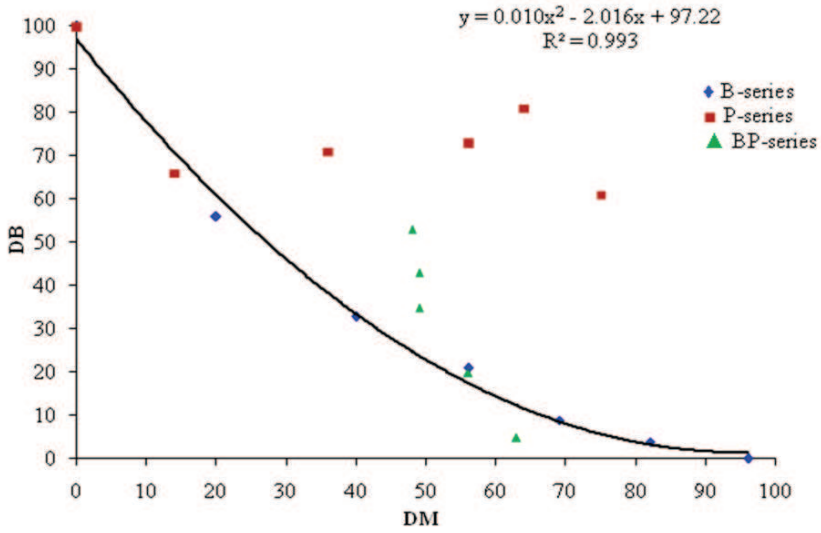


Fig. 12. DB values versus DM for the different HG series

The values reported for DB were determined in duplicate (standard error < 8%).

A polynomial relationship between DB and DM can be observed for HG samples from the B-series, DB values decreasing rapidly when DM increases. On the opposite, DB values observed for samples from the P-series are high and don't seem to depend upon DM in the range observed. HG-B20 exhibits a DB value close to DB values observed for HG samples from the P-series. Two intermediate samples (HG-B77-P63, HG-B76-P56), cannot be differentiated from the B-series. In those samples the difference between the DM of the "mother HG" (initial DM, DMi = 76 or 77) and the final DM (DMf) is small.

For the other three intermediate samples, DB values are observed between B and P series. Those samples are the ones in which the difference between DMi and DMf is important. In order to get a better insight into the DB values and potentially

get a better differentiation between the different series, DMi was tentatively taken into account.

In Fig. 13, this claim is indeed magnified since initial DM (DMi) of all series has been taken into consideration.

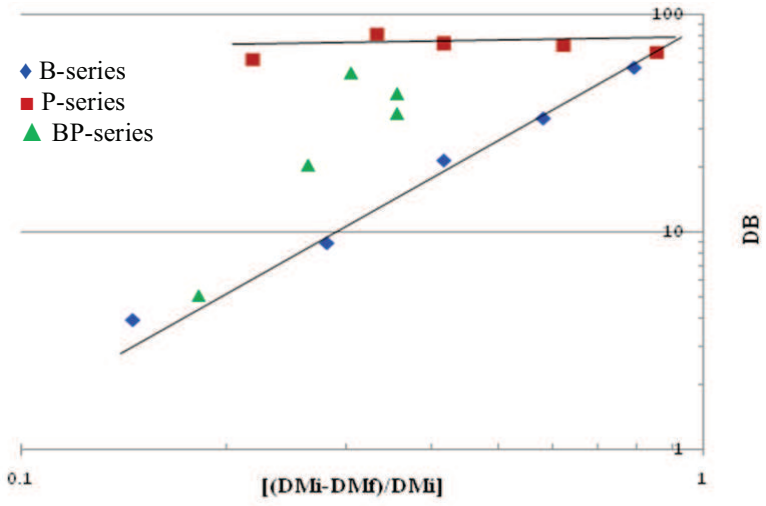


Fig 13. DB values versus (DMi-DMf)/DMi values for all samples.

As presented in Fig. 13, HG-B77-P63 is still not differentiable from B-series. For HG-B76-P56 a significant difference is observed from B-series while HG-B82-P48 is close to P-series.

According to some authors, DB_{abs} is more informative than DB to illustrate the impact of blockiness on rheology of pectin solution, especially in the presence of Ca^{2+} (Ström *et al*., 2007; Slavov *et al*., 2009).

As shown in Fig. 14, for both B- and P- series DB_{abs} values raise by decreasing DM and for BP-series these values are in intermediate phase.

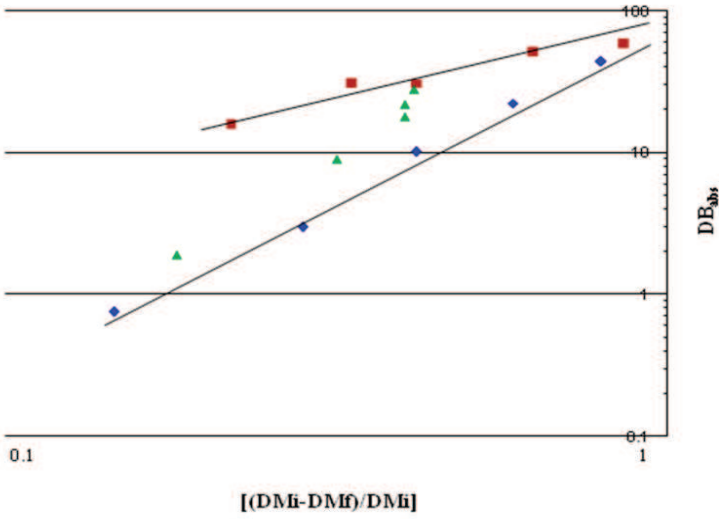


Fig. 14. DB_{abs} values versus (DMi-DMf)/DMi values for all samples.

The values reported for DB_{abs} were determined in duplicate (standard error < 8%). As observed in Fig. 14, DB_{abs} values give roughly similar information to DB values. It is however noteworthy that HG-B77-P63 can be differentiated from B-series trend.

The reactivity toward calcium ions is another approach used to determine the distribution pattern of methyl groups over HG domains (Ralet *et al*., 2001; Löfgren *et al*., 2005; Ström *et al*., 2007; Slavov *et al*., 2009). Since the length of HG is similar for all samples, the only intrinsic parameter that can control calcium binding properties is DM and its distribution pattern (Lutz *et al*., 2009).

The values of γCa^{2+} of HGs from the B-series (Table 3) are in the range reported by Ralet *et al*. (2001) for pectin samples deesterified with alkali while values obtained for HGs from the P-series are much lower than those reported by the same authors for pectin samples deesterified by plant-PME. This indicates that p-PME treated HGs generated in this work can bind calcium ions more tightly than p-PME treated pectins. This difference belongs to the different DM (96 vs 82) of mother samples used for p-PME deesterification. This may emphasize again that the initial DM is very important and the average length of demethylated blocks generated by p-PME increases with increasing the DM of the mother sample. The dependency of calcium activity upon DM for the different HG series is presented in

Fig. 15. The values reported for γCa^{2+} were determined in duplicate (standard error < 3%).

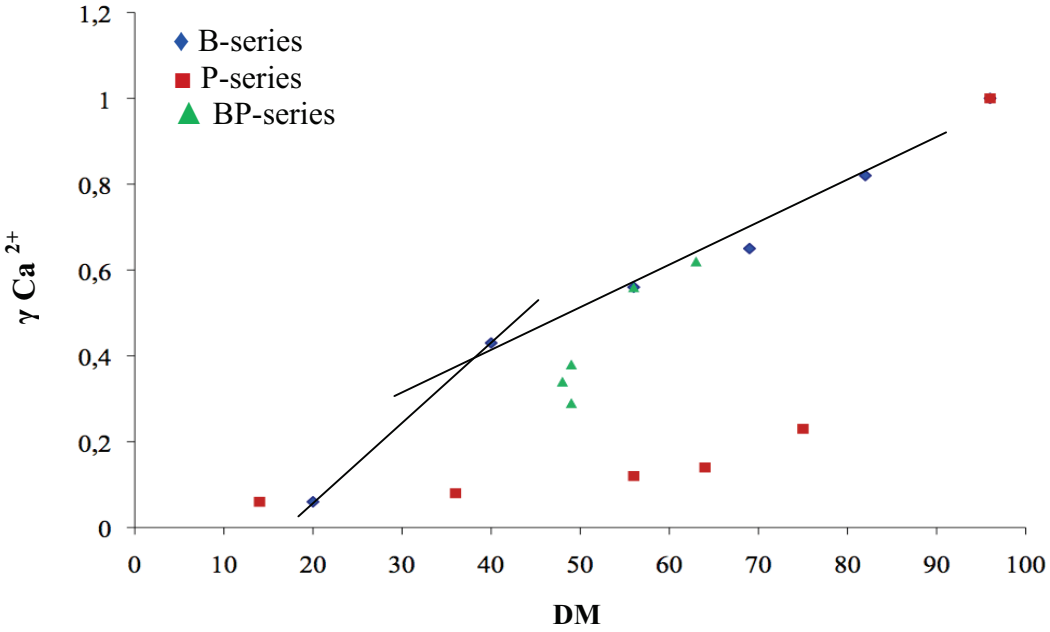


Fig. 15. γCa^{2+} versus DM of all HG samples

There is a linear relationship between DM40 and DM90 for B-series. For B-series a drop in γCa^{2+} is shown when DM decreases below 40, which is consistent with previous reports (Ralet *et al*., 2001). Statistically, for a random process, blocks of non-methylated GalA are indeed generated for DM < 40 (Ralet *et al*., 2001). The dependency of γCa^{2+} values upon DM is much lower for samples from the P-series compared to samples from the B-series. This shows that intermolecular binding of calcium ions to carboxyl groups of two molecules leading to the formation of dimers exists for all HG samples from the P-series, whatever their DM. The formation of demethylated blocks long enough to induce calcium bridging of two HG molecules is also obvious for HG-B20, in agreement with previous findings (Ralet *et al*., 2001). The trend of the B-series curve is almost the same as expressed by Ralet and co-workers (2003) for citrus pectin of B-series. For BP-series, 2 samples (HG-B77-P63 and HG-B76-P56) cannot be differenciated from B-series, the rest are placed in between of B- and P-series. This result is in total accordance with what was observed for DB and DB_{abs}.

The physicochemical properties of polyelectrolytes, in aqueous solutions, such as electrical conductivity, interfacial activity, and formation of microdomains are mainly determined by the linear charge density. Most useful information comes from the determination of the free fraction of counterions to characterize a polyelectrolyte in the absence of external salt. Its theoretical value is directly related to the charge parameter and the valency of the counterion. The relations depend on the charge parameter value and on the valency of the counterions (Manning, 1974; Rinaudo, 2009). The degree of binding of the counterions can be determined by conductimetry and spectrophotometry (Ralet *et al*., 2001).

The values of experimental to theoretical γCa^{2+} (exp/theo) are roughly constant for samples from the P-series highlighting the formation of HG dimers through calcium ions, and hence the presence of long demethylated GalA stretches, at all DM (Fig. 16).

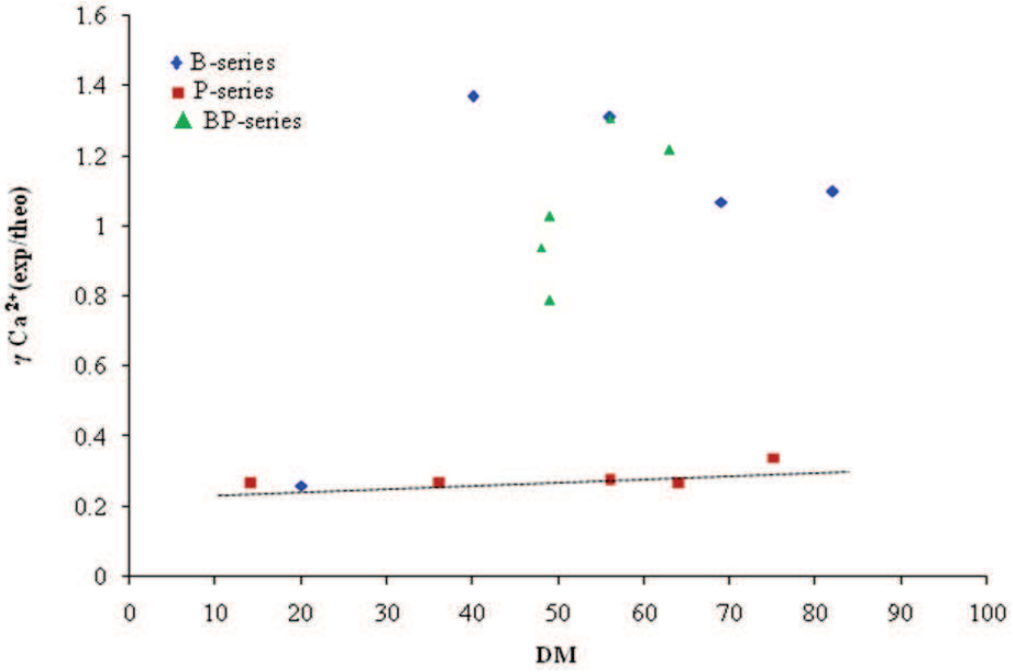


Fig. 16. γCa^{2+} (exp/theo) versus DM for all HG samples

As presented in Fig. 16, γCa^{2+} (exp/theo) of 2 samples of BP-series (HG-B77-P63 and HG-B76-P56) are not differentiable from B-series, which is in good agreement with the results of $\gamma Ca^{2+}$and DB.

1.4. Discussion

Not only DM but also the distribution of methyl esters upon HG domains has a great impact on pectin functionality. Differences in distribution can be observed within one pectic molecule (intramolecular level) or between different molecules (intermolecular level). There are discernible differences between the methyl-esterification patterns on pectin produced by the action of p-PME and fungal PME or base catalysis. Base-catalysed deesterification shows the best fit for a theoretically random deesterification process while f-PME deesterification pattern is more ordered but still close to random or homogeneous (Limberg *et al*., 2000b). P-PMEs appear to be processive enzymes, introducing demethylated blocks of GalA into the HG region (Limberg *et al*., 2000a; Limberg *et al*., 2000b; Ralet and Thibault, 2002). Since pectin is a very complex molecule, designing HG models could simplify the complexity of the molecule and illustrate in more details methyl ester distributions over the HG backbone (Guillotin *et al*., 2005). In this regard, three series of HGs with different patterns of methyl groups were prepared from HG isolated from citrus pectin by alkali (B-series) and p-PME (P-series) treatment. A third series was prepared by using p-PME on selected samples from the B-series (BP-series).

Blockiness is correlated to calcium binding properties of HM pectin in an egg-box like system and in modelling for junction zones interactions (Braccini and Perez, 2001). So, the possibility of designing the functional properties of pectin by manipulating the size and distribution of blockiness in the HG region *via* reaction with various PMEs under controlled conditions could create the potential to obtain desired engineer properties of pectin.

A minimum number of 9 consecutive non-esterified GalAs were determined for an interaction to occur (Liners *et al*., 1992). Other reports estimated a minimum block size of 8-12 GalA units (Powell *et al*., 1982), or even 15-20 GalA units (Kohn and Luknar, 1977; Braccini and Perez, 2001).

Recently, by limited endo-PG digestion combined with chromatographic analysis, it has been said that a minimum block size contains approximately 6-13 GalAs for calcium interaction to occur (Luzio and Cameron, 2008). It is also said that 2-4 block numbers on each molecule appears to be conductive for calcium

interaction. That shows very few blocks need to be present to be incorporated into the calcium sensitivity (Luzio and Cameron, 2008).

In Fig. 17, values of γCa^{2+} were compared to the values reported by Ralet and co-workers (2001) depending on the difference between initial DM of the mother HG or pectin and final DM reached. It is noteworthy to say that the mother HG in this study has DM96 while the mother pectin used by Ralet *et al*. (2001) had DM81.

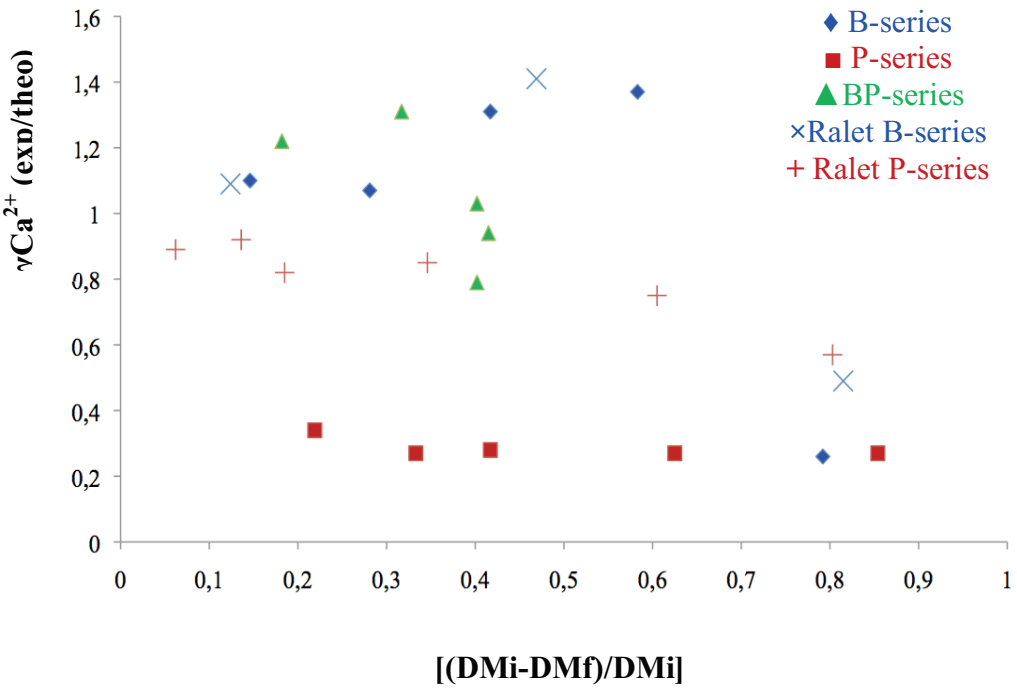


Fig. 17. γCa^{2+}(exp/theo) versus (DMi-DMf)/DMi for all HG samples and values of Ralet *et al*. (2001)

Manning's theoretical values calculated for P-series must however be considered cautiously as this theory uses a model of uniform charge density along the polyion. Furthermore, pectins are known to be heterogeneous with respect to sugar composition, molar mass and charge density.

As observed, γCa^{2+}(exp/theo) values of B-series fit very well with values for random distribution over whole pectins as reported by Ralet *et al*. (2001). Compared to the results reported for P-series pectin by Ralet *et al*. (2001), new P-series exhibit very low values of exp/theo for all DM, hence very long demethylated blocks were generated from HG96. For BP series, very short blocks of GalAs were observed for

HG-B77-P63 and HG-B76-P56 while short blocks of GalAs exist in HG-B82-P48, HG-B76-P49a and HG-B76-P49b, which also showed intermediate values of γCa^{2+}(exp/theo). Fig. 18, represents a graphical overview of p-PME process on different HG of various DM producing non-methylated stretches that differ in length. The more initial DM, the more possibility to have longer non-methylated GalAs exist.

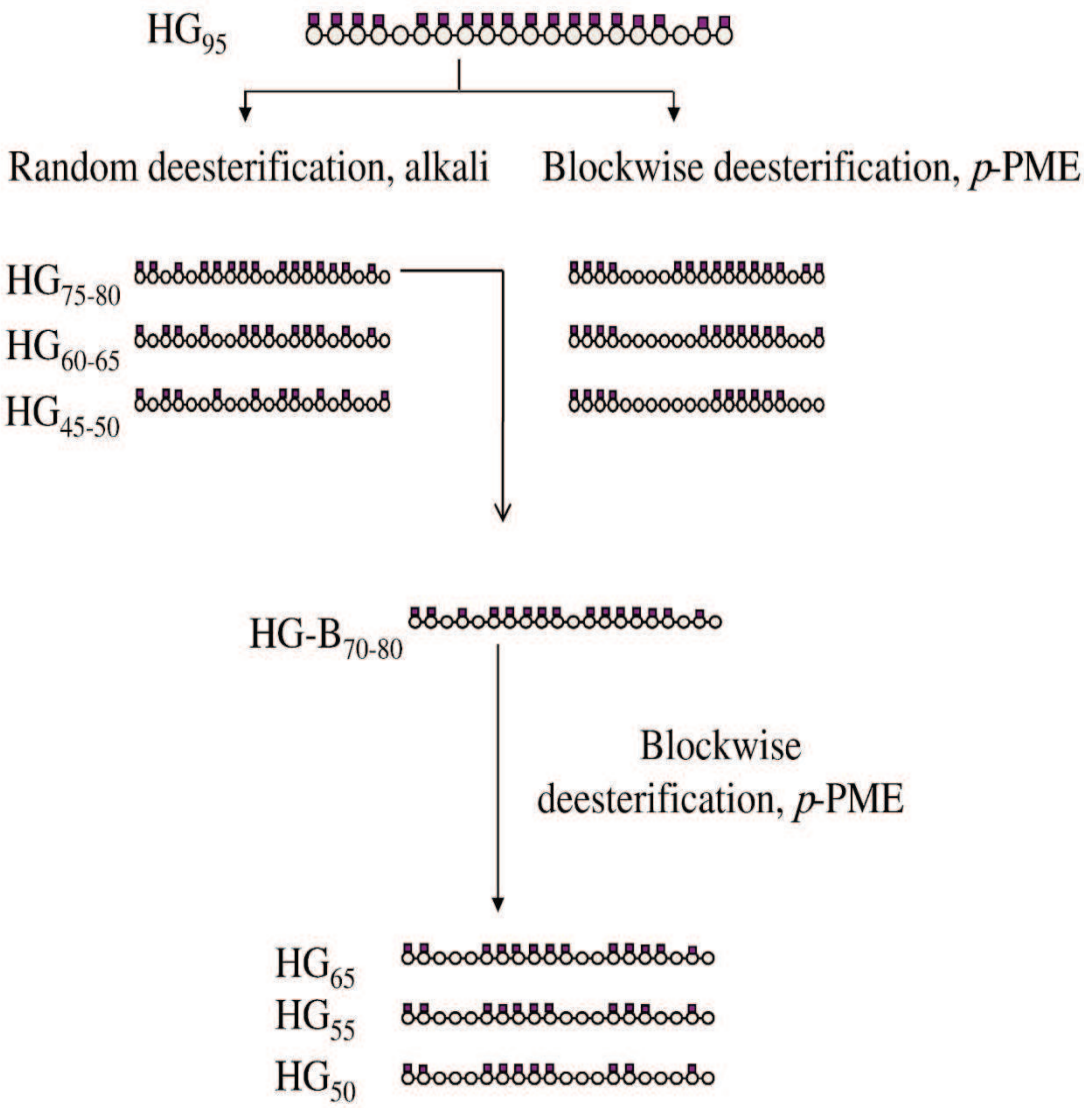


Fig. 18. Shematic representation of *p*-PME action on different substrates of different DM distribution pattern

Since DM of the initial mother sample was obviously important, we considered the impact of initial DM of the substrate on final products at molecular level. Taking

into account initial DM allowed a differentiation between HG samples with subtle differences in distribution patterns of methylesterification.

In this work, we could find good correlation between DB, γCa^{2+} and experimental γCa^{2+} over theoretical values (Ralet *et al*., 2001). It is also claimed that the value of DB_{abs} correlates well with the reactivity of pectin toward calcium ions (Ström *et al*., 2007) since charge distribution over the pectin backbone has a great impact on formation of "egg-boxes model" between calcium ions and pectin molecules (Ralet *et al*., 2001; Löfgren *et al*., 2005; Lutz *et al*., 2009).

Analysis of complete digestion of pectin molecules with *endo*-PG and quantification of monomer, dimer and trimer on the basis of total GalA, as described by Daas *et al*., (1999) gives relatively uniformative data for blocky structure (Guillotin *et al*., 2005). That's why the term "DB absolute" (DB_{abs}) was introduced by Guillotin and co-workers (2005), which quantify the oligomers amount on the basis of free GalA. Despite of all these reports we could find a harmonic correlation between all parameters of DB, DB_{abs}, γCa^{2+} and γCa^{2+}(exp/theo).

In this regard, Fig. 19 represents the values of γCa^{2+}(exp/theo) versus DB% to magnify the difference between the block sizes of P- and BP-series.

As shown in Fig. 19, HG-B82-P48, HG-B76-P49a and HG-B76-P49b have shorter blocks comparing to P-series and HG-B20. So, HG can be deesterified resulting in numerous but short deesterified blocks of GalAs while it is possible to create less but larger deesterified ones by manipulating the initial DM of HG or pectin.

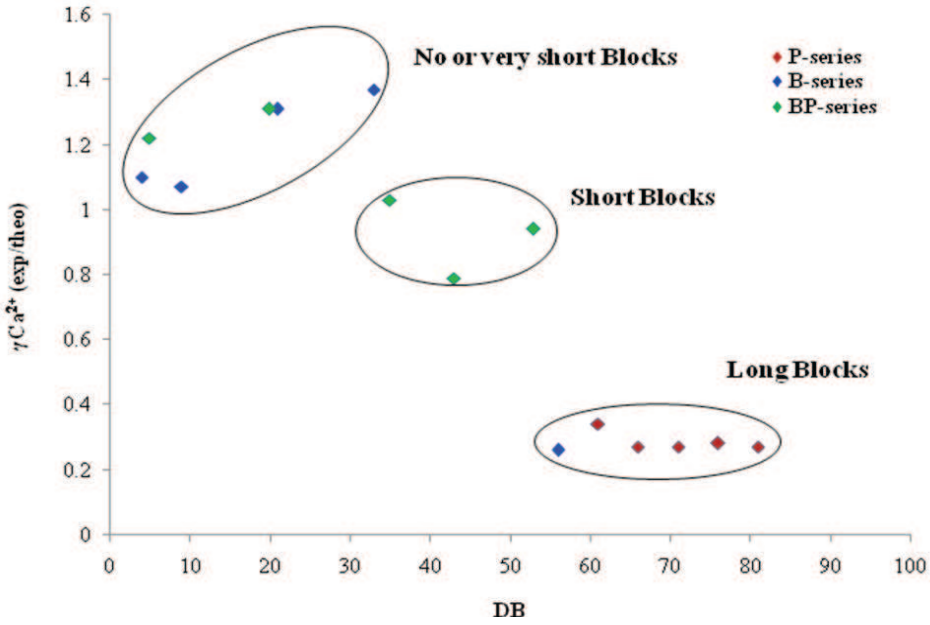


Fig. 19. γCa^{2+}(exp/theo) versus DB of all HG models

1.5. Conclusion

As previously said, we could generate three types of HG according to their distribution pattern of methylesters. By using p-PME on alkali deesterified HGs, shorter blocks of non-esterified GalAs were generated, which can still be calcium sensitive. The generated HGs were prepared as standard models within different range of DM based on various deesterification mode of action. That allowed us to take new approach over different size of non-methylated and methylated GalA stretches in a subtle range of DM. In special, for HG with DM> 50, we could find models with short blocks of non-methylated GalAs and long blocks of methylated GalAs that could not be produced by random process of alkali deesterification. In order to observe that claim, we needed to purify an enzyme suited for HM pectin digestion, which is pectin lyase.

2. Purification and mode of action of pectin lyase

2.1. Introduction

Pectin lyases (PL) are the only enzymes capable of depolymerizing highly esterified pectin into oligomers without prior action of other enzymes (Delgado *et al*., 1993). They cleave pectin by a ß-elimination mechanism that results in the formation of 4,5- unsaturated oligogalacturonates (Targano and Pelosof, 1994). The action of PL is therefore limited to fully or partly methyl esterified regions on the HG domains. Investigation on pectin lyases has been initiated using fungal resources. They are all endo-acting enzymes and have been classified together with the majority of pectate lyases within family 1 of the polysaccharide lyases (Couthino and Henrissat, 1999).

There are two main types of PL, PLA and PLB (Mayans *et al*., 1997; Scavetta *et al*., 1999; Yadav *et al*., 2009). The activity of PLA depends on the DM of the substrate. Absence or presence of methyl esterification plays a crucial role in substrate recognition by PLA (Voragen, 1972). PLA is capable to cleave the bond between a methyl-esterified and a non-methylesterified galacturonic acid residue, where the newly formed 4,5-unsaturated non-reducing end residue always contains a methyl-ester. The distribution of the methyl-esters, random versus blockwise, is very important as demonstrated by Voragen (1972). Blocks of methyl-esters will function as good substrates for PLA. When the methyl-esters are randomly distributed, the effect of unesterified galacturonic acid residues is stronger as a stretch of ideal substrate is hardly available. As a consequence, activity will be reduced more than it will be using the same low DM pectin with blockwise methyl-ester distribution (van Alebeek *et al*., 2002, 2003). PLB is unusual because the protein can be isolated in two conformational states, one at pH 6 and the other at pH 7.5. Unlike the pH 6 state, the activity of pH 7.5 state is dependent upon ionic strength and undergoes reversible but inactivating change (Kester and Visser, 1994). The activity of PLB is highly dependent on the distribution of the methyl esters and its activity decreases significantly as the degree of esterification decreases (Yadav *et al*., 2008).

Here we report on the purification and determination of the mode of action of a PL. Model HGs of tailored DM and methyl distribution were used to get a better understanding of the mode of action of the purified PL.

2.2. Materials and methods

2.2.1. Pectin lyase purification

2.2.1.1. Preparation of initial enzymatic solution

Peclyve (industrial pectin lyase of *Aspergillus niger*, N° 2305) contains 30% glycerol, 1.4 g/kg potassium sorbate and 3 g/kg sodium benzoate. 20 mL of enzyme were dialyzed against ultra pure water at 4°C for 8h changing water every 75 min and dialysis tubing every 150 min.

2.2.1.2. Ammonium sulfate sequential precipitation

The dialysed initial enzymatic solution was saturated at 50% ammonium sulfate and centrifuged 10 min at 18000 g at 4°C. The supernatant was then saturated at 60% ammonium sulfate prior to centrifugation in the same conditions. Precipitation was performed for ammonium sulfate concentrations of 50, 60, 80 and 100%. The different precipitates recovered were solubilized in a minimal volume of ultra pure water and dialyzed against ultra pure water at 4°C for 8h changing water and dialysis tubing every 2h. Activity tests were performed on Peclyve and all the precipitates.

2.2.1.3. Hydrophobic Interaction Chromatography

Fractions were dialysed twice 90 min against 500 mL of 50mM phosphate buffer pH6. Ammonium sulfate was added to reach a final concentration of 1.5M. Hydrophobic Interaction Chromatography (HIC) was performed on a Äkta™ Purifier 10 system, driven by Unicorn™ 3.21 software (Amersham Pharmacia Biotech). Samples (1 mL), conditioned in 50mM phosphate buffer 1.5M $(NH_4)_2SO_4$ pH6, were loaded onto a Resource ether (GE Healthcare, Amersham Bioscience, 0.86 mL) column previously equilibrated with 50mM phosphate buffer 2M $(NH_4)_2SO_4$ pH6. Elution was performed at a flow rate of 1 mL/min using the following gradient:

- 0-7 mL: 100% B
- 7-25 mL: linear gradient 100 to 0% B
- 25 mL: 100% A

(B: 50mM phosphate buffer 2M $(NH_4)_2SO_4$ pH6; A: 50mM phosphate buffer pH6)

0.5 mL fractions were collected. Appropriate fractions were pooled, dialysed overnight against 1L of ultra pure water then for 24h against 10mM acetate buffer pH5. Activity tests were then performed on the different pools.

2.2.1.4. Anion Exchange Chromatography

Anion exchange chromatography (AEC) was performed on a Äkta^{TM} Purifier 10 system driven by Unicorn^{TM} 3.21 software (Amersham Pharmacia Biotech). Sample (2 mL), conditioned in 50mM acetate buffer pH5, was loaded onto a MonoQ (GE Healthcare, Amersham Bioscience, 1 mL) column previously equilibrated with 50mM acetate buffer pH5. Elution was performed at a flow rate of 1 mL/min using the following gradient:

- 0-6 mL: 100% A
- 6-26 mL: linear gradient 100 to 50% A, 0 to 50% B
- 26-37 mL: linear gradient 50 to 0% A, 50 to 100% B.

(A: 50mM acetate buffer pH5; B: 50mM acetate buffer 1M NaCl pH5)

0.5 mL fractions were collected. Appropriate fractions were pooled and dialysed against 50mM acetate buffer pH5. Activity tests were then performed on the different pools.

2.2.1.5. Enzymatic assays

- *Lyase activity*

To assess the activity of pectin and pectate lyase, raw lemon pectin (Grinsted 3450) and polygalacturonic acid were used as substrates, respectively. Activity was followed using 2.5 mg/mL of samples in 50mM acetate buffer pH5 at 40°C. The reaction was initiated by addition of enzymatic fraction (190µl of sample solution + 600µl of acetate buffer + 10µl of enzymatic fraction adequately diluted) and the activity measured at 20 and 30 min. The rate of reaction was determined spectrometrically by measuring the rate of formation of double bond (C=C) by its absorbance at 235 nm ($\varepsilon = 5500\ M^{-1}\ cm^{-1}$ as the molar absorption coefficient of C=C at 235 nm) (eq. 9) (Mutenda *et al*., 2002).

$$nkat/mL = \Delta DO/\ 5.5 \times 10^{-3} \times [(Vol_{enzyme+\ substrate})/\ Vol_{enzyme}] \times [Vol\ final/\ (Vol_{enzyme+\ substrate} \times 1200)] \quad (9)$$

- *Galactanase activity*

Galactanase activity was assessed on potato galactan as substrate (2.5 mg/mL) in 50 mM acetate buffer pH 5, based on the photometric detection of color (in an alkaline medium, the pseudoaldehydic group of sugars reduces the cupric ions Cu^{2+}. The latter react with the arsenomolybdic reagent producing a blue colour) at λ= 600 nm (Nelson, 1944).

- *Endo-PG activity*

To assess the PG activity, polygalacturonic acid was used as substrate (2.5 mg/mL) in 50 mM acetate buffer pH 5 based on the photometric detection of color (thibarbituric acid in acidic media reacts with GalA to form red dye) at λ= 600 nm (Nelson, 1944).

2.2.1.6. Protein determination

The Bradford assay relies on the binding of the dye Coomassie Blue to protein that has an absorbance maximum at 595 nm. For protein determination, the reactant (Bio-Rad) was added to the substrate at a ratio of 1 to 4. After 10 to 30 min, 250µl of the mixture was added to ELISA plaques and the absorbance was read at 595 nm. Bovin serum albumine was used as standard (0-25µl/mL). A_{595} of the samples and standards against the reagent blank was measured (Bradford, 1976).

2.2.1.7. SDS-PAGE Electrophoresis

This technique is used to separate proteins according to their molecular weight. The buffer used in this method is sodium dodecyl sulphate (SDS) (Laemmli, 1970), β-mercaptoethanol and bromophenol for staining the protein bond. The polyacrylamide gel (BioRad 10-20%, 0.375M, Tris-HCl) was placed in Mini-PROTEIN ® (BioRad), at 200 V and 60 mA. The standard mixture (Kaleidoscope BioRad) contains proteins with molecular weights of 7-204 kDa. Staining was done by Coomassie blue (EZBlue, Sigma).

2.2.2. Estimation of purified pectin lyase mode of action

2.2.2.1. Enzymatic hydrolysis

15 mg of samples (HG-P36, HG-P64, HG-B40 and HG-B69) were solublized in 5 mL 50mM acetate buffer pH 5 (3mg/mL). 30µl of pectin lyase (57.6 nkat/mL) were added to samples before incubation for 24h at 40°C. Samples were concentrated to ~ 1 ml under vaccum and desalted on a Sephadex G10 column (78 × 4.4 cm) eluted at 90 mL/h with water. The elution was monitored with refractometric detection.

2.2.2.2. Size Exclusion Chromatography

Dessalted enzymatic digests were concentrated to less than 1 mL under vaccum at 40°C and then injected to a chromatographic system constituted of a Biogel P6-extra fine (92 × 5 cm) column and a Biogel-P4- fine (86 × 5 cm) column mounted in series. The elution was performed by 100 mM acetate buffer pH 3.6 at a flow rate of 10 mL/h. Fractions were collected and analyzed for their GalA content by the automated *meta*-hydroxydiphenyl method (Thibault, 1979). Appropriate fractions were pooled and either extensively dialysed against distilled water (polymeric pools), either concentrated under vacuum at 40°C to ~ 3 ml , desalted on a Sephadex G10 column (78×4.4cm) and concentrated again to reach a final concentration of ~ 150 µg/mL.

2.2.2.3. GalA and DM determination

Colorimetrical DM determination of HGs was performed in 100 µL of 200 mM Tris-HCl buffer pH 7. 40 µL (3 mg/mL) of methylbenzothiazolinone-2-hydrazone (MBTH, Sigma M8006-1G), 50 µL of sample or H_2O (as standard) and 20 µL of alcohol oxidase (E.C. 1.1.3.13, Sigma, A2404) (0.02 UI/µL) were added for oxidation of the released methanol. After addition of the alcohol oxidase, the samples were incubated for 20 min at 30 °C, and then 200 µL of a solution containing 5 mg/mL each of ferric ammonium sulfate and sulfamic acid was added. After 20 min at room temperature 0.6 mL of H_2O was added and the tubes were vigorously vortexed to assure oxidation. The absorbance at 620 nm was determined (Anthon and Barrett, 2004; Slavov *et al.*, 2009).

GalA measurements were performed by *m*-hydroxydiphenyl assay (Thibault, 1979).

2.2.2.4. HPAEC analyses

Purification of each peak eluted from SEC was estimated using high-performance anion-exchange chromatography (HPAEC) on a Dionex system equipped with a Carbopac PA1 column (2mm) with pulsed amperometric detection as explained in RESULT, chapter 1.

2.2.2.5. Mass spectrometry

2,5-dihydroxybenzoic acid (DHB) was purchased from Sigma-Aldrich Co. (Saint Quentin Fallavier, France). N,N-dimethylaniline (DMA) was purchased from from Fisher Scientific (Fair Lawn, NJ, USA). Milli-Q water (Millipore, Bedford, MA, USA) was used in preparation of all solutions. All chemical reagents used were HPLC grade. DMA/DHB was prepared by dissolving 100 mg of DHB in 1 mL of H_2O/ACN (1:1). 20 µL of DMA (purchased from from Fisher Scientific (Fair Lawn, NJ, USA)) was added in matrix solution. 1 µL of sample was directly mixed with 1 µL of matrix solution on polished steel target plate.

Mass spectrometry acquisition was performed on an Autoflex III MALDI TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a Smartbeam laser (355 nm) in positive reflector mode. Laser power was adapted for each sample. Mass spectra were automatically processed by FlexAnalysis (Bruker Daltonics, Bremen, Germany).

2.3. Results

2.3.1. Purification of pectin lyase from Peclyve enzymatic mixture

2.3.1.1. Peclyve and ammonium sulfate precipitates

To find out the activity of PL and possible contamination with some other pectin-degrading enzymes, Peclyve was tested to determine PG, galactanase and pectin lyase activities (Table 5).

Table 5. Protein measurement and different enzyme activities of Peclyve and ammonium sulfate precipitates

		activity (nkat)				activity (% initial)		
	proteins (mg)	pectin lyase	galactanase	Endo-PG	pectin lyase/endo-PG	pectin lyase	galactanase	PG
Peclyve	**29**	**12921**	**1605**	**152000**	0.09	**100**	**100**	**100**
60%	3.56	1881	52	728	2.58	15	3	<1
80%	8.93	4114	213	7190	0.57	32	13	5
100%	10.12	6061	0	59988	0.10	47	0	39
Total						94	16	44

As shown in Table 5, Peclyve contains PL activity but accompanied by high amounts of PG activity and moderate amounts of galactanase activity. In order to purify the PL, ammonium sulfate sequential fractionation was performed with different saturation. In ammonium sulfate 50%, no precipitation was achieved.

Altogether, 96% of the PL activity was recovered in the other ammonium sulfate precipitates (60%, 80% and 100%). The more ammonium sulfate concentration used, the more PL activity was obtained (47% in 100%, 32 in 80%, 15 in 60%). Most of the galactanase and PG activity were eliminated since they were not precipitated.

Although the 60% saturation of ammonium sulfate was not the best with respect to the total amount of PL recovered, it was kept for further purification steps as PL/ PG activity was the highest.

2.3.1.2. Hydrophobic Interaction Chromatography

In order to separate the proteins according to the hydrophobicity of amino acid side chains, HIC was used. The chromatograms obtained for each ammonium sulfate precipitate are presented in Fig. 20.

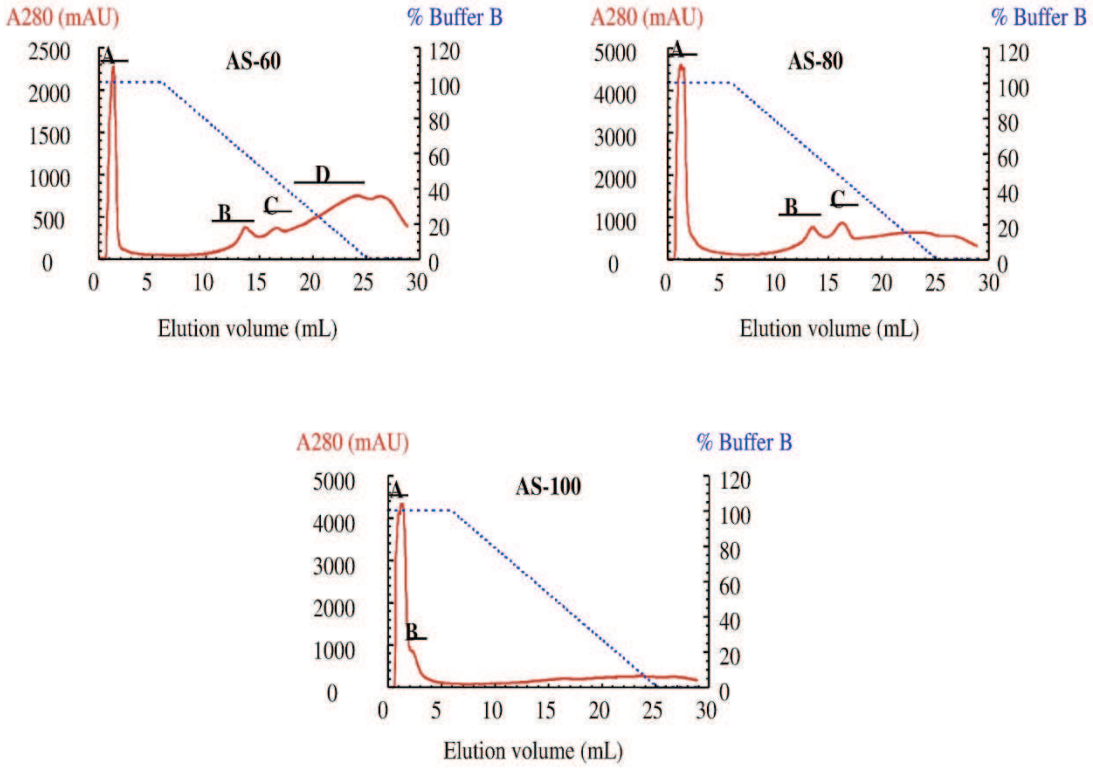


Fig. 20. HIC chromatograms of the different ammonium sulfate fractions

Following the absorbance at 280nm, 4 peaks were detected for the 60% ammonium sulfate precipitate (A60, B60, C60, D60), 3 peaks for the 80% ammonium sulfate precipitate (A80, B80, C80) and 2 peaks for the 100% ammonium sulfate precipitate (A100, B100). Appropriate fractions were pooled; protein measurement and enzymatic activity tests were performed on each pool (Table 6).

PL activity was the highest in B80, followed by A80, A60 and B60. PG activity was the highest in A80. B80 fraction was obviously the best choice according to high PL and low PG activity but since no pectate lyase activity was detected in A60 pool, this pool was chosen for futher purification steps.

Table 6. Protein measurements and enzyme activity tests of HIC pools

Fraction		activity (nkat)			
	proteins (µg)	pectin lyase	galactanase	Endo-PG	pectate lyase
A60	**273**	**175**	**2**	**115**	**0**
B60	90	118	1	1	nd
C60	54	12	3	3	nd
D60	143	8	0	9	nd
A80	**854**	**180**	**10**	**838**	**6**
B80	**261**	**202**	**7**	**5**	**4**
C80	133	27	5	4	nd
A100	900	21	0	2743	nd
A'100	103	2	15	923	nd

nd: not determined

2.3.1.3. Anion Exchange Chromatography

The elution profile of A60 on MonoQ HR5/5 is shown in Fig. 21. The chromatogram shows four well separated peaks at 0 M, 0.125 M, 0.3 M and 0.375 M NaCl.

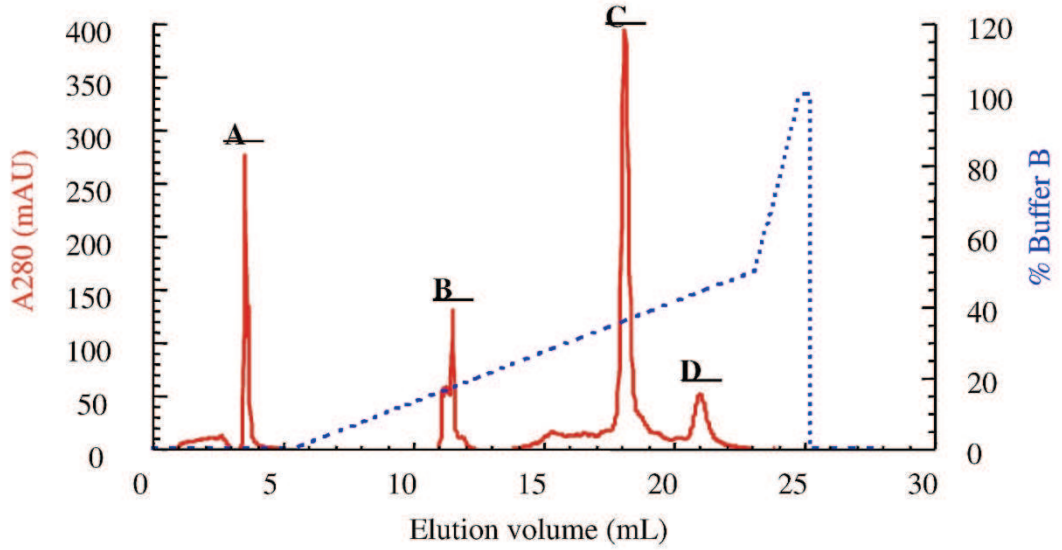


Fig. 21. AEC chromatogram of HIC A60 fraction

Pools corresponding to each of the 4 peaks (A60a, b, c and d) were dialysed against distilled water and activity tests were performed on each of them (Table 7).

Table 7. Enzyme activity tests of AEC-separated fractions

Fraction	activity (nkat)		
	pectin lyase	galactanase	Endo-PG
A60a	2.5	0	0.2
A60b	2.0	0	0
A60c	**547**	**0**	**0**
A60d	37.0	0	5.6

The third fraction (A60c) eluted between 0.3-0.33 M NaCl was collected as the targeted fraction.

2.3.1.4. Purification summary

The whole procedure of PL purification is shown in Fig. 22 and Table 8.

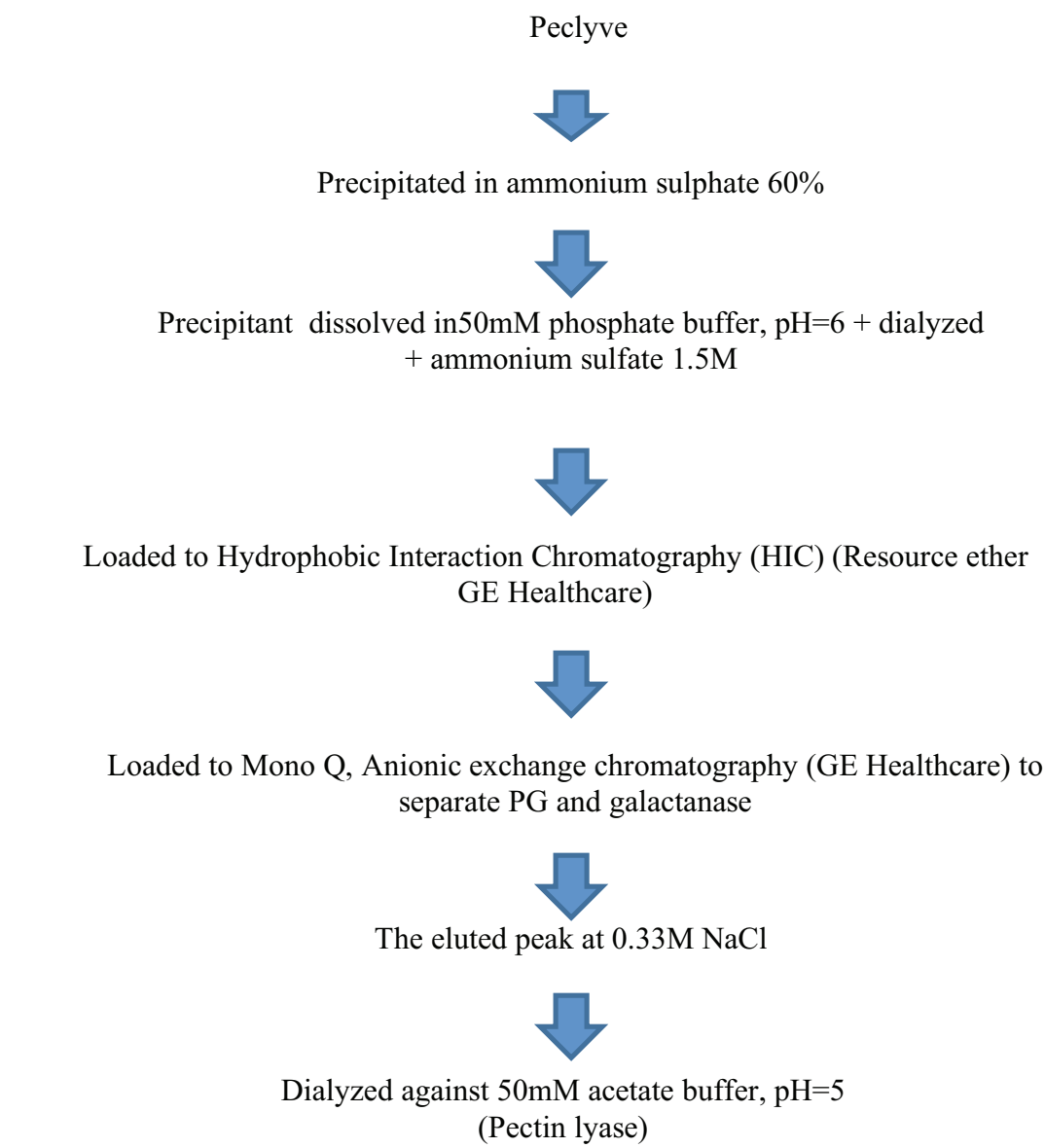


Fig. 22. PL purification procedure

Table 8. PL purification procedure

Fraction	volume (mL)	activity	nkat total	activity yield (%)	protein total (ug)	protein yield (%)	specific activity (nkat/mg)	purification rate
Peclyve crude	20				29000	100		
		PL	12921	100			446	1
		galactanase	1605	100				
		Endo-PG	152000	100				
60% ammonium	4.4				3564	12.3		
sulfate precipitate		PL	1881	14.6			528	1.2
		galactanase	52	3.2				
		Endo-PG	728	0.5				
HIC								
Excluded fraction	9.6				820	2.8		
		PL	524	4.1			639	1.4
		galactanase	6	0.4				
		Endo-PG	345	0.2				
AEC								
Fraction 0.3M NaCl	9.5				152	0.5		
		pectin lyase	**547**	**4.2**			**3600**	**8.2**
		galactanase		0				
		PG		0				

2.3.1.5. SDS-PAGE electrophoresis

Peclyve, ammonium sulfate 60% precipitate, HIC excluded fraction A60 and AEC fraction A60c were analyzed by SDS-PAGE (Fig. 23). Purified PL estimated M_w was 38 kDa. SDS-PAGE revealed that PL is homogenous. Specific activity of purified PL was 3600 nkat/mg and the purification rate was 8 times.

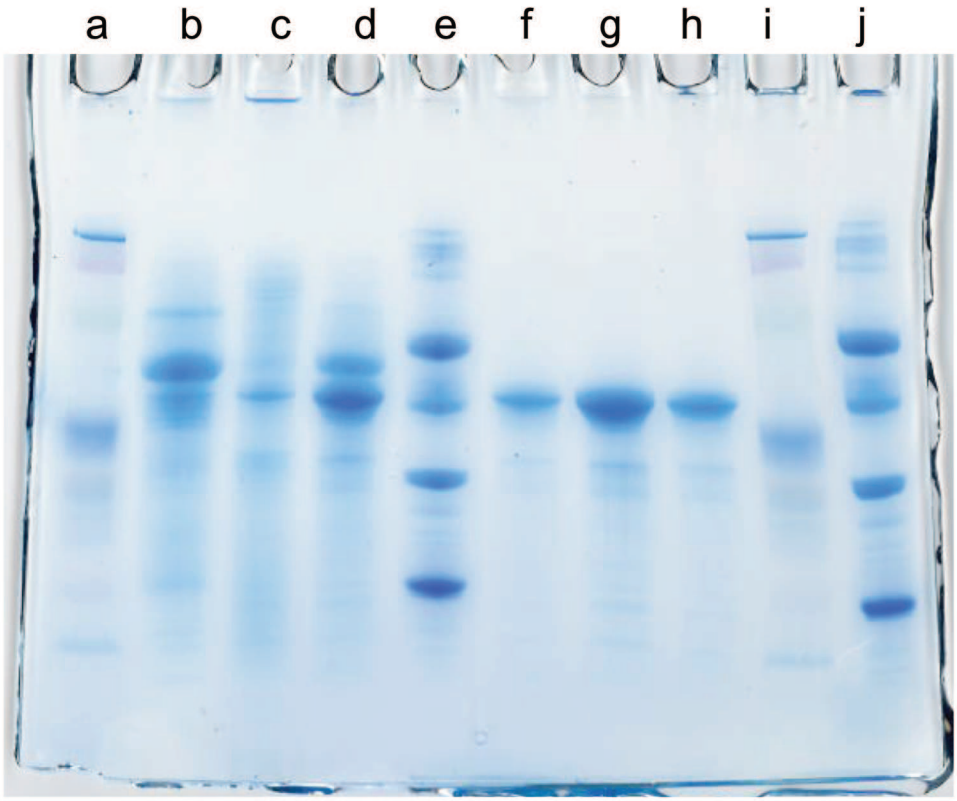


Fig. 23. SDS-PAGE electrophoresis of PL purification stages. (a) and (i): kaleidoscope kit; (e) and (j): ribonuclease A, chymotrypsinogen A, ovalbumin, albumin; (b) Peclyve; (c) Ammonium sulfate 60% precipitate; (d) HIC excluded fraction A60; (f), (g) and (h) AEC fraction A60c (5, 10 and 5 µL, respectively).

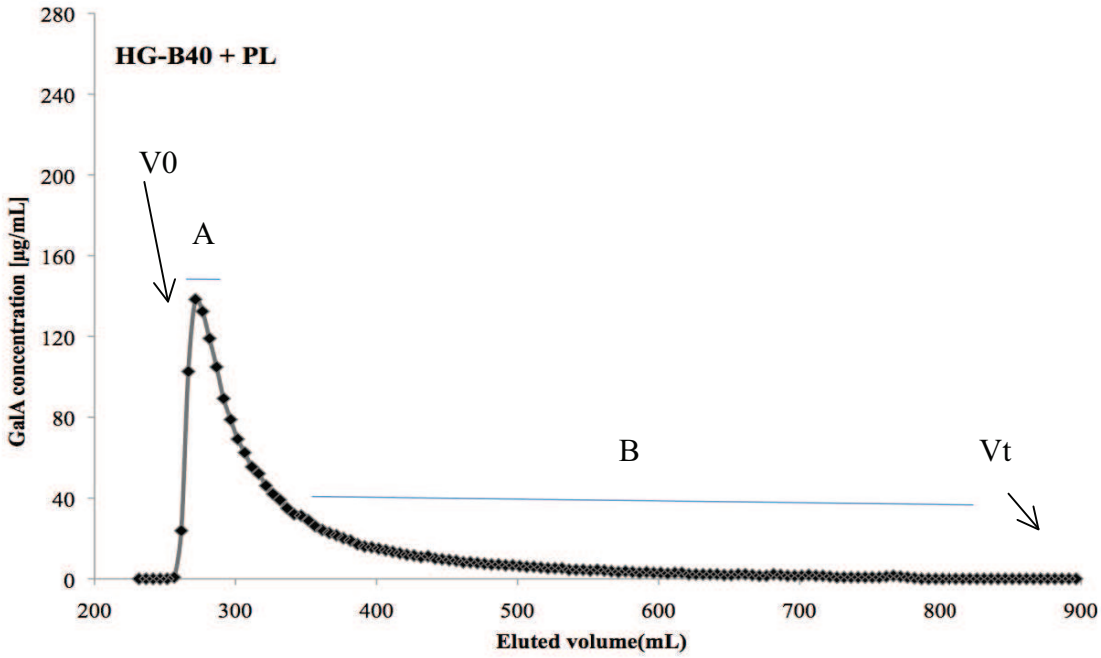
2.3.2. Estimation of purified pectin lyase mode of action

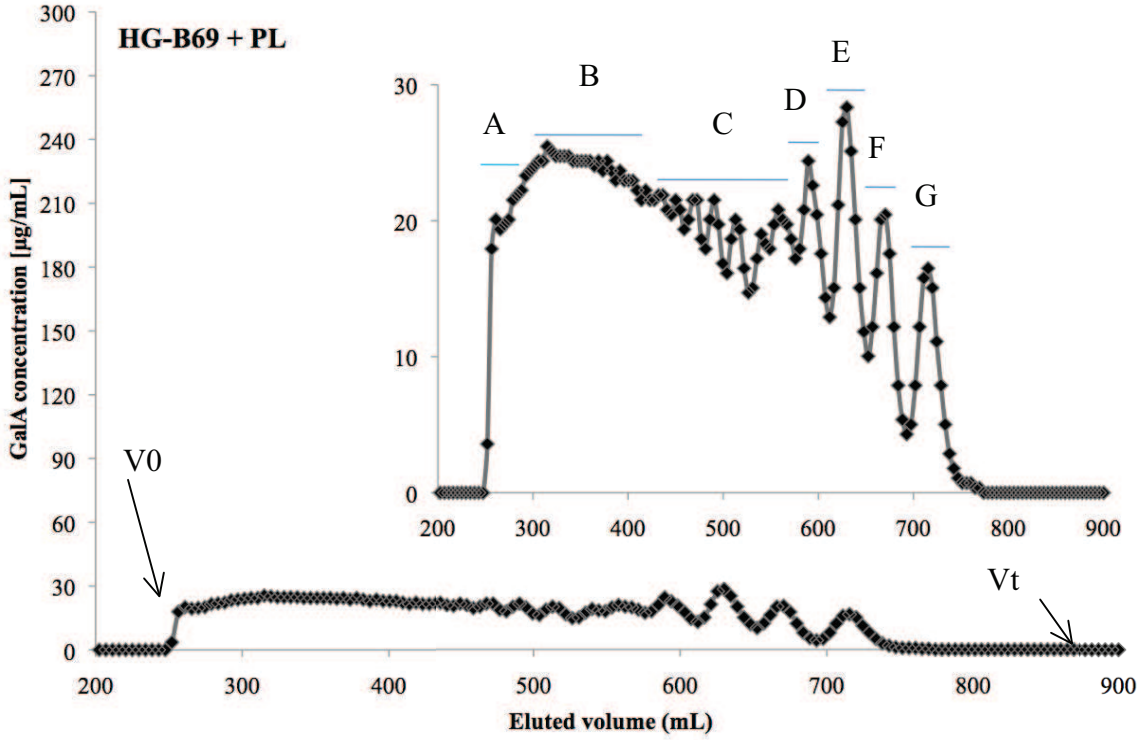
The different model HGs produced (see RESULTS AND DISCUSSION Chapter 1) were used to investigate the mode of action of purified PL. The specifity of the enzyme is reflected in different oligomer patterns as separated by SEC and further analyzed by MS.

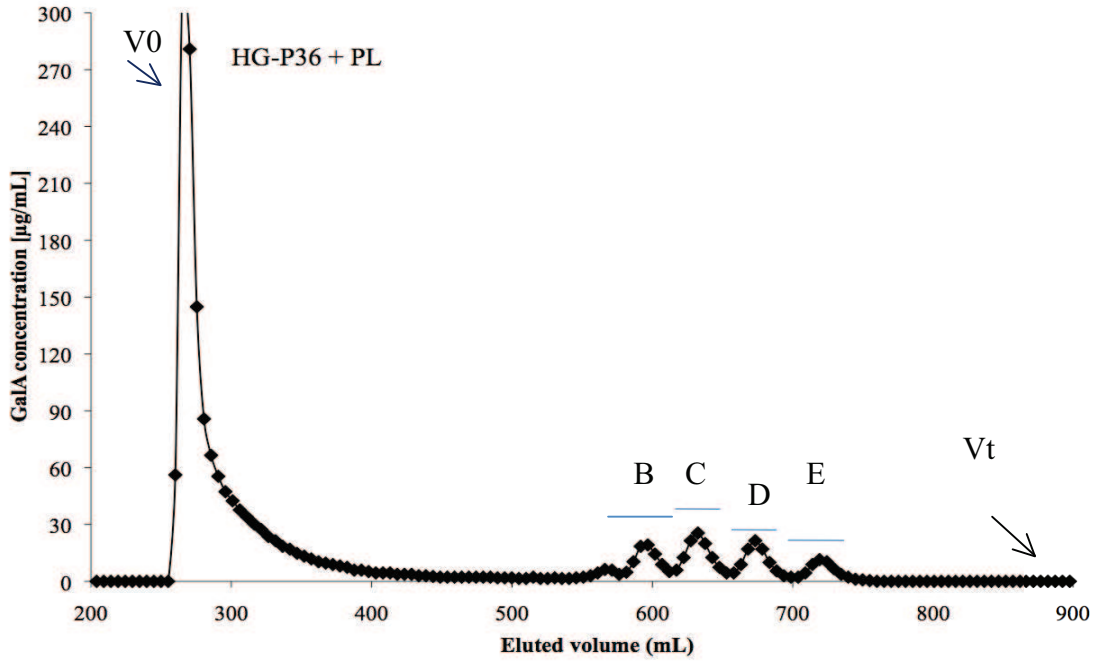
In order to estimate the mode of action of purified PL, HGs of different DM (LM and HM) and various distribution patterns of methylesters (random and blocky) were used for digestion. To identify the optimum hydrolysis time, hydrolysis was carried out for 3 days.

HPAEC analysis revealed that the various oligomers concentrations did not drastically differ between 1 day and 3 days of hydrolysis, in agreement with van Alebeek *et al*. (2002) findings showing that 24 hours is needed for full digestion by PL.

HG 24h-PL-hydrolysates were fractionated by low pressure SEC. The elution patterns of digested HGs are presented in Fig. 24.




HG-B69 + PL
GalA concentration [µg/mL]
Eluted volume (mL)
V0
Vt
A
B
C
D
E
F
G


HG-P36 + PL
GalA concentration [µg/mL]
Eluted volume (mL)
V0
Vt
B
C
D
E

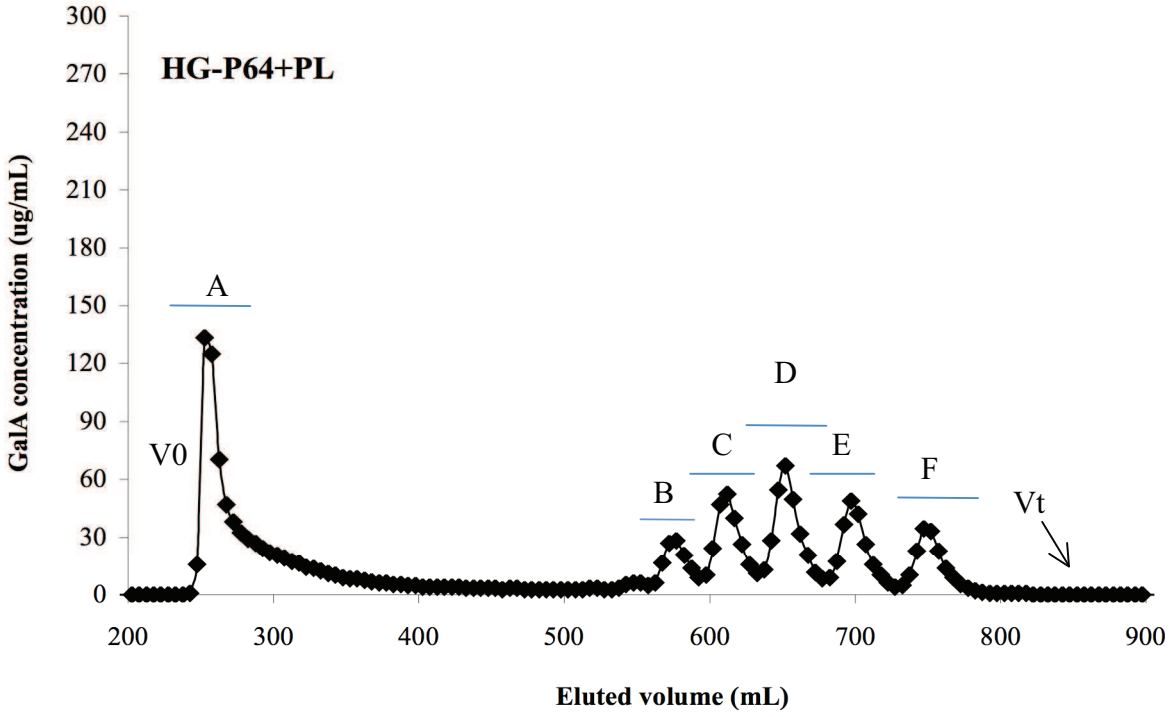


Fig. 24. Elution patterns of different HGs+ PL

All chromatograms exhibit a polymeric fraction eluting at the void volume representing non-hydrolysed or very partially hydrolysed HG stretches. It is obvious that DM distribution pattern has a great impact on PL hydrolysis since the chromatograms of B- and P-series are totally different.

For HG hydrolysates from the B-series, chromatograms differ widely depending on the DM of the sample. HG-B40 hydrolysate exhibits a major polymeric peak eluting at the void volume indicating that this HG sample was not hydrolysed by PL. HG-B69 exhibits a radically different elution pattern with only limited amounts of material eluting at the void volume together with a large range of oligomeric peaks.

For HG hydrolysates from the P-series, chromatograms are very much alike whatever the DM of the sample with a polymeric fraction eluting at the void volume together with 4 to 5 well resolved oligomeric peaks eluting for elution volumes between 550 and 780 mL. The total amount of polymeric fraction decreases and the amount of oligomeric fractions increases as the DM increases. P-series HGs thereby

showed very similar oligomer chain length distribution together with a constant decrease in oligomer concentrations with decreasing DM. PL can digest HG-P36 to some extent demonstrating that even at that low DM, there are long enough blocks of methylated GalAs to interact with PL active sites, which is in good agreement with findings of Limberg *et al*. (2000a) on pectin samples.

Each SEC fraction was collected, concentrated and desalted prior to analysis of GalA content and DM.

Table 9. Yields, chemical composition and degree of polymerization of the fractions recovered after size-exclusion chromatography

Sample	Yield%	DM	dp
HGB40			
A	78	34	nd
B	22	38	nd
HGB69			
A	36	58	>16
B	23	61	11 to 16
C	19	66	7 to 10
D	6	81	6
E	8	77	5
F	5	87	4
G	3	81	3
HGP36			
A	75	8	nd
B	7	> 95	6
C	11	> 95	5
D	5	> 95	4
E	2	> 95	3
HGP64			
A	43	8	nd
B	1	nd	8
C	6	nd	7
D	13	> 95	6
E	15	> 95	5
F	13	> 95	4
G	9	> 95	3

nd: not determined

As presented in Table 9, HG-B40 is not hydrolysed by PL and showed fractions A and B with DM close to the initial DM of 40. In HG-B69, fractions A, B and C represent partially hydrolysed fractions, which exhibit a DM close or slightly lower than that of the initial HG sample (69). In P-series, fractions A are non-hydrolysed or partially hydrolysed and exhibit a very low DM. These fractions represent long stretches of non-methylated GalAs not suitable for PL digestion. In P-series, the rest of fractions are highly methylated and correspond to the degraded long blocks of methylated GalAs. In HG-B69, hydrolysed fractions (D to G) showed lower DM compared to P-series indicating the heterogeneity of methyl distribution patterns over HG stretches. The hydrolysable part of HG-B69 is close to HG-P36, which reveals the impact of methyl distribution pattern of methylesters over HG stretches in PL digestion despite of huge difference of DM (van Alebeek *et al.*, 2002). HG-P64 was the most hydrolysable substrate for PL.

Oligomeric fractions arising from HG-B69 and HG-P36 were analyzed by MALDI-TOF-MS in positive mode. The dp of GalA oligomers present in the different SEC fractions was thereby assessed (Table 9), together with their DM fine distribution (Table 10). MS spectrum of HG-B69 E is fully described in Fig. 25 for clear illustration of the observed ions.

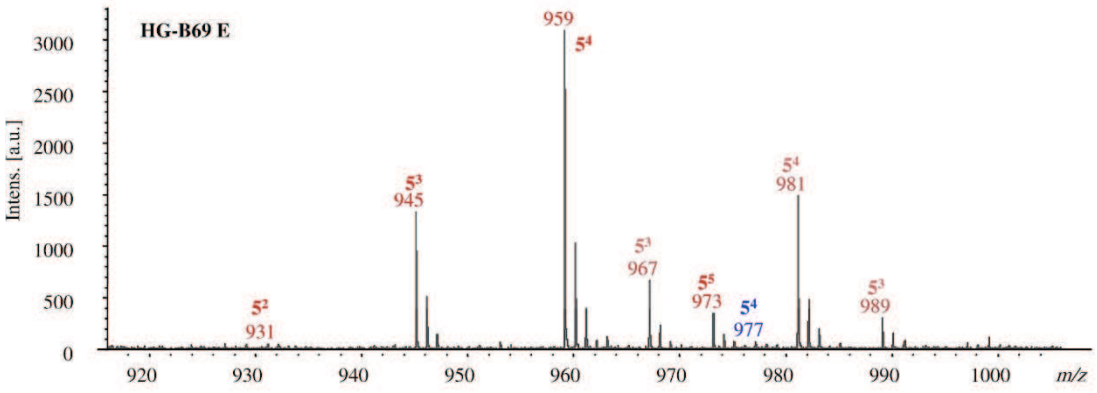


Fig. 25. MALDI-TOF-MS spectrum in the positive mode of pentamers of GalA produced by PL.

The MS spectrum of HG-B69 E was characterized by the predominance of sodium adducts representative of unsaturated GalA oligomers of dp5 bearing 2 to 5 methyl groups (*m/z* 931, 945, 959 and 973). Sodiation of the carboxylic functions

was systematically observed (Fig. 25). The major species (5^4, *m/z* 959) exists also in the saturated form (*m/z* 977). This ion relates to oligomers arising from the non-reducing end of HG molecules, thereby bearing no unsaturation.

Mass spectra of the different SEC fractions (annexe 3) were similarly interpreted and results obtained are summarized in Table 10.

Table 10. Unsaturated oligogalacturonates detected by MALDI-TOF MS in the different fractions. Base peaks appear in bold.

Samples	**SEC fractions**	**Oligomers (dp^{DM})**	***m/z* (sodium adducts)**
HG-B69	B	16^8, $\mathbf{16^9}$, 16^{10}, 16^{11}	2953, **2967**, 2982, 2996
		15^7, 15^8, $\mathbf{15^9}$, 15^{10}	2763, 2777, **2791**, 2805
		14^7, $\mathbf{14^8}$, 14^9	2586, **2600**, 2615
		13^6, 13^7, $\mathbf{13^8}$, 13^9	2393, 2410, **2424**, 2439
		12^6, $\mathbf{12^7}$, 12^8	2220, **2234**, 2248
		11^6, $\mathbf{11^7}$, 11^8	2044, **2058**, 2072
	C	10^5, 10^6, $\mathbf{10^7}$	1853, 1867, **1881**
		9^5, $\mathbf{9^6}$, 9^7	1677, **1691**, 1705
		8^4, $\mathbf{8^5}$, 8^6	1487, **1501**, 1515
		7^4, $\mathbf{7^5}$, 7^6	1311, **1325**, 1339
	D	6^3, 6^4, $\mathbf{6^5}$, 6^6	1121, 1135, **1149**, 1163
	E	5^2, 5^3, $\mathbf{5^4}$, 5^5	931, 945, **959**, 973
	F	4^2, $\mathbf{4^3}$, 4^4	755, **769**, 783
	G	3^1, $\mathbf{3^2}$, 3^3	565, **579**, 593
HG-P36	B	6^4, 6^5, $\mathbf{6^6}$	1135, 1149, **1163**
	C	5^3, 5^4, $\mathbf{5^5}$	945, 959, **973**
	D	4^2, 4^3, $\mathbf{4^4}$	755, 769, **783**
	E	3^1, 3^2, $\mathbf{3^3}$	565, 579, **593**

Although the same species were observed for HG-B69 and HG-P36-derived oligomers of dp 3 to 6, their relative intensities differ widely, as illustrated in Fig. 26 for HG-B69 E and HG-P36 C, both consisting of oligoGalA of dp 5.

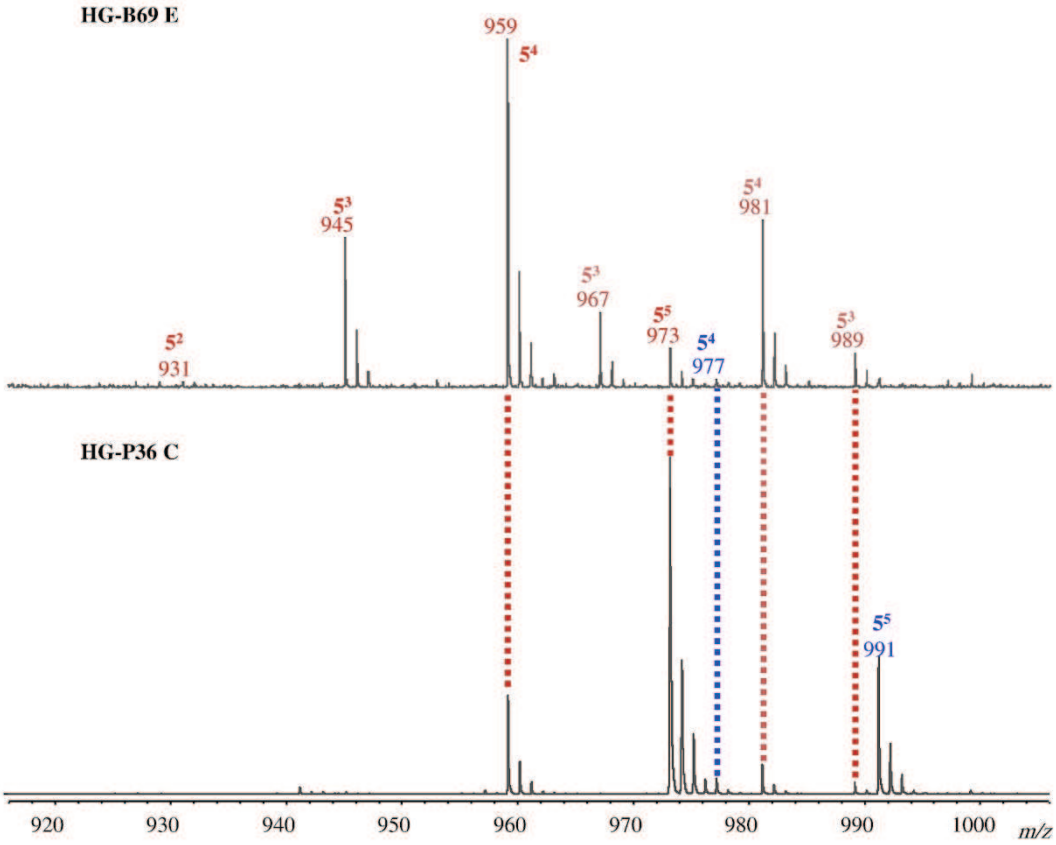


Fig. 26. Different populations of dp5 in HG-B69 and HG-P36

The relative amounts of the different oligomers present in one fraction cannot be assessed by ions intensity since ionization rates vary widely from one chemical species to the other. Relative intensities comparison between HG-P36 and HG-B69 fractions of identical dp can however be achieved using fully methylated oligomers as internal standards (arbitrary intensity value of 100) (Table 11).

Table 11. Relative intensities of demethylated species using fully methylated unsaturated oligogalacturonates as internal standards

		Chemical species (dp^{DM})		
dp	SEC fraction	n^{n-2}	n^{n-1}	n^{n}
dp 3	HG-B69 G	123	2029	100
	HG-P36 E	2	91	100
	Relative amounts (B/P)	**62**	**22**	**1**
dp 4	HG-B69 F	66	259	100
	HG-P36 D	0.3	29	100
	Relative amounts (B/P)	**220**	**9**	**1**
dp 5	HG-B69 E	328	654	100
	HG-P36 C	1.5	29	100
	Relative amounts (B/P)	**226**	**23**	**1**
dp 6	HG-B69 D	226	397	100
	HG-P36 B	10	72	100
	Relative amounts (B/P)	**23**	**6**	**1**

Singly and doubly demethylated oligomers of dp 3 to 6 appeared to be much more frequent in HG-B69 than in HG-P36, in agreement with the overall DM values calculated for the corresponding SEC fractions.

Beside oligomers of low dp, HG-B69 PL hydrolysate also contains oligomers of higher dp recovered as HG-B69 C (dp 7-10) and B (dp 11-16) fractions (Table 9). For HG-B69 C and for HG-B69 B, the base peaks consisted in oligomers corresponding to n^{n-2} and n^{n-3} and to n^{n-4} to n^{n-8} (dp^{DM}), respectively (annexe 3, Table 10). Those oligomers of higher dp were absent in HG-P36 PL hydrolysate.

2.4. Discussion

The purification procedure summarized in Table 6 shows that 8-fold purification with 4% yield and 3600 nkat/mg specific activity from the Peclyve has been achieved. The purification procedure involved in the present work has only three steps: ammonium sulfate fractionation, hydrophobic interaction chromatography on a Resource Ether column and anion exchange chromatography on a MonoQ HR5/5 column. Yadav and co-workers (2008) also used a three steps procedure to purify PL from *A. flavus*. This procedure included ammonium sulfate precipitation, DEAE-cellulose column chromatography followed by Sephadex G-100 column chromatography. The specific activity of their purified PL was 305.5 nkat/mg, lower than the specific activity achieved in the present work.

In order to specify the mode of action of purified PL, two HGs of each B- and P-series were treated by PL to find out the impact of methyl ester distribution patterns on PL digestion as done by previous authors (Voragen, 1972; van Alebeek *et al*., 2002). The results show that random distribution of methylesters can block PL activity even when DM is high. In this case, blocky LM HG (HG-P36) can be hydrolysed more specifically than random HM HG (HG-B69).

HPAEC with pulsed amperometric detection is used for separation of the oligomer carbohydrates (Voragen *et al*., 1993) but it is quite difficult to identify the nature of an unknown compound on the basis of its position in a chromatogram. In this regard, extensive purification steps such as preparative size exclusion chromatography followed by desalting steps are required prior to characterization by MS to avoid unambiguous structural identification.

The most rapid, specific, and sensitive technique to determine the sequence of nonesterified and methyl-esterified residues in oligogalacturonates was obtained by MS. Ionization in the negative ion mode gave good results but completely methyl-esterified oligomers could not be detected in negative ion mode (Körner *et al*., 1999).

As presented in Table 10, fully methylated unsaturated oligomers are dominant in HG-P36 while partially methylated unsaturated oligomers are abundant in HG-B69. This claim is magnified in Fig. 24 and Table 11. In HG-P36, fully methylated unsaturated oligomers or with one non-methylated GalA are detected.

In HGs treated by endo-PL (purified from *A. niger*, Pektolase ^{TM}, Danisco) no monomer was detected in the oligogalacturonates as reported by Limberg *et al*., 2000a. They also found that during short incubation time, PL liberated mainly higher and fully methylated oligomers of dp 4-15 from HM pectin and thus had a preference to cleave in areas with 100% methyl esterification. Extended PL digestion and the results obtain by ESI ion trap MS/MS sequence analysis of oligomers by Körner *et al*. (1999) demonstrated that some oligomers contained free GalA on the newly formed reducing end. The cleavage point for PL is therefore not limited to adjacent methyl esterified GalAs but can also be positioned between a free acid and a methyl esterified one.

van Alebeek *et al*. (2002) investigated the impact of PL on fully methylated oligomers. They could observe the saturated and unsaturated digestion products, which is consistent with our results. Körner *et al*. (1998) have studied pectin lyase digests by MALDI-MS and found that these digests were very heterogeneous with respect to dp and DM of the formed oligomers. They could find pentamer with only two methylester groups from a PL digest of pectin DM 58 but they did not state the type of PL.

It is revealed in this study that random distribution of methylesters compared to DM parameter has greater impact on decreasing the activity of the enzyme. It is said by Mutenda *et al*. (2002), who used PL on 3 types of pectin (p-PME treated, f-PME treated and base treated pectin), that pectins with DM< 50% generated by base and f-PME de-esterification (random) are not suitable substrates for PL.

2.5 Conclusion

The purification of PL from industrial pectolytic enzyme was achieved. The impact of methyl ester distribution patterns of alkali and p-PME treated HGs over PL digestion has been highlighted. The dominant oligomer population in P-series is fully methylated, while for B-series produced oligomers are more heterogenous with respect to DM.

3. Appraisal of degree of blockiness of model HG using *endo*-polygalacturonase and pectin lyase

3.1 Introduction

Distribution of methyl esters along the HG backbones has always been a matter of debates since DM and demethylated block number and size are related to pectin functionality (Guillotin *et al*., 2005; Hellín *et al*., 2005). Enzymatic fingerprinting with pectolytic enzyme with the help of chromatography paved the way to find out more information about methyl esterified and non-methyl esterified sequences detection and their separation (Hotchkiss *et al*., 2002; Cameron *et al*., 2008). Extensive analysis for the intramolecular methyl distribution has been estimated using exo-PG, PL and endo-PG by Limberg *et al.* (2000a, b). Daas *et al*. (1998) developed a method where HPAEC was performed on a CarboPac PA1 column (Dionex) at pH 5 on endo-PG digested pectin samples, followed by post addition of alkali to enable PAD detection by measuring the amount of liberated GalA after digestion. Another way is loading the digested enzyme on size exclusion chromatography to separate the oligomers. Several methods have been used to design the methyl distribution pattern over the pectic backbone such as enzymatic or chemical treatments (Limberg *et al*., 2000a, b).

In the present work, as few long blocks in the HG P-series and numerous short blocks in HG-BP- series were recognized (RESULTS, chapter 1), we were up to discriminate the blocks of non-methylated GalAs of each series. In this regard, saturated and unsaturated oligomers of GalAs were prepared as standards for HPAEC and complete enzymatic digestion made by PL and endo-PG were achieved. Comparison of different oligomer populations of both digestions for each series was the aim of this work in order to probe into methyl esters distribution onto model substrates and get insight into the pectin molecules. Biogel SEC (see previous chapter) gave accurate results but is very time consuming. Quantitative HPAEC (much more rapid) for PL digests and comparison with PG digests was proposed.

3.2. Materials and methods

3.2.1.1. Saturated and unsaturated oligogalacturonic acid standards preparation

3.2.1.1 Saturated oligogalacturonic acid standards

Polygalacturonic acid (Sigma, Orange P3889) (4.4 g in 440 mL water) was autolysed at 100°C for 25 h under magnetic stirring. After centrifugation (20000g × 15 min, 20 ºC), the supernatant containing oligogalacturonates was recovered and 2 volumes of absolute ethanol were added. The mixture was left at 4°C overnight to ensure precipitation of oligomers of very high dp. After centrifugation (10000g × 15 min), the insoluble fraction was suspended in 100 mL of distilled water, concentrated under vaccum to discard alcohol and freeze-dried. This fraction (1.06 g) contains oligomers of dp ~ 12. The soluble fraction in ethanol was concentrated to 60 mL and dialysed twice against distilled water for 40 and 70 min to eliminate dp 1,2 and 3 before being freeze-dried. This fraction (1.11g) contains oligomers of dp ~ 5 (Thibault *et al.*, 1993). Alcohol-soluble fraction was solubilized in 10 mL water, concentrated to less than 1 mL and loaded onto preparative size exclusion chromatography Biogel-P6 (extra-fine) (92 × 5)-P4 (fine) (86 × 5) eluted by 100mM acetate buffer pH 3.6 to fractionate different dp with a flow rate of 10 mL/h and 5 mL per tube. Then two series of peaks were pooled, peaks corresponding to dp 3, 5, 7, 9 and peaks corresponding to dp 4, 6, 8, 10. Those pools were fractionated again on the same chromatographic system.

Each tube of the second fractionation was analysed for GalA concentration by automated *meta*-hydroxydiphenyl method (Thibault, 1979). Adequate tubes were pooled, concentrated under vaccum to ~ 1 ml at 40°C, desalted through Sephadex G10 and concentrated again to less than 0.5 mL. Each dp fraction was individually injected to semi-preparative HPAEC (Dionex-PA100) with an elution flow rate of 3 mL/min using the gradient shown in Table 12.

Table 12. HPAEC separation conditions for saturated GalAs oligomers

Time (min)	1M NaOAc%	0.5M NaOH %
0-20	25-50	20
20-30	50-60	20
30-31	60-25	20
31-60	25-25	20

Each saturated oligomer purified by semi-preparative HPAEC was neutralyzed by HCl and desalted on G10. Desalted solutions were analysed for their GalA concentration.

3.2.1.2 Unsaturated oligogalacturonic acid standards

HG-P64 PL digest's dessalted SEC fractions B, C, D, E, F and G, corresponding to unsaturated GalA of dp 3 to 8 (see RESULTS AND DISCUSSION, Chapter 2) were further purified by HPAEC (Dionex-PA200) at an elution flow rate of 0.4 mL/ min using the gradient given in Table 13.

Table 13. HPAEC separation conditions for unsaturated GalAs oligomers

Time (min)	1M NaOAc%	0.5M NaOH %
0-20	25-25	20
20-25	50-50	20
25-26	50-25	20
26-55	25-25	20

Individual dp fractions were neutralized, desalted and analysed for their GalA concentration as described above (3.2.1.1).

3.2.2 Degradation of model HGs with endo-PG or PL

Substrates were solubilized at a concentration of 1 mg/mL in 2 mL 50mM acetate buffer (pH 4 for *endo*-PG and pH 5 for pectin lyase). 24µL of PL (28.7 nkat/mL) were added to samples for 24 h at 40°C and 12 µL of endo-PG was added to samples (7100 nkat/mL, diluted 1/200) at 40°C for 3 days.

3.2.3 HPAEC quantification of HGs degradation products

20µl of the digested samples were injected to the anion resin column CarboPac PA1 (2 × 250 mm Dionex). Chroméléon Software (Dionex) software was used for collecting and processing the data. The mobile phases were all degassed with helium in order to prevent absorption of carbon dioxide and transformation to carbonate. Columns used were thermostated at 20 °C (Quéméner *et al.*, 2003).

The elution rate was 0.25 mL/min and concentration of 0.5M NaOH was kept constant at 100 mM. The gradient of 1M NaAOc is described in Table 8. Identification and quantification of oligomers was done according to the retention times obtained for the saturated and unsaturated standards previously prepared.

3.3. Results

3.3.1. Oligogalacturonic acid standards

Saturated oligogalacturonic acids derived from polygalacturonic acid autolysis were loaded to Biogel P6 (extra-fine)-P4 (fine). Peak separation was detected by automated *meta*-hydroxydiphenyl method which quantifies GalA concentration in each tube of eluted solution.

The peaks corresponding to dp 3, 5, 7, 9 and dp 4, 6, 8, 10 (Fig. 27) were collected separately and reinjected again to Biogel P6 (extra-fine)-P4 (fine) to have a more precise separation as presented in Fig 28.

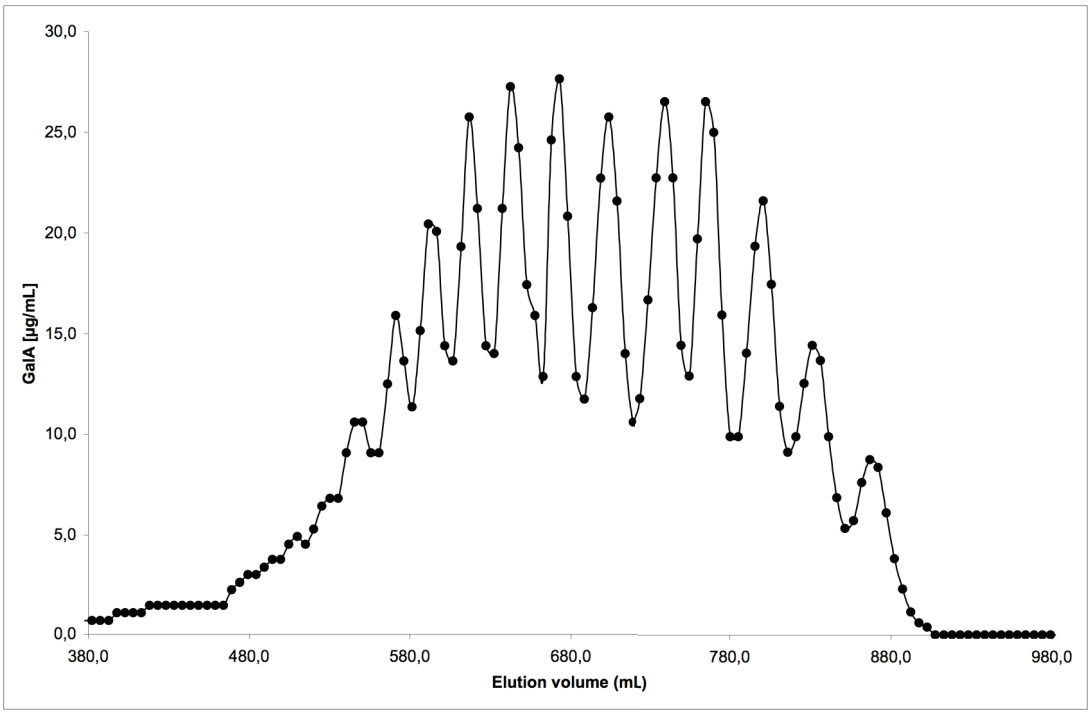
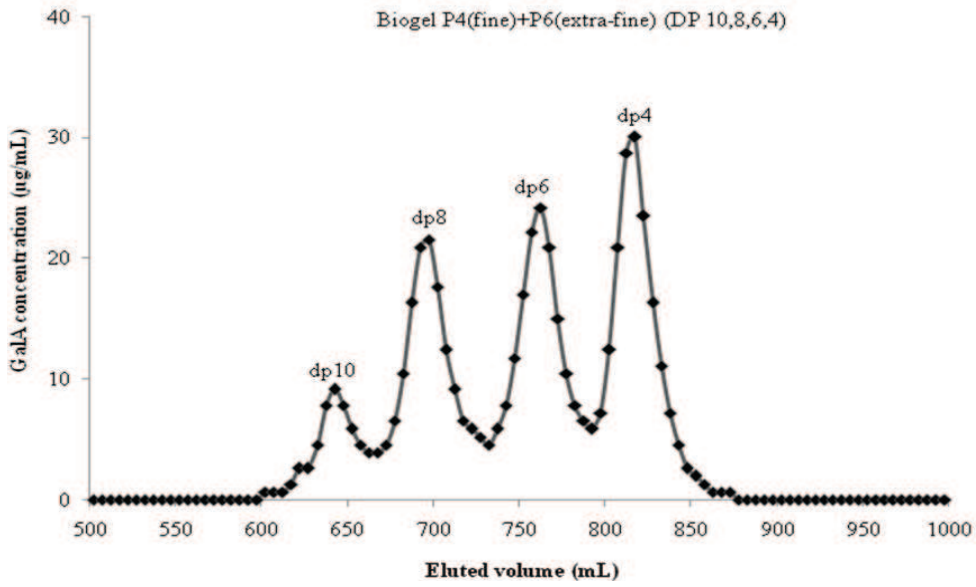


Fig. 27. Biogel P6-P4 elution pattern of saturated oligomers of GalA, derived from autolysis of PGA.



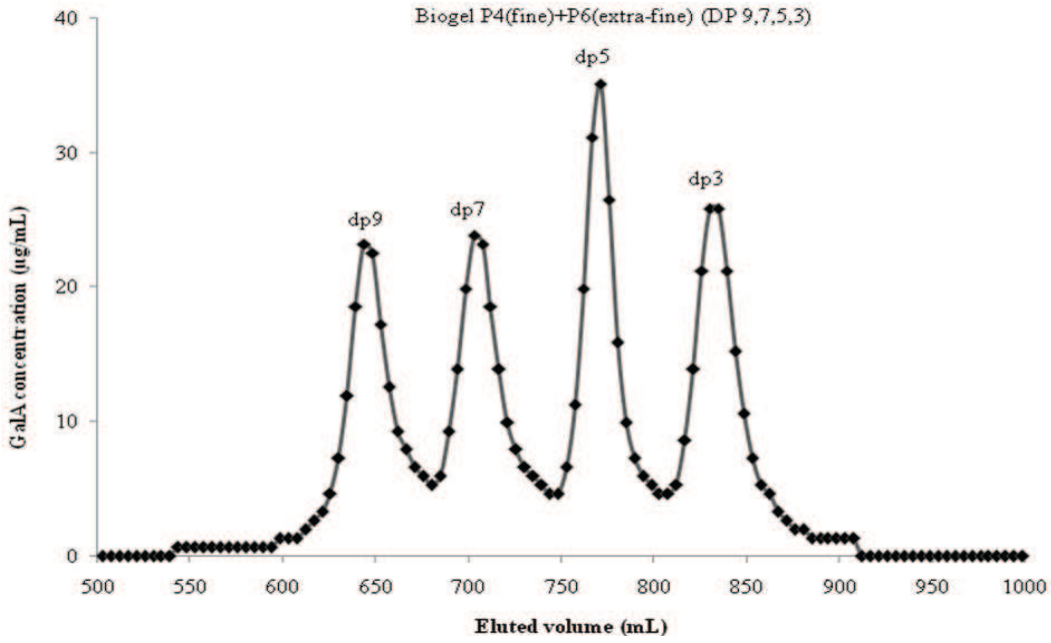


Fig. 28. Biogel P6-P4 elution patterns of saturated oligomers of GalA, dp 3 to 10

The eluted peaks were pooled, desalted through G10 Sephadex in order to avoid any salt interference in retention times in HPAEC and reinjected to semi-preparative HPAEC-PA100 for further purification. At the same time, GalA concentration of each sample was quantified by automated *meta*-hydroxydiphenyl method.

Each dp was loaded onto analytical HPAEC Carbopac PA1 for response factor (frp) calculation. frp for each saturated oligomer was calculated as follows:

$$frp = Area / [GalA] \quad (11)$$

After this determination, frp dependence on dp was described by fitting the data to an exponential function as presented (eq.12; Fig. 29):

$$y = 0.41042 \times dp^{(-1.4646)} \qquad R^2 = 0.99335 \quad (12)$$

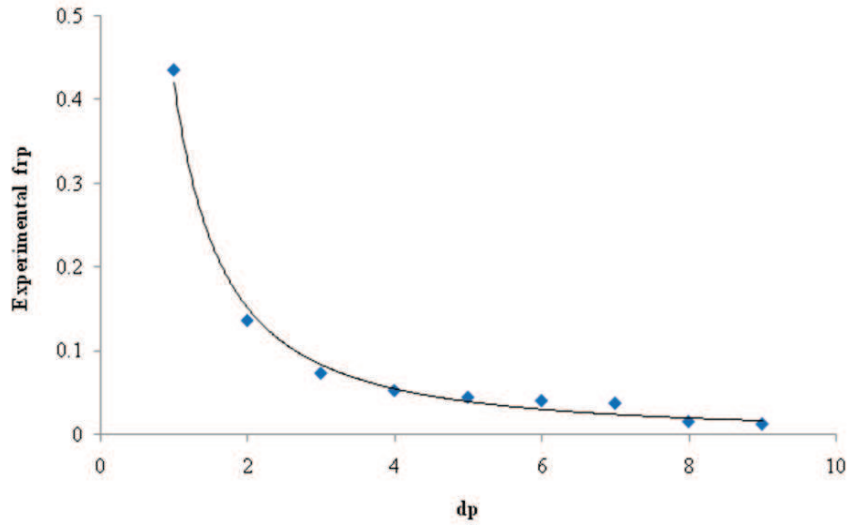


Fig. 29. frp of saturated oligomers of GalAs versus dp

The reduced PAD response of acidic oligosaccharides has been reported previously (Hotchkiss and Hicks, 1990; Campa *et al*., 2004). However, the literature did not provide exact relationships between dp and frp.

Calculated and experimental response factors and retention times of all dps have been obtained as described in Table 14.

Table 14. Retention times and response factors of standard saturated oligomers

Oligomer (Saturated)	Retention time (min)	frp (calculated)	frp (experimental)
dp1	2.9	0.410	0.436
dp 2	5.3	0.149	0.136
dp 3	10.5	0.082	0.073
dp 4	15.3	0.054	0.052
dp 5	19.1	0.039	0.044
dp 6	22.2	0.030	0.040
dp 7	24.7	0.024	0.037
dp 8	26.9	0.020	0.015
dp 9	29.3	0.016	0.012
dp 10	31.7	0.014	
dp 11	34.3	0.012	
dp 12	36.7	0.011	
dp 13	38.9	0.010	
dp 14		0.009	
dp 15		0.008	

The contamination of each oligomer was less than 3%.

For having unsaturated standards of GalA, the PL digest of HG-P64 was loaded to Biogel P6 (extra-fine)-P4 (fine) (GalA was quantified by automated *meta*-hydroxydiphenyl method as described in previous chapters). Each eluted peak was collected, desalted and further purified by semi-preparative HPAEC-PA200. Recovered fractions were desalted and concentrated subsequently. The desalted dp fractions were analysed by analytical HPAEC (Carbopac PA1) for frp calculation.

For unsaturated galacturonic acid oligomers as standards, frp was calculated as done for saturated ones by fitting the data to an exponential function as presented (eq.13; Fig. 30):

$$y = 0.30731 \times dp^{(-1.292)} \qquad R^2 = 0.98943 \qquad (13)$$

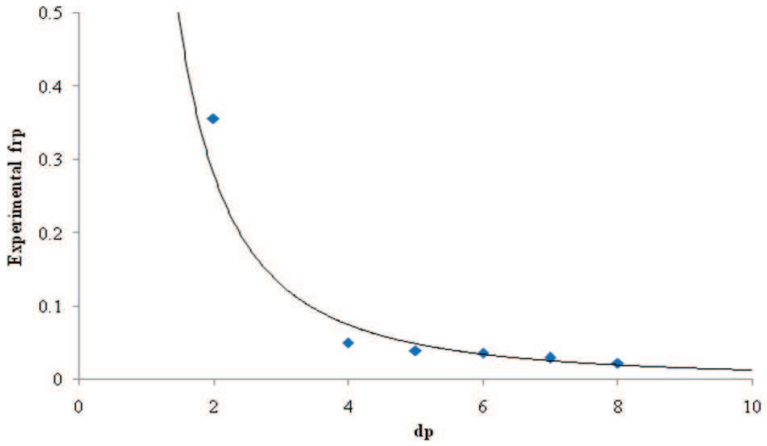


Fig. 30. frp of unsaturated oligomers of GalAs.

Calculated and experimental response factors and retention times of all dps have been obtaind as described in Table 15. As already pointed out (Hotchkiss and Hicks, 1990), unsaturated oligomers were eluted later from the CarboPacPA1 column than the corresponding dp saturated counterparts.

Table 15. Retention times and response factors of standard unsaturated oligomers

Oligomer (Unsaturated)	Retention time (min)	frp (calculated)	frp (experimental)
dp1			
dp 2	14.5	0.307	0.356
dp 3	20.3	0.126	
dp 4	24.5	0.074	0.051
dp 5	28	0.051	0.040
dp 6	31.5	0.038	0.037
dp 7	34.9	0.030	0.030
dp 8	38.2	0.025	0.023
dp 9	41.2	0.021	
dp 10	43.9	0.018	
dp 11	46.3	0.016	
dp 12	48.5	0.014	
dp 13	50.3	0.012	
dp 14	52.1	0.011	
dp 15	53.5	0.010	

The contamination of each oligomer was less than 3%.

3.3.2. HPAEC quantification of HGs degradation products

3.3.2.1. PL digestion

In PL digestion of an ideal substrate (HG96), dp 3-7 were recovered as major hydrolysis products and dp2 and dp8 as minor ones. The overall recovery, using the frp calculated with our home-made standards was 97.5, which shows that the results are reliable. So we will consider dp 2-8 as hydrolysable parts of HG by PL.

For HG-P36 and HG-P64 PL digests, there is a good agreement between overall dp 2-8 quantification by HPAEC and overall quantification after SEC fractionation (24/25 for HG-P36 and 61/57 for HG-P64). No oligomers were detected by HPAEC for HG-B40 and HG-B20 PL hydrolysates, which is also in agreement with SEC results. For HG-B69, it is more complicated as we had reasonable SEC separation only up to dp6. If dp 2-6 is considered, it results in a ratio 22/18.3, which is reasonable. It indicates that HPAEC results for dp 2-8 are reliable.

While comparing different series according to dp populations, for dp 2-8 (Fig. 31) we could find that for P-series we are close to an ideal linear relationship dp 2-8 = DM. This result confirms that samples from this P-series generated from a very highly methylated mother sample consist of long blocks of methylated GalAs (fully degradable by PL) interspersed with long blocks of non-methylated GalAs (fully hydrolysable by PG, results and discussion chapter 1).

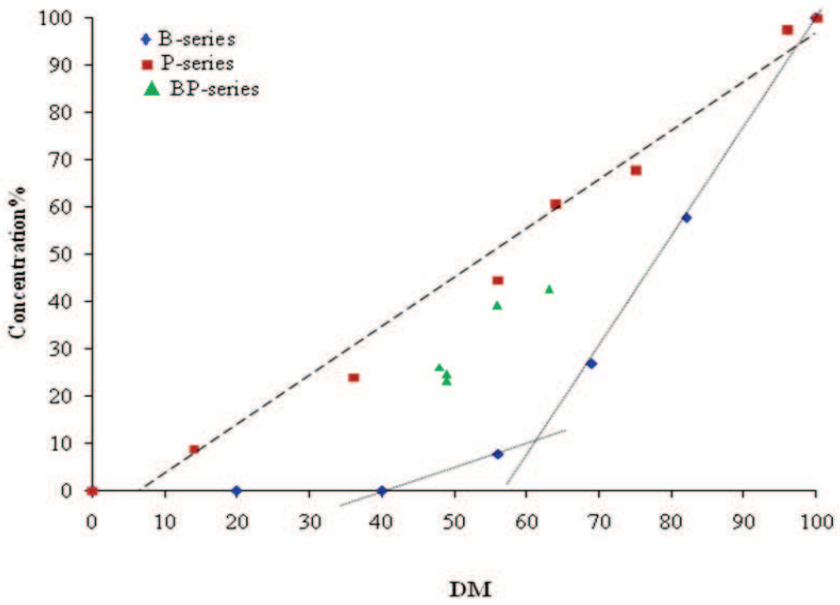


Fig. 31. dp 2-8 of PL digestion of HGs

For B-series we have a polynomial relationship between dp 2-8 and DM. From DM100 down to DM 65, there is a roughly linear relationship dp 2-8 = 0.43 DM + 57. This gives values to van Alebeek's findings (2002) who explained that when the methyl-esters are randomly distributed, the effect of unesterified GalA is

stronger as a stretch of ideal substrate becomes more and more hardly available as the DM decreases. As a consequence, activity is reduced more (2.3 times more) than while the same low DM pectin with blockwise distribution is used.

All BP samples exhibit values that are intermediate between B and P ones of similar DM. The trend of P-series (dp 2-8 = DM) is now roughly followed from the mother HG point (DM ~ 80). This proves that DM decreased in a blocky fashion for BP samples that can hardly be differentiated from P-series on the basis of the amount of dp 2-8 released by PL. It may imply that the BP-series contain long blocks of highly methyl esterified GalAs, which is a good substrate for PL since PL can cleave not only between two methyl-esterified residues of GalAs but also between an esterified and a non-esterified GalA (Körner *et al*., 1999; Limberg *et al*., 2000a).

3.3.2.2. Endo-PG digestion

The concentration of GalA% of each oligomer generated by endo-PG digestion of different B-series, P-series and BP-series are plotted in Fig. 32 for dp 1 to 3, Fig. 33 for dp 4 to 12 and Fig. 34 for dp>12.

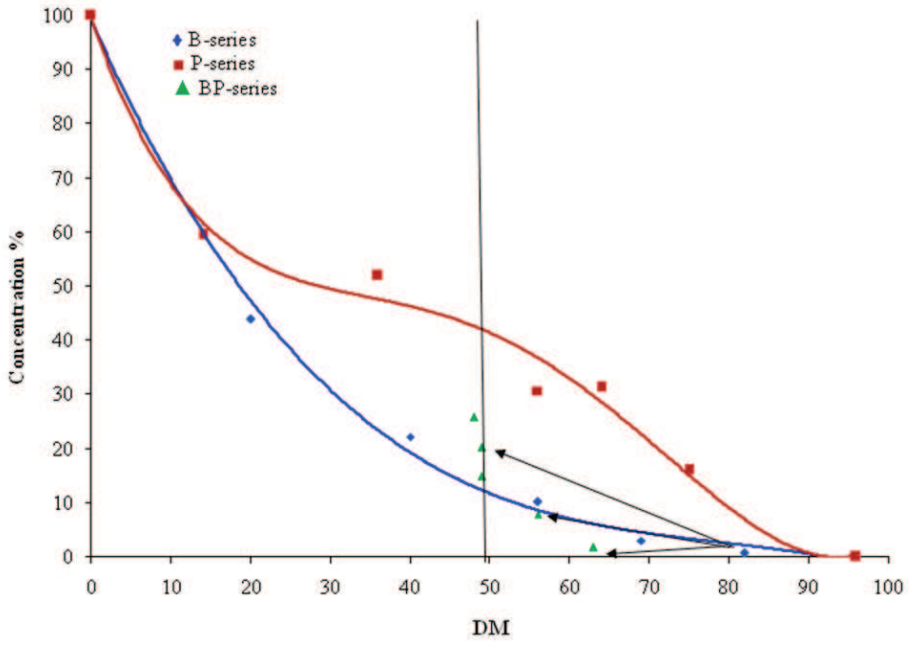


Fig. 32. dp 1-3 concentration versus DM for all HGs

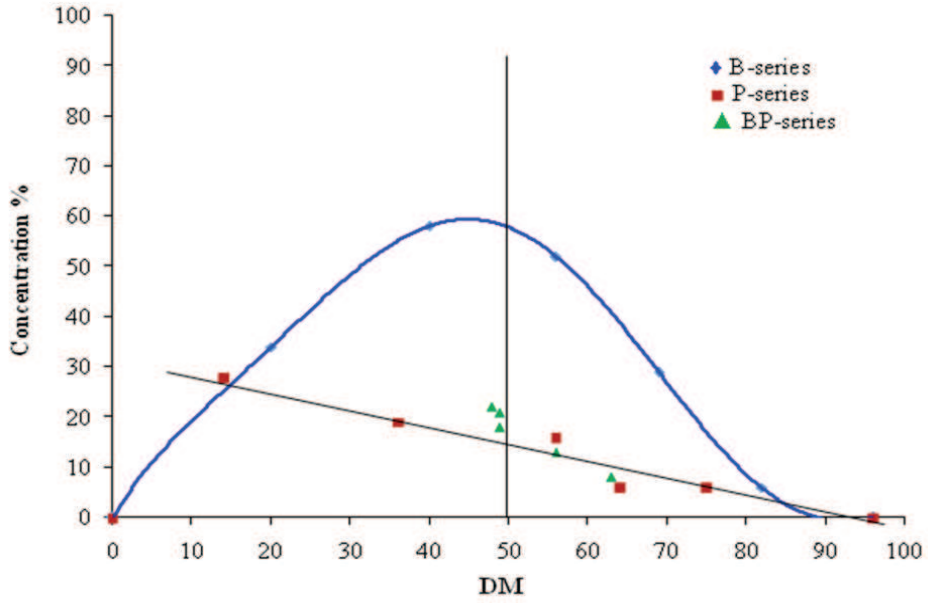


Fig. 33. dp 4-12 concentration versus DM for all HGs

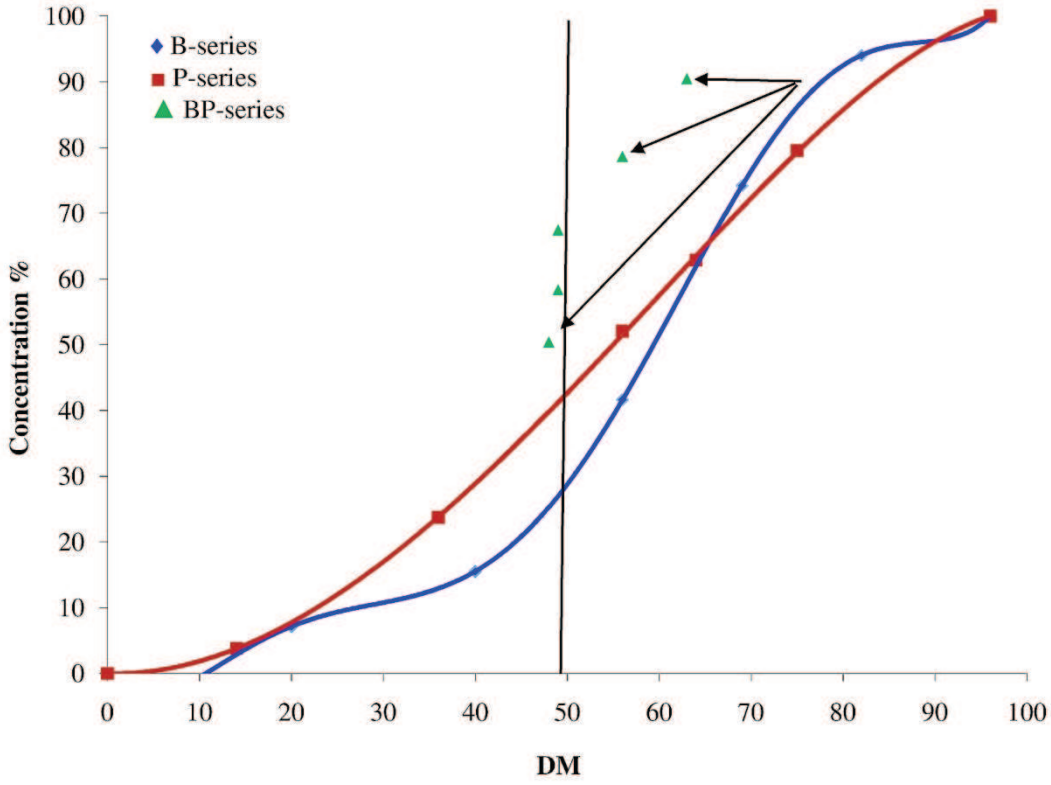


Fig. 34. dp > 12 concentration versus DM for all HGs

For Fig. 32, concentration of dp 1-3 is values of DB_{abs}. P-series, showed a polynomial order 4 fit and B-series a polynomial order 3 fit. No difference between B and P samples was observed for DM values > 85 and < 15. Between DM 15 and

DM 85, higher values were observed for P-series, which is in agreement with the findings of Limberg *et al*. (2000a). Maximal difference was observed between P and B at DM 50. For BP series, values are very close to B series. For BP samples of high DM, even lower values of dp 1-3 were observed comparing to B-samples of corresponding DM. There is in fact no difference (virtually) in the amount of PG-hydrolysable stretches between the mother B samples (DM ~ 80) and the BP derived samples of DM63 and 56. This means that the deesterification mode applied to the mother samples HG-B~80 generates less hydrolysable stretches than a random process does from HG96. While deesterification is continued to DM 48-49 for BP-series, differences between B and BP series are observed showing that more hydrolysable stretches than a random process were generated now.

In Fig. 33 for B-series, when the DM decreased from 100 to 45-50, more partly hydrolysable stretches, which consist of partly methylated oligomers of dp 4-12, were generated. While deesterification is continued to DM< 50, there is more chance to generate fully hydrolysable stretches (that are recovered as dp1-3) and thereby less chance of generating parly methylated oligomers of dp 4-12. For P-series, deesterification mode doesn't promote the generation of dp 4-12 partly methylated oligomers. The chance of generating those oligomers is very low between DM100 and DM80. Then this chance increases as the DM decreases and samples from the B and P series cannot be distinguished for DM values below 15. For BP-series, virtually no difference between the mother sample HG-B~80 and the HG-BP sample of DM63 was observed while the rest of BP-series followed the trend of P-series. This means that the deesterification mode applied to HG-B~80 does not promote the generation of dp 4-12 partly methylated oligomers. Using those results, HG-BP samples cannot be differenciated from HG-P samples.

As presented in Fig. 34, no difference was observed in the amount of PG-non hydrolysable long stretches between the mother B samples (DM82) and the BP-sample of DM63. For the other samples of BP-series, the amount of dp > 12 oligomers began to decrease as DM decreased. This is the image in a mirror of what was found for PL dp 2-8. The concentration of dp > 12 for BP-series DM 48-49 was the highest followed by P-series at corresponding DM. The concentration of dp > 12 of BP-series DM48-49 is like HGP64 and HGB69. Since endo-PG strict requirement is non-esterified GalAs on both cleavage sides (Körner *et al*., 1999), this means that

in BP-series, individual short blocks of non-methylated GalAs are scatered along HG stretches.

3.4. Discussion

Fingerprinting with PL and endo-PG has been used previously for detection of the differences in the deesterification mechanism by p-PME, f-PME and base catalysed treatment (Limberg *et al*., 2000a). In order to find a clue to differentiate model HGs, we used both of these enzymes and compared the digested products according to dp of oligomers . Since frp values for unsaturated dp > 10 are very low, studies were performed for PL digestion on dp 2-8 and for endo-PG digestion on dp 1-3, dp 4-12 and dp > 12 as 100- (dp 1-3 + dp 4-12). Fig. 35 represents the concentration of PG dp 1-3 over PL dp 2-8 to compare model HGs in both digests.

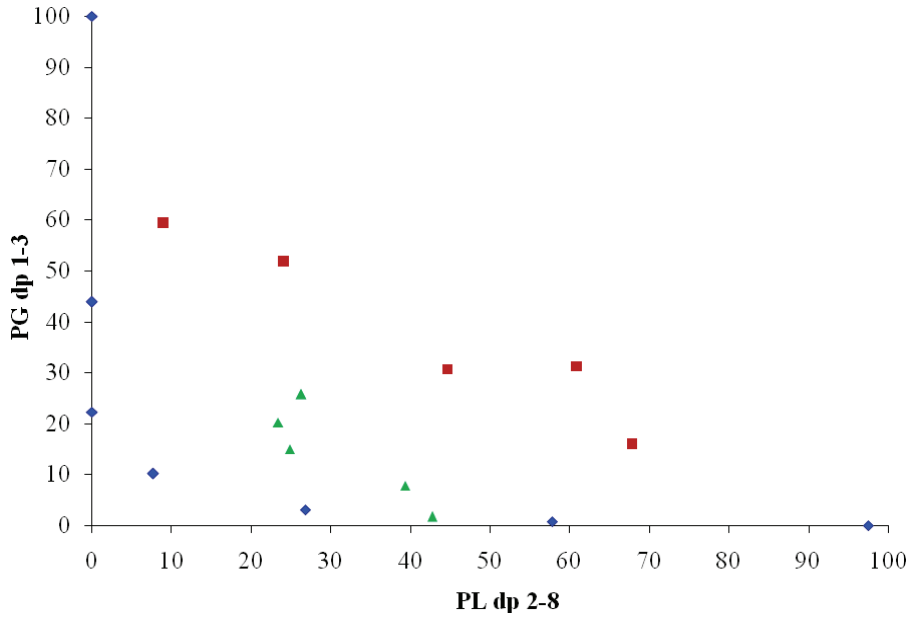


Fig. 35. Concentration of PG dp 1-3 versus PL dp 2-8 for all model HGs

Clear differences exist between B- and P-series at high DM level (DM> 50). For BP-series, HG-B76-P56, HG-B76-P49a, HG-B76-P49b and HG-B82-P48 present intermediate values while for HG-B77-P63, this value is matched to B-series. This result reconfirms that for four intermediate BP-samples shorter blocks than the ones present in P-series are generated while no blocks are detectable for HG-B77-P63.

In Fig. 36, ratio of PL dp 2-8/ PG dp 1-3 is plotted versus DM for all model HGs.

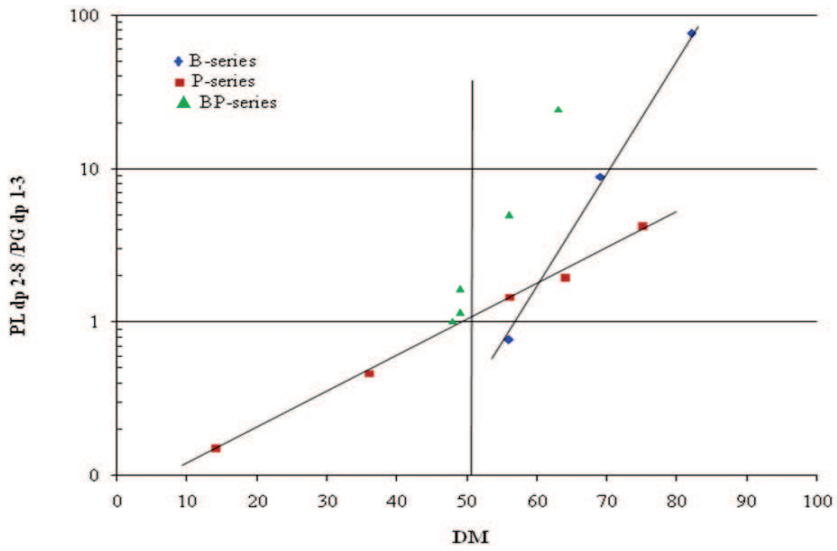


Fig. 36. PL dp 2-8/PG dp 1-3 concentration versus DM for all HGs

As presented in Fig. 36, till DM 60 this ratio is higher for P-series than for B-series while at DM ~ 60 this ratio turns the other way round. For BP-series of DM>60, there are long blocks of highly methylated GalAs together with very short blocks of non-methylated GalAs, which cannot hamper PL digestion but are not favored by PG. For HG-B76-P56, HG-B76-P49a, HG-B76-P49b and HG-B82-P48, values are closer to P-series than B-series while for HG-B77-P63 this ratio is even higher than B-series at the corresponding DM, which represents more methylated packed GalA blocks as PL favorite.

3.5. Conclusion

It is shown that dp population in each B- and P-series digested by PL and endo-PG followed different trends. For four samples of BP-series, HG-B76-P56, HG-B76-P49a and b and HG-B82-P48, intermediate blocksize of GalA (shorter than in P-series) was confirmed while for HG-B77-P63 very short blocks were detected, which is consistent with our previous findings (chapter 1 of result section). BP-series were differentiable from B-series by dp 2-8 of PL digestion and dp 4-12 and dp > 12 generated by endo-PGII. BP-series were differentiable from P-series by oligomers of dp 1-3 and dp > 12 generated by endo-PGII. These findings could be very helpful to develop our knowledge over more complex molecules like pectin in further researches.

4. Characterization of commercial pectins and extracted pectin from lime peels and application in ADD

A part of this chapter is a manuscript draft expected to be published in *Food Hydrocolloids*.

4.1. Introduction

Acidified dairy drinks (ADD) is a class of popular beverages that include drinking yogurt, beverages containing both milk and fruit juice, and soft drinks containing milk solids as a minor ingredient. Doogh is a traditional Iranian fermented milk drink. The main ingredients consist of yogurt, salt, water and/or mineral water. It is possible to add CO_2 (less than 0.6 g/100mL), which is favoured by Iranians. The pH must be less than 4.5 (generally 3.7-4.0), fat percentage not less than 0.5%, salt percentage not more than 1% and the milk solid non-fat must be more than 3.2% for all types of this drink (generally 4-6%) (ISIRI 2009, No. 10528). While storing, the visual appearance is clearly affected by whey separation and sedimentation and the texture typically becomes unpleasant (grainy and slimy).

HM- pectin is widely used to stabilize these drinks, where it prevents the sedimentation problem and whey separation caused by flocculation of the milk protein (Glahn and Rolin, 1996; Janhøj *et al*., 2008; Nakamura *et al.*, 2003). Pectin for this purpose is extracted from citrus peels especially lime and lemon (Albersheim *et al.,* 1996; Willats *et al*., 2001). According to FAO (2007), Iran is the 9th country in the world for lime and lemon production. *Citrus aurantifolia* as the major variety is produced over 615000 tons/year. As 30% of this production goes to juice factory and considering 50% (w/w) of waste peels, around 100000 tons of pectin is estimated to be producable annually.

The stability of ADD depends on the concentration and the type of pectin, the concentration of casein, the ionic strength and the pH. In addition, thermal treatment and homogenisation during processing influence ADD stability (Glahn and Rolin, 1996). At molecular level, pectin stabilizing process can be improved by proper pectin with high degree of methylation, high molar mass, high surface coverage, blockwise distribution of anchor points, strong adsorbing terminal with little affinity for the solvent and voluminous dangling end with high solvent affinity (Boulenguer

and Laurent, 2003; Herbstreith and Fox KG Corporate Group, 2003; Janhøj *et al.,* 2008; Sedlmeyer *et al.,* 2004; Tholstrup Sejersen *et al.,* 2007; Syrbe *et al.,* 1998; Tromp *et al.,* 2004; Ye, 2007). For pectin, the importance of the distribution pattern of non-methylated HG segments, termed as degree of blockiness (DB), especially in the presence of calcium, termed as calcium sensitivity, has been highlighted (Ström *et al*., 2007).

In this study, we made attempts to evaluate the impact of blockiness of HM pectin in Doogh. In this regard, three types of pectin as non-calcium sensitive pectin (NCSP), medium calcium sensitive pectin (MCSP) and high-calcium sensitive pectin (HCSP) were selected as standard samples in market for this purpose. Chemical composition and physicochemical properties of commercial pectins offered for stabilization process in ADD were evaluated in order to have a better insight into the pectin structure at molecular level. With respect to our findings about commercial pectins, new pectins were extracted from *Citrus aurantifolia* peels by different acid extraction methods. The different pectins were applied in Doogh to evaluate their stabilizing efficiency. Pectins were compared with model HGs in order to have an interpretation about their distribution patterns of methylesters.

4.2. Manuscript draft

Commercial Pectin and Extracted Pectin of Citrus aurantifolia in Iranian Acidified Dairy Drink

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Abstract

With their refreshing natural taste and high nutritional value, drinkable yogurts enjoy great popularity. Without the addition of stabilizers in this product, protein flocculates after a short time and whey separation with sandy mouth-feel occurs. To prevent this behavior and to enrich texture, pectin may be added as a protecting colloid. Pectin is a heterogeneous polysaccharide and its fine structure varies depending on the plant source and extraction conditions. Generally, commercial pectins are divided into low-methoxyl pectin with a degree of methylation (DM)< 50% and high-methoxyl pectin (DM>50%). High-methoxyl pectin can minimize protein-protein interactions, thereby reducing protein coagulation. For this purpose, not only the DM but also the distribution of methyl esters on pectins, are of the great importance in the stabilization process. The aim of this research is to broaden our knowledge about the structure of (i) suitable commercial apple and citrus pectins that exist in the market for this purpose and (ii) extracted pectins from *Citrus aurantifolia*. Studying their intra and inter-molecular properties and applying these pectins in drinkable yogurt assisted us to magnify that not only the distribution patterns, but also the necessity of minute estimation of the size and number of blocks

of deesterified galacturonic acids in pectin backbones is needed to explain pectin role as stabilizer in drinkable yogurt.

Keywords: Pectin, acidified dairy drink, stability, blockiness

1. Introduction

Acidified Dairy Drink (ADD) refers to plenty of products containing fermented milk or fruit juices in milk (Nakamura, Furuta, Kato, Maeda, Nagamatsu 2003). According to the Institute of Standards and Industrial Research of Iran (ISIRI), Iranian ADD is made of diluted yogurt, fermented whey or buttermilk but diluting yogurt is the routine procedure in Iran. The main ingredients consist of yogurt, salt, water and/or mineral water. CO_2 is usually added (< 6 g/L), which is favoured by Iranians. The pH must be less than 4.5 (generally 3.7-4.0), fat percentage not less than 0.5, salt percentage not more than 1 and the milk solid non-fat must be above 3.2% for all types of this drink (generally 4-6%) (ISIRI No. 10528, 2009), which means they can be regarded as suspensions of colloidal casein gel fragments. All ADD share a common problem since the pH of these products is in the range of 3.4 to 4.6 (Nakamura *et al*. 2003). The casein aggregates settle out during storage wheying off or aggregate into large compact clusters (sandy mouth feel) upon pasteurization. With pectin, these product defects can be overcome with some limitations, since the pH range of stabilization with pectin is restricted to 3.5-4.2 (Boulenguer and Laurent 2003; Liu, Nakamura, Corredig 2006).

Pectin is a group of complex anionic polysaccharides whose major consistuent is α-(1-4) D-galacturonic acid (GalA) units with some methyl esterified carboxyl groups as homogalacturonan (HG). The rhamnogalacturonan I (RG-I) backbone consists of [→2)-α-L-Rha*p*-(1→4)-α- D-Gal*p*A-(1→] repeats (McNeill *et al*., 1984; Albersheim *et al*., 1996) covalently linked to HG domains (Round *et al*., 1997, 2001, 2010). The RG-I backbone is decorated mainly with arabinan and arabinogalactan I and II side chains. It is said that 80-90% of citrus pectin dry weight is galacturonic acid. The majority of GalA is found in HG as smooth region (Ridley, O'Neill, Mohnen 2001).

Commercially, pectins are extracted by treating apple pomace or citrus peels with hot dilute mineral acids. Pectin with a high degree of methylation DM>50% is known as high methoxyl pectin (Rolin 2002). Pectin's functional properties and specially reactivity toward calcium is largely dependent on the amount of methylated galacturonic acids and their distribution pattern within the HG region as recognized random or ordered (blockwise) distribution (Powell, Morris, Gidley, Rees 1982; Willats *et al.* 2001). At pH› 3.5, the negatively charged pectin is absorbed onto the positively charged milk proteins (Pereyra, Schmidt, Wicker 1997). It has been demonstrated that pectin associates with caseins at pH‹ 5.5 during acidification in emulsions stabilized by sodium caseinate (Bonnet, Corredj, Alexander 2005). High methoxyl pectin has relatively low affinity for the surface of casein particles due to the high degree of methyl esterification. Blocks of low affinity areas on the pectin molecules may protrude from the surface of the casein micelles like the mechanism caused by ***κ***-casein at high pH values termed as steric stabilization (Tholstrup Sejersen, Salomonsen, Ipsen, Clark, Rolin, Balling Engelsen 2007; Tromp, Kruif, van Eijik, Rolin 2004). In addition to steric repulsion, it has been suggested that a network of casein/pectin complexes, in which pectin coated proteins, are connected to each other. Pectin-pectin links has indeed been claimed to be necessary to achieve stability in acid milk dispensions (Tholstrup Sejersen *et al.* 2007; Tromp *et al*. 2004; Laurent and Boulenguer 2003). It has also been proved that a network of pectin/casein complexes rather than a pectin network provide stability (Tromp *et al.* 2004). Since electrosorption is responsible for the attachment of pectin onto casein micelles, this adsorption has a multi-layering nature (Tuinier, Rolin, de Kruif 2002). Not only the overall charge of the pectin but also its charge distribution on the chain determines its functionality as an ingredient in foods (Lam, Shen, Paulsen, Corredig 2007). For pectin, the importance of the distribution pattern of non-methylated pectin galacturonic acid residues over HG-backbones, termed as degree of blockiness (DB), especially in the presence of calcium, termed as calcium sensitivity, has been highlighted (Ström, Ribelles, Lundin, Norton, Morris, Williams 2007). The formation of calcium-pectin networks is described by egg box model with calcium ions trapped between the carboxyl groups of different pectin chains (Braccini and Perez 2001). To make this stable junction zone, a specific range of adjacent carboxyl groups is needed (Powell *et al.* 1982; Luizo and Camreron 2008). Although the calcium sensitive pectin provides more network than the non-calcium sensitive ones

and stabilizes greater particle sizes, calcium sensitivity is not the only criterion governing stability in acidified milk drinks (Laurent and Boulenguer 2003). The aim of the present work is to evaluate and compare the chemical composition and physicochemical properties of commercial pectins offered for stabilization process in ADD in market and those extracted from *Citrus aurantifolia* in the south of Iran by acid extraction method. The efficiency of those pectins for Iranian ADD stabilization was assessed and tentatively linked to the methyl distribution pattern of the selected pectins.

2. Materials and methods

2.1. Materials

Three commercial high methoxyl citrus pectins (i) non-calcium sensitive (NCS), (ii) medium-calcium sensitive (MCS), (iii) high-calcium sensitive (HCS) were kindly provided by Degussa (France).

2.2. Methods

2.2.1. AIR preparation

The peels of *Citrus aurantifolia* (lime) were collected from industrial juice extraction in September 2007 from Shiraz, Iran. The peel samples were air-dried at 30˚C or oven-dried at 55˚C for 5 days and 2 days, respectively. The dried samples were ground with a hammer mill and the particles <300μm were discarded to avoid trouble during filtration. 100 g of each types of peel was immersed in boiling ethanol 96% (v/v) for 20 min. The slurry was filtered through G3 sintered glass and insoluble material was left under magnetic stirring for 30 min with ethanol/water (70/30 v/v) and filtered again. This step was repeated until the filtrate gave a negative reaction to the phenol sulphuric acid test (Dubois, Gilles, Hamilton, Reberts, Smith 1956). The residue was dried by solvent exchange (ethanol/acetone) and left overnight at 40˚C to give alcohol insoluble residue (AIR).

2.2.2. Pectin extraction

Four different extraction conditions were considered on AIR: HCl 0.05, 0.03 and 0.025 M at 85˚C for (3× 20 min) and also water (pH 4.5; pH was decreased by HCl 0.1 M) at 100˚C for (3× 20 min).

5 g of sample were added to 150ml of extracting solution for 20 min under mild stirring. The slurry was then centrifuged and the liquid phase filtered through G3 sintered glass. Extraction was performed twice further. All the extracts were filtered again and the pH was adjusted to 5 by NaOH 0.1M, prior to concentration under vacuum at 40˚C to get ~1/5 of the initial volume. The pectin was coagulated by 3 volumes of ethanol 96% (v/v) and left overnight at 4˚C. The coagulated pectin was recovered by centrifugation and washed several times with ethanol 70% (v/v) then ethanol 96% (v/v) and acetone before drying at 40˚C. In pectin extraction from peels, 30g of peels were mixed with 1 L of HCl 0.03M at 85˚C for 3× 20 min. Soluble pectin was precipitated by 2 volumes of ethanol 96% (v/v) and the procedure continued as done for AIR.

2.2.3. Analytical

The uronic acid content of AIR, extracted pectins, residues and commercial pectins was determined by the automated *m*-phenylphenol method (Thibault, 1979). The individual neutral sugars were analysed as their alditol acetate derivatives by gas chromatography (GLC) after acid hydrolysis. Pectins were hydrolysed with 1M H_2SO_4 (3h, 100˚C). AIR and residues were pre-hydrolysed with 13M H_2SO_4 (30min, 25˚C), diluted to 1M H_2SO_4 and heated (2h, 100˚C). The individual neutral sugars obtained were reduced with $NaBH_4$, acetylated and analysed by GLC (Blakeney, Harris, Henry, Stone 1983). Inositol was used as internal standard.

Methoxyl and acetyl contents of pectin were quantified by HPLC. Methanol and acetic acid released by alkaline de-esterification (1N NaOH) for 1h at 4˚C in the presence of $CuSO_4$, were quantified by HPLC on C_{18} Superspher eluted at room temperature with sulphuric acid solution at pH 3.5, at a flow rate of 0.7mL/min. Isopropanol was used as internal standard. The degree of methylation (DM) and the degree of acetylation (DA) were calculated as the molar ratio of methanol and acetic acid to galacturonic acid, respectively (Levigne, Thomas, Ralet, Quemener, Thibault 2002b).

DM was also determined by titrimetry. All samples were recovered in acidic form by passing through a strong H^+-exchanger (Rohm and Hass Amberlite IR 120) ($25cm^3$). Free carboxylic acid functions were quantified at the neutralization point by conductimetric titration with NaOH of known molarity and total carboxylic functions

were determined by colorimetry on the same solutions after saponification (Ralet and Thibault, 2002). DM was calculated as shown in eq. 1:

DM= 100 × (total carboxylic functions- free carboxylic functions)/ total carboxylic functions

Degree of blockiness of pectins was estimated using high-performance anion-exchange chromatography (HPAEC) on a Dionex system equipped with a Carbopac PA1 column and pulsed amperometric detection. The column was eluted with 0.5M NaOH, 1M sodium acetate and water at 0.25 mL/min. Borwin Software (JMBS Developments, Grenoble, France) was used for data acquisition and processing. Monomer, dimer and trimer of GalA were used as standards (Dass, Voragen, Schols 2000; Guillotin, Bakx, Boulenguer, Mazoyer, Schols, Voragen 2005; Bonnin, Le Goff, Körner, Vigouroux, Roepstorff, Thibault 2002). Pectin samples were hydrolysed by a purified *endo-PGII* of a cloned *Asp. Niger* provided by *Novozymes (Bagsvaerd, Denemark)*. 6 µl of poly-galacturonase (*endo*-PGII; 36 nkat/mL) (the enzyme purified as described by Sakamoto *et al*. (2003) from a liquid preparation of *Aspergillus niger*, Novozymes) was added to 3 mL of HG solution (~1mg/mL) in 50mM acetate buffer pH 4. Hydrolysis was performed at 40°C for 3 days, fresh enzyme (6 µl) being added at 24h and 48h. After 3 days, hydrolysates were filtered (0.45µm Millipore) and injected to HPAEC. The degree of blockiness (DB) is the amount of mono-, di- and trigalacturonic acid released by the *endo*-polygalacturonase related to the amount of free galacturonic acid present in the sample. The absolute degree of blockiness (DB_{abs}) is the amount of mono-, di- and trigalacturonic acid released by the *endo*-polygalacturonase related to the total amount of galacturonic acid (free and methyl-esterified galacturonic acid) present in the sample.

2.2.4. Molar mass determination

Pectin average molar masses and intrinsic viscosities were determined using high-performance size-exclusion chromatography (HP-SEC) as described by Ralet, Dronnet, Buchholt, Thibault (2001). HP-SEC was performed at room temperature on a system constituted of one Shodex OH SB-G pre-column, followed by two columns in series (Shodex OH-Pack SB-804 HQ and OH-Pack SB-805 HQ, Shodex, Showa Denko KK, Miniato, Japan), eluted with 0.05M $NaNO_3$ buffer, containing 0.02% NaN_3 as preservative, at a constant flow rate of 42 mL/h. Detectors used were a

refractometer (RI) (ERC 7517A), a differential viscosimeter (T-50A, Viscotek, Houston, TX) and a multiple angle laser light scattering device (MALLS) (Mini dawn, Wyatt, Santa Barbara, CA), operating at three angles (41˚, 90˚ and 138˚). 0.5 mL of pectin solution in water (~5mg/mL) were added to 0.5 mL of 0.1M $NaNO_3$, containing 0.04% NaN_3, mixed five times in 3h then filtered (0.45µm) before injection of 50µL. Data for molar mass determinations were analysed using Astra Software (Wyatt, Santa Barbara, CA), taking a d_n/d_c of 0.146. Data for viscosimetry determinations were analysed using Tri SEC Software (Version 3.0, Viscotek, Houston, TX).

2.2.5. ADD preparation

Preparation of the drinkable yogurt was carried out by diluting yogurt (MSNF 11%, fat 1.5%), homogenizing at 200 kg/cm^2 and adding 0.075% of potassium sorbate to prevent fungal growth. The pectin stock solution was prepared by dissolving 1.2 g of pectin under stirring in 150 mL of water at 50˚C. The pectin solution was added to yogurt to make different pectin concentrations from 500-4000 ppm and have 5.5% MSNF in the final drink. The pH was adjusted to 4.2, 4.0 and 3.7 by lactic acid or NaOH 10%. All the samples were kept for 2 hours at 4˚C before use, as recommended by Laurent and Boulenguer (2003).

2.2.6. Measurement of precipitate quantity

After 20 g of each acidic beverage had been centrifuged at 2000*g* for 20 min, the supernatant was decanted and the residue allowed standing for 20 min. After the remaining supernatant was decanted, the weight percentage of precipitate was measured (Nakamura *et al.* 2003).

2.2.7. Stability measurements

100 mm of ADD samples in tubes of 1.5 cm diameter were stored at 4°C for 21 days and the serum separation was measured daily for 1 week (in mm of the layer of clear supernatant at the top of the sample in the bottle) and then weekly to monitor instability (Quemeneur, Haluk, Hardy 1995). If the layer of clear serum was more than 1mm of the total sample height, the sample was considered unstable (Tromp *et al.* 2004).

2.2.8. Chromatography

Chromatography on DEAE-Sepharose CL-6B was performed on a column (31×2.6 cm) equilibrated with 0.05M sodium succinate, pH 4.5, at a flow rate of 90mL/h. Pectin samples (20mL of a solution at 1mg/mL) were loaded onto the column and eluted with a linear NaCl gradient (0-0.6M). Fractions (14mL) were collected and analyzed for GalA and neutral sugar content (Thibault 1979; Tollier and Robin 1979).

2.2.9. Data Analysis

Data analysis was performed on experimental parameters using ANOVA (one-way analysis of variance) of SPSS ver.9 and Harvard Graphic 98 to show the results of the evaluation of different pectin types and concentrations at different pH on the stability of ADD. According to the ANOVA analysis, the interaction was significant for $p< 0.05$. Data were compared using Duncan test at the 95% confidence level.

4. Result and discussion

4.1. Yield and analytical figures of commercial and extracted pectins and residues

The analytical results of the commercial pectins, lime AIRs and their corresponding pectins are summarized in Tables 1 and 2.

The AIRs represented 63-65% of the lime peel dry weights, which is in agreement with previously published data (Yapo, Lerouge, Thibault, Ralet 2007a; May 1990). 71-72.5% of the AIRs dry weight consists of carbohydrate, which is in close agreement with the amounts of 67.4% in lemon dietry fibers (Ralet and Thibault 1994), 69.9% in lemon pulp (Brillouet, Rouau, Hoebler, Barry, Carré, Lorta 1988) and 81.7% in lime peels reported by Yapo *et al*. 2007a.

Lime AIR carbohydrates consist of mainly GalA, glucose and arabinose. The GalA content was in the range 344-350 mg/g, the DM in the range 80-86% and the DA in the range 15-20%, which is in consistency with previous reports (Ros Schols, Voragen 1998; Koubala, Kansci, Mbome, Crépeau, Thibault, Ralet 2008b; Yapo *et al.* 2007a).

The pectin yield for water extraction was in the range of 5.8-9% (w/w), which is in agreement with previously reports (Yapo *et al.* 2007a and Koubala *et al*. 2008b).

Higher pectin yield was obtained using acidic extraction conditions (35-41.1% w/w). The yield increased significantly with decreasing pH, as reported by Levigne, Ralet, Thibault (2002a) and Yapo, Robert, Etienne, Wathelet, Paqout (2007b) for sugar beet pulp. The uronic acid content of the extracted pectins was in the range of 570-640 mg/g , in agreement with literature (Yapo *et al*. 2007a). Acid extracted pectins were poorer in neutral sugars compared to the water extracted ones, in consistency with results of Koubala *et al.* (2008a and b). The rhamnose content of acid-extracted pectins was higher than the water extracted pectins of air-dried AIR, suggesting that air-dried AIR acid extracted pectins are richer in hairy regions. The molar ratios of GalA to rhamnose were in the range of 29-47, which is close to the values reported by Yapo *et al.* (2007a). The GalA contents of the residues of acid extractions showed that 87-93% of the pectins present in AIRs were extracted by acidic treatments. Water extracted pectin of oven-dried AIR had the highest rhamnose content, comparable with water extracted pectin of air-dried AIR. It seems that oven-dried pectin is more sensitive to be broken down by acid, compared to the air-dried one. All the extracted pectins possessed high DM% but low DA%, which is consistent with the results of Yapo *et al.* (2007a).

Degree of blockiness represents the amount of non-methyl-esterified mono-, di- and tri-galacturonic acid released by endo-polygalacturonase relative to the total amount of non-methylesterified galacturonic residues present in the pectin (Daas, Meyer-Hansen, Schols, De Ruiter, Voragen 1999). The absolute degree of blockiness (DB_{abs}) is the amount of mono-, di- and trigalacturonic acid released by *endo*-polygalacturonase related to the total amount of GalA (free and methyl-esterified GalA) present in the sample. DB% values were in the range of 6.7-13.8% for commercial pectins and 5.4-16.4% for extracted pectins, which fit in the range mentioned by Dass *et al.* (1999) and Guillotin *et al.* (2005) for high methoxyl citrus pectins. DB% of 11% is considered for a blockwise distribution of free GalA and DB% of 1% is introduced as a non-blocky random distribution of non-methylated GalA in high methoxyl pectin (Guillotin *et al*. 2005). DB_{abs} of commercial pectins was in the range of 2.4-5.2 and for acid extracted pectins of 2.4-5.3, which is in the range of calcium sensitive HM pectins studied by Guillotin *et al.* (2005).

It is said that the strength of Ca^{2+}-pectin gels correlates better with the DB_{abs} values than with DM and DB. By this explanation all the acid extracted pectins

appear slightly blocky and close to the commercial types. It should be mentioned that pectins having similar DM and DB values may still differ in the size and number of blocks (Guillotin *et al.* 2005).

4.2. Macromolecular characteristics

Pectin weight-average molar mass and intrinsic viscosity values were in a specific range of 176-320 (kg/mol) and 740-1210 (mL/g), respectively for commercial and extracted pectins (Table 3).

The values are in agreement with values reported by Zykwinska *et al.* (2008) on acid extracted citrus pectin of similar DM (M_w: 180 kg/mol and η= 900 mL/g). The polydispersity index was less than 2 for almost all pectins (data not shown). Polydispersity Index ‹2 for pectins reveals that the fractions are relatively homogenous with respect to molar mass (Hellìn, Ralet, Bonnin, Thibault 2005).

4.3. Fractionating by analytical Anion Exchange Chromatography

To understand more about the charge density of the pectins used in ADD, ion exchange chromatography (AEC) on DEAE-Sepharose CL-6B was used to fractionate pectins. GalA and neutral sugar recoveries were > 95% for all samples. Ralet and Thibault (2002) represented the correlation between ionic strength of the eluant in AEC fractions and Debye length (i.e., eluant ionic atmosphere thickness) versus DM, which is linear. For HCS pectin, about 46% of pectin eluted at an ionic strength of NaCl 0.12 M (DM around 90%) and the rest at an ionic strength of NaCl 0.24 M (DM of around 70%). These values for NCSP at the same NaCl ionic strength were 67% and 33%, respectively and for the pectin of air-dried peels, 51.3% and 48.7%, respectively. For MCSP, the distribution was 57.3% and 42.7% at ionic strength of NaCl 0.14 M and 0.22 M, respectively. NCSP and MCSP were eluted as a very thin peak at the beginning regarded to the highly methylated GalA units over HG domains with a random scattered non-methylated GalA generated by alkaline or fungus-PME deesterification (Denès, Baron, Renard, Péan, Drilleau 2000). HCSP and pectin extracted from air-dried peels were eluted as a broader second peak compared to NCSP and MCSP. As reported by Guillotin *et al.* 2005 the DB is not sufficient to explain the elution behavior of the sub-populations on anion exchanger.

4.4. Sample selection for ADD application

Acid extraction by HCl 0.03M was chosen to apply on air-dried peels according to DM and DB values, which were in good agreement with DM and DB values of commercial pectins. 30g of peels were treated by acid. The extraction conditions in each step were the same as performed on AIR, except pectin recovery steps with alcohol. The chemical composition and macromolecular characteristics are summarized in Table 4. This pectin and commercial pectins were used for application in ADD.

4.5. Measurement of precipitate quantity

Fig. 1 shows the precipitate quantities of Iranian ADD after low speed centrifugation that contains 0.05-0.4% pectin at pH 3.7, 4.0 and 4.2. The low precipitate quantities generally indicate good stability of milk protein under acidic conditions (Nakamura *et al.* 2003).

Pectin is more effective as a protective hydrocolloid at pH=4 compared to pH 3.7 and 4.2 but statistically the NCSP, MCSP and pectin extracted from air-dried peels were the same as a protective hydrocolloid at pH 4 and 4.2. Since pectin interacts with casein particles *via* electrostatic interactions, its stabilization behaviour is pH-dependent. As shown by Liu *et al.* (2006), HM pectin with DM≥ 67% had a monomodal distribution of particle sizes at pH≥3.8 and for pectin of DM 64% the dispersion showed a bimodal distribution of particles at pH≤3.8. Bimodal distribution of particles corresponded to be prone to serum separation, which is in consistency with our results. For CSP, statistically there was no difference between pH 3.7, 4.0 and 4.2. It seemed that this kind of pectin plays the same role in a broad range of pH. It is probably expectable since Löfgren, Guillotin, Evenbratt, Schols, Hermansson (2005) showed that two pectins with different internal distributions of methoxyl groups responded completely differently to Ca^{2+} and pH changes. It is said that the blockwise internal arrangement allows stronger and more effective intermolecular interactions that could overcome the pH changes (Lutz, Aserin, Wicker, Garti 2009). As the pH decreased from 4.1 to 3.5, the adsorbed amount of pectin onto the casein micelles increased (Tuinier *et al*. 2002). That's why we had more sedimentation values for HCS pectin compared to the others, which is also in consistency with reports of Joye and Luzio (2000), who noted that HCS pectin yields a greater sediment

weight than NCS pectin. Joye and Luzio (2000) attributed this result to the fact that HCS pectins tend to retain more water in the gel than NCS ones do. It is also observed that suitable type of pectin at a concentration of 0.4% can stabilize milk proteins under acidic conditions over the entire pH range tested, which is in consistency with Nakamura *et al.* (2003) results. The statistical analysis showed that for NCSP only, the stabilizing effect of pectin concentration at 0.3% and 0.4% was the same. NCSP could stabilize the drinkable yogurt of 5.5% MSNF more effectively at 0.3% pectin concentration compared to the other pectins. For the pectin of air-dried lime peels the trend of curves is neither like NCS nor HCS commercial pectin but close to MCSP. This can be explained by the result of Ros *et al.* (1998) that pectin extracted by an acid process are normally high methoxyl pectin and contain a mixture of both calcium sensitive and non-calcium sensitive pectins. The trends of curves are alike to Sedlmeyer, Brack, Rademacher, Kulozik 2004 and Willats *et al*. (2006). It may be hypothesized that for HCS curves slope are more sharp due to the matter that when a polymer is attached by a single terminal points (HCS pectin) to the particle, the layer formed is thicker than that formed when the polymer is attached at several points around the particles as happens for NCS pectin (Laurent and Boulenguer 2003).

4.6. Stability measurements

Aggregation and settling of casein particles during storage results in serum separation and the formation of casein clusters, which contributes to sandy mouth-feel (Syrbe, Bauer, Klostermeyer 1998). The development of serum separation with time for ADD made with different pectin concentrations and stored at 4°C is shown in Fig. 3. Since sour taste is more favored by Iranians, pH=4.0 was selected for further investigations. For NCS, MCS and HSC pectin at a concentration of ≥ 0.2% virtually no serum separation occurred during the first 7 days of storage. For NCS pectin, clear serum separation at pectin concentration of 0.1% appeared in the 7th day but for MCS pectin the serum separation appeared in the second week. Liu *et al.* (2006) claimed that pectin of DM around 68-71% is more effective to stabilize ADD of 8.5% MSNF than DM around 64%. Similar results were observed by Lam *et al.* (2007) who compared a pectin of DM<60% and a pectin of DM around 68-71%. Statistical analysis showed that the major serum separation occurred in the first week of storage for

all pectins studied, which is close to the results of Lucey, Tamehana, Singh, Munro 1999. It is possible that the less MSNF we have in ADD, the less charge density is needed to stabilize the proteins. For HCS pectin at a concentration of 0.2% the serum separation appeared clearly in the second week. Samples appeared cloudy during the first week. For pectin of air-dried peels at a concentration of 0.2%, serum separation occurred in the 7^{th} day of storage. It was observed that at a concentration of 0.3% of pectin, stabilization occurred during the whole shelf life of ADD (5 weeks, data not shown) without any serum separation or sedimentation whatever the pectin used. This concentration was lower than the one observed by Lucy *et al.* (1999) for 6.5% of MSNF, Nakamura *et al.* (2003) for 8% MSNF and Tromp *et al.* (2004) for 8.5% MSNF. It can be observed that many criteria can influence ADD stability. It is important to note that the effect of the pectin charge (DM) also depends on the type of protein employed. Processing history of the protein, its composition and its colloidal properties strongly affect the interactions of the protein with the pectin. The net charge of the adsorbed surface layer can also affect the stability of the final drink (Dickinson 2003). The more M_w pectin has, the more pectin in needed to stabilize according to effective coverage onto casein micelles as observed by Du, Jing, Zhang, Huang, Chen, Zhou (2009) for CMC in ADD. Beside that pectin requires a high molecular weight to stabilize ADD (Liu *et al.* 2006; Hotchkiss *et al.* 2002).

5. Conclusion

In order to stabilize Iranian ADD of 5.5% MSNF, high methoxyl pectin has to be added as much as 0.4% for stabilization during the whole shelf-life of 5 weeks. The chemical characteristic results also admitted through practical use of pectin in ADD, which demonstrated that commercial pectins are not very different in Ca^{2+} sensitivity. It is also concluded that many factors affect on the stabilizing ability of pectin. The combination of DM% in a range of 65.9-68.4 and DB% of 6-14 is quite effective to protect ADD against unstability of protein at a concentration $\geq$ 0.3%. It may also be hypothesized that the less MSNF we have, the less charge density we need in pectin for effective stabilization of ADD. These results may also highlight that it is not a matter of great importance if there is more fractions of high DM% but the most important thing is to have a

very few long enough blocks of free GalA in a large proportion of molecules, capable to be incorporated into the calcium sensitive structure and playing a role as a stabilizer, which is in consistency with Luzio and Cameron (2008). It can also be concluded that calcium sensitivity is not the only criterion governing stability in ADD (Laurent and Boulenguer 2003).

6. Acknowledgements

We thank Mr. Boulenguer for providing the commercial pectin samples from Cargill. The financial support from French government by CROUS is gratefully acknowledged.

Table 1

Yield (% w/w), chemical composition (mg/g; % w/w), degree of methoxylation and acetylation (% mol) of commercial pectins, AIRs and extracted ones from lime AIRs

Sample	Characteristics				
	Originated from	Yield%	GA	DM%	DA%
NCS	Citrus	-	762.5 ^{d}	68.4 ^{b}	1.2 ^{a}
MCS	Citrus	-	774.4 ^{d}	67.5 ^{b}	1.8 ^{a}
HCS	Citrus	-	812.4^{e}	65.9 ^{b}	1.1 ^{a}
Air- dried AIR	Lime-peels	64.3^{g}	344.8^{a}	80.2 ^{d}	20.5 ^{c}
Oven –dried AIR	Lime-peels	63.5 ^{g}	348.7 ^{a}	85.7 ^{d}	30.4 ^{d}
Air- dried AIR water	Lime-AIR	5.8 ^{a}	605.2^{c}	73.8 ^{c}	4.7 ^{b}
Air- dried AIR HCl 0.025%M	Lime-AIR	35.8 ^{c}	638.4 ^{c}	65.8 ^{b}	2.5 ^{a}
Air- dried AIR HCl 0.3% M	Lime-AIR	39.4^{e}	638.9 ^{c}	64.1 ^{b}	2.6 ^{a}
Air- dried AIR HCl 0.05%M	Lime-AIR	41.4^{f}	617.8 ^{c}	59.8^{a}	2.6 ^{a}
Oven- dried AIR water	Lime-AIR	9 ^{b}	633.5 ^{c}	67.6 ^{b}	4.1 ^{b}
Oven- dried AIR HCl 0.025% M	Lime-AIR	35 ^{c}	610.8 ^{c}	64.0 ^{b}	2.3 ^{a}
Oven-dried AIR HCl 0.3%M	Lime-AIR	37.6 ^{d}	588.4 ^{b}	66.8 ^{b}	2.6 ^{a}
Oven- dried AIR HCl 0.05% M	Lime-AIR	39.2 ^{e}	575.8 ^{b}	67.0 ^{b}	2.7 ^{a}

Values are the average of two replicates

Values in the same column followed by different superscripts are significantly different at (*$p<0.05$)

Table 2

Chemical composition (mg/g; % w/w), degree of blockiness (% mol) of commercial pectins and extracted ones from lime AIRs

Sample	Characteristics						
	Originated from	NS			Molar Ratio%	Degree of blockiness	
		Rha	Gal	Ara	GalA/Rha	Oligomers of 1&2&3DP [1]	Absolute [2]
NCS	Citrus	160 [b]	400 [c]	160 [a]	41 [b]	6.7 [a]	2.4 [a]
MCS	Citrus	150 [b]	330 [b]	200 [b]	43 [b]	10.5 [b]	3.4 [b]
HCS	Citrus	140 [b]	260 [a]	250 [b]	47 [b]	13.8 [c]	5.2 [c]
Air- dried AIR	Lime-peels	110 [a]	390 [c]	940[g]	26 [a]	-	-
Oven –dried AIR	Lime-peels	110 [a]	370 [c]	690 [e]	26 [a]	-	-
Air- dried AIR water	Lime-AIR	110[a]	660 [e]	770[f]	47 [b]	6.9 [a]	1.8 [a]
Air- dried AIR HCl 0.025%M	Lime-AIR	150 [b]	320 [b]	570 [d]	35 [a]	8.9 [b]	2.9 [a]
Air- dried AIR HCl 0.3% M	Lime-AIR	150 [b]	330 [b]	520 [d]	35 [a]	10.6 [b]	3.7 [b]
Air- dried AIR HCl 0.05% M	Lime-AIR	160 [b]	360 [c]	440 [c]	32 [a]	9.3 [b]	3.7 [b]
Oven- dried AIR water	Lime-AIR	180 [c]	470 [d]	700 [e]	29 [a]	5.4 [a]	1.8 [a]
Oven- dried AIR HCl 0.025% M	Lime-AIR	170 [c]	270 [a]	690 [e]	40 [b]	15.3 [c]	5.3 [c]
Oven-dried AIR HCl 0.3% M	Lime-AIR	140 [b]	270 [a]	630 [e]	35 [a]	16.4 [d]	5.3 [c]
Oven- dried AIR HCl 0.05% M	Lime-AIR	140 [b]	310 [b]	510 [d]	34 [a]	15.6 [d]	5 [c]

1 -Amount of mono-, di- and tri-GalA released by the enzyme related to the amount of free GalA present in the sample

2 -Amount of mono-, di- and tri-GalA released by the enzyme related to the total amount of GalA [(100-DM)×DB/100]

Values are the average of two replicates

Values in the same column followed by different superscripts are significantly different at (*$p<0.05$)

Table 3

Weight average molar mass (kg/mol), intrinsic viscosity [η] (ml/g) of pectins

Sample	M_w	[η]
NCS	190[a]	740[a]
MCS	195[a]	760[a]
HCS	184[a]	790[a]
Air- dried AIR water extraction	320[b]	1210[c]
Air- dried AIR HCl 0.025%M extraction	257[a]	940[b]
Air- dried AIR HCl 0.3% M extraction	251[a]	930[b]
Air- dried AIR HCl 0.05% M extraction	233[a]	740[a]
Oven- dried AIR water extraction	191[a]	750[a]
Oven- dried AIR HCl 0.025% M extraction	176[a]	850[a]
Oven-dried AIR HCl 0.3% M extraction	190[a]	780[a]
Oven- dried AIR HCl 0.05% M extraction	217[a]	820[a]

Values are the average of two replicates.

Values in the same column followed by different superscripts are significantly different (*$p<0.05$).

Table 4

Yield (% w/w), chemical composition (mg/g), degree of methoxylation and acetylation (% mol), Weight average molar mass (kg/mol), intrinsic viscosity [η]

Characteristics	Air-dried HCl 0.03 M extraction
Originated from	Air-dried peels
Yield%(w/w)	21.1 [a]
GalA (mg/g)	618 [a]
DM%	64.7 [b]
DA%	2.3 [a]
Rha (mg/g)	16.8 [b]
Ara (mg/g)	53.6 [a]
Gal (mg/g)	38.6 [a]
Molar ratio% (GalA/Rha)	31 [a]
DB%	6 [a]
DB% absolute	2.7 [a]
M_w (Kg/mol)	370 [b]
η (ml/g)	840 [b]

Values are the average of two replicates.

Values in the same row followed by different superscripts are significantly different (*p<0.05).

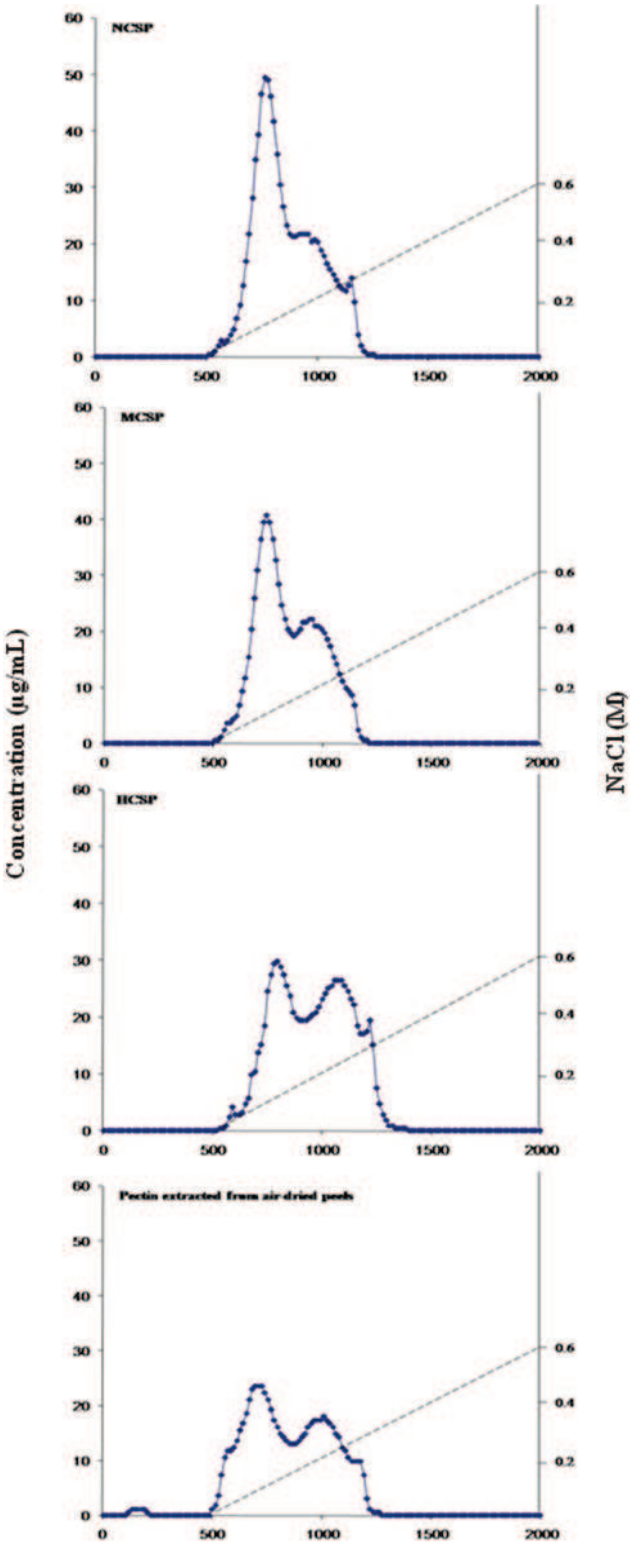

Fig. 1. Elution profiles of the pectins of similar DM but differing in their distribution of methyl esters (NCSP, MCSP, HCSP and pectin extracted from air-dried peels) on ion exchange chromatography (DEAE- Sepharose CL-6B): (♦) galacturonic acid; (•) neural sugars; (-) NaCl molarity

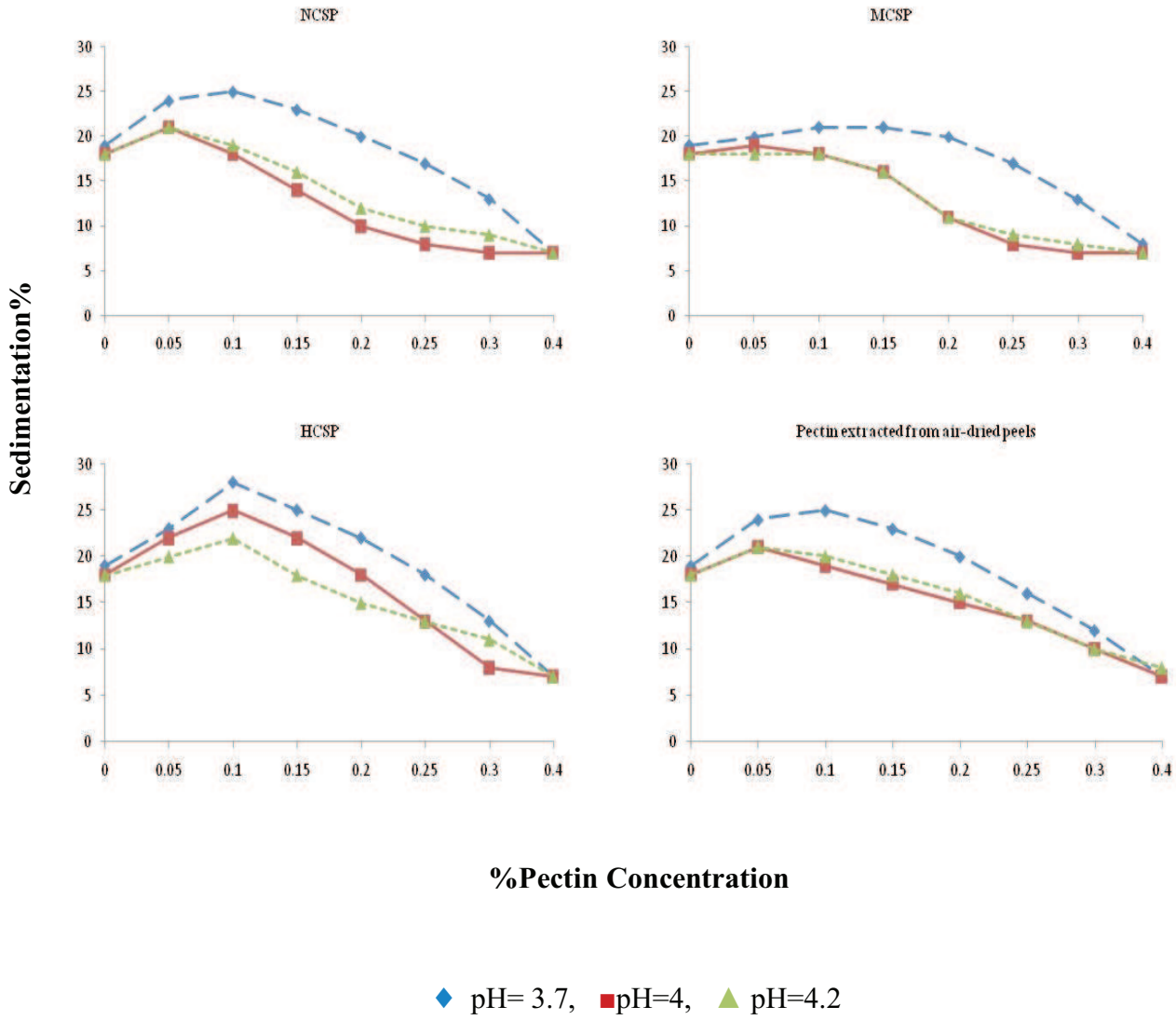

Fig. 2. Effect of the type and concentration of pectin at different pH on the precipitation quantity of Iranian ADD.

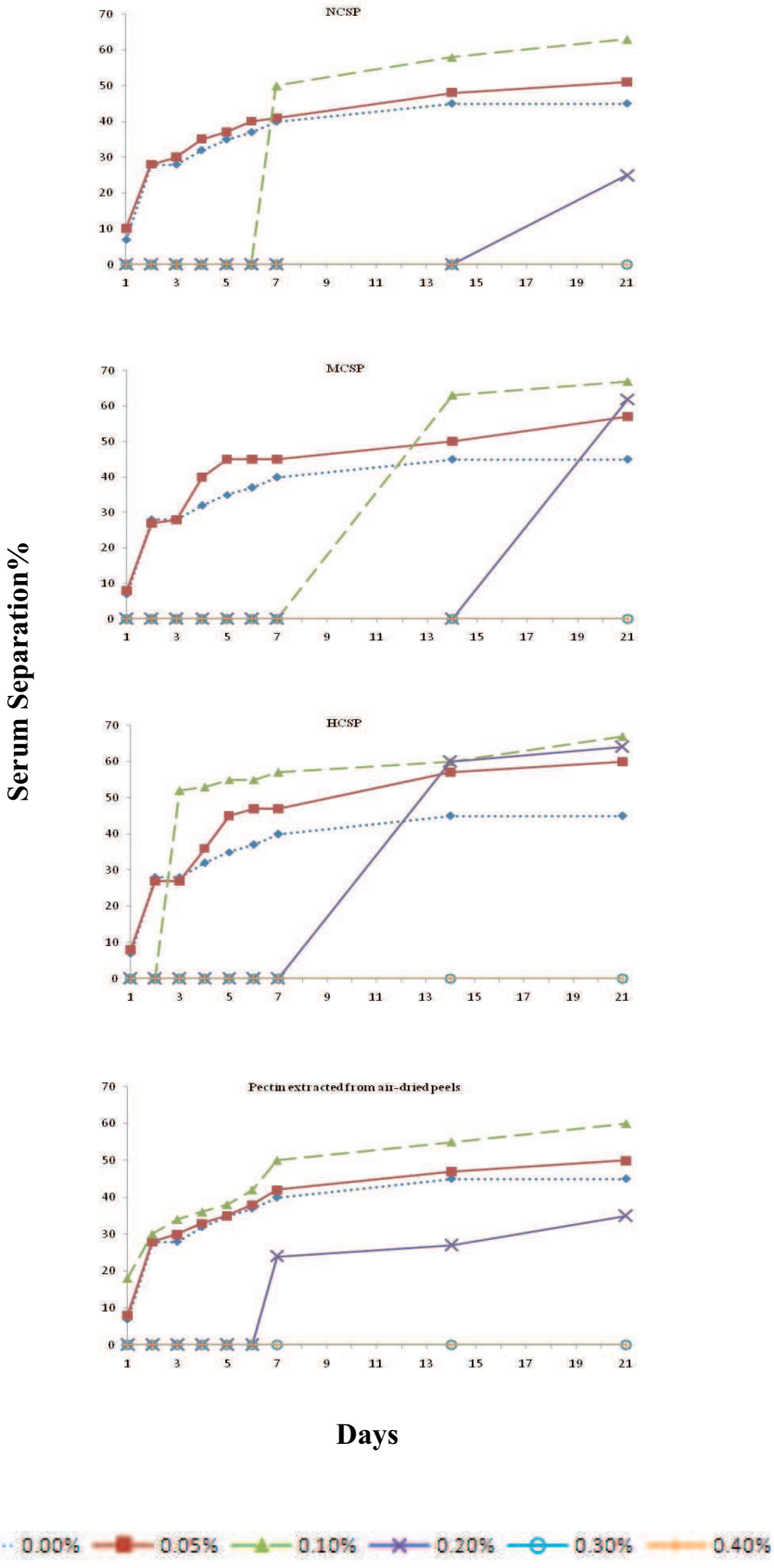

Fig. 3. Effect of the type and concentration of pectin at pH=4 on the serum separation of Iranian ADD.

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4.3. Complementary results

4.3.1. Endo-PG and PL digestion of commercial and extracted pectins

In order to have a logical comparison between model HGs and citrus pectins, we have to consider that citrus pectin consists of ~ 93% of HG and 7% of RG (RG domains are not methylated) (Ralet *et al*., 2008b; Yapo *et al*., 2007). DM values for NCSP, MCSP, HCSP and pectin A (extracted pectin) (calculated as DM/0.93), are 73.6, 72.6, 70.9 and 69.6%, respectively.

Hydrolysis rate of pectins by endo-PG and PL are presented in Table 16 on an HG-derived GalA basis (g $GalA_{oligomers}$/ 100 g $GalA_{HG}$; with $GalA_{HG} = GalA_{pectin} \times 0.93$).

As shown, pectins are suitable substrates for PL digestion. It has to be noted that PL hydrolysable parts do not necessarily represent PG non-hydrosable parts.

Table 16. Endo-PG and PL hydrolyzed parts of pectins

Sample	Hydrolysis % by endo-PG (dp 1-3)	Hydrolysis % by PL (dp 2-8)
NCSP	9.1	55.4
MCSP	12.9	57.6
HCSP	15.6	74.6
Pectin A	8.2	86.0

HCSP had the highest endo-PG and PL digestion yields since non-methylated and methylated stretches of GalAs are favored by both enzymes, respectively. Although Pectin A has the lowest DM, its digestion yield by PG was very moderate.

PL digestion (dp 2-8) is plotted versus DM in Fig. 37. In order to have a good interpretion about pectins, HG model samples are also pointed.

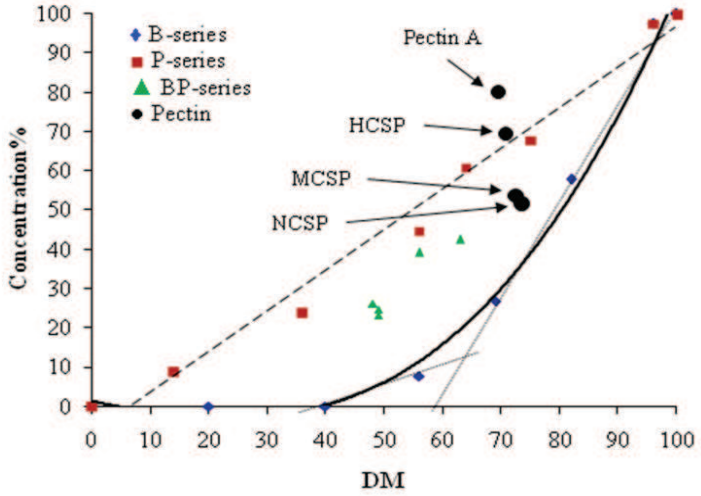

Fig. 37. Concentration of dp 2-8 of PL digestion versus DM

Pectin samples do not have random distribution patterns of methylesters like B-series. HCSP and Pectin A had even more PL hydrolysable parts than P-series. It may indicate that few long blocks of non-methylated GalAs exist, as in P-series, while other blocks of non-methylated GalAs are within the PL digested parts. For NCSP and MCSP even these few long blocks do not exist as they showed an intermediate behavior between B- and P-series. These results confirm the AEC fractionations, since we hypothesized that few but long blocks of non-methylated GalAs might explain the elution patterns of pectins in AEC.

Fig. 38, shows endo-PG II dp 1-3 digestion of pectins versus DM accompanied by other HG samples.

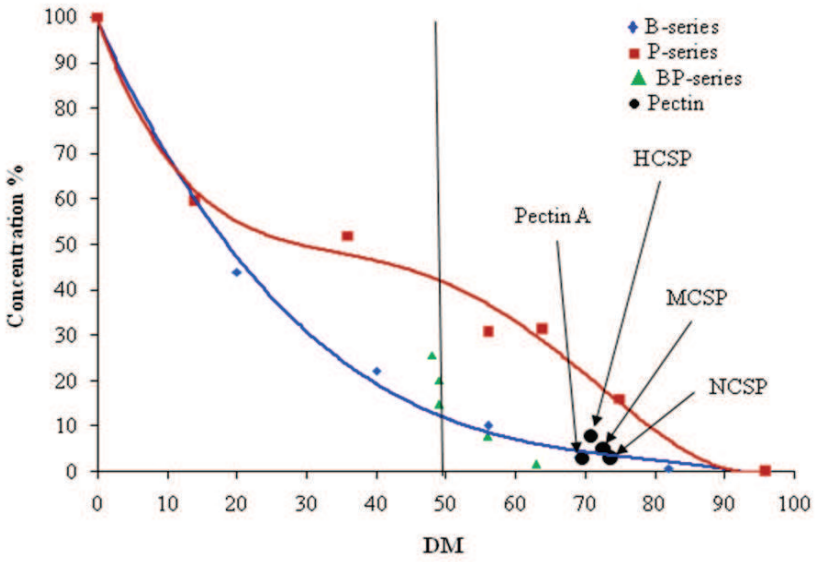

Fig. 38. Concentration of PG dp 1-3 versus DM for all HGs and pectins

Pectins showed a B-series-like behavior. It may confirm that DB values cannot fully explain subtle differences in blockiness of pectin molecules, especially at high DM values. Differences are the most obvious at DM ~ 50.

To have a better interpretation about PG digestion of pectins, dp 4-12 and dp > 12 of PG digestions were plotted versus DM in Fig. 39 and 40.

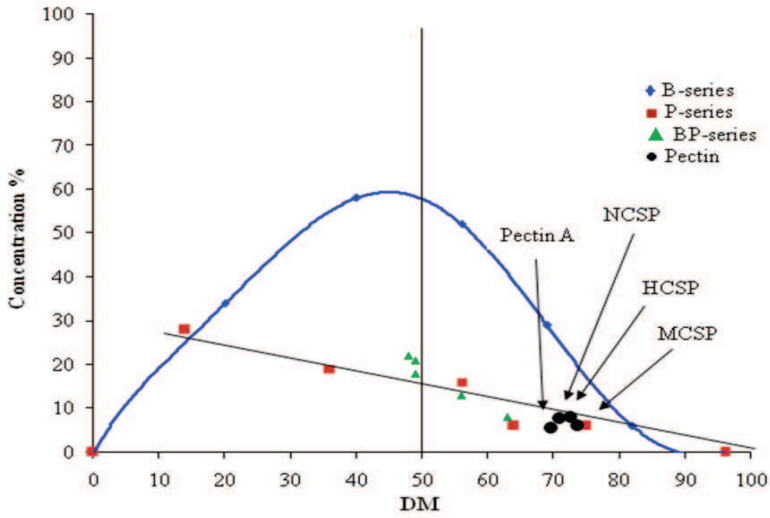

Fig. 39. Concentration of PG dp 4-12 versus DM for all HGs and pectins

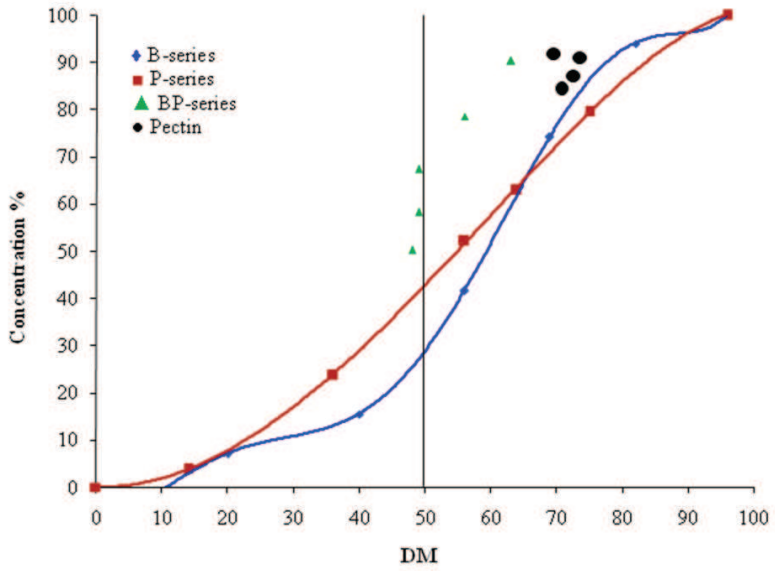

Fig. 40. Concentration of PG dp > 12 versus DM for all HGs and pectins

Fig. 39 showed the partially hydrolyzable part of pectins and HG samples. As observed, pectins do not have a random distribution of methylesters like B-series. The blocks of non-methylated GalAs are short enough not to be present in PG digests of dp 1-3 as exist for P-series HGs of corresponding DM. PG non-hydrolyzable parts of pectins (Fig. 40) showed a BP-series-like behavior, meaning that short blocks of non-methylated GalAs, not long enough to stick to PG subsites, are scattered over HG domains.

4.4. Discussion

In order to have a good interpretion about pectins and their differences in distribution pattern of methylesters over HG domains presented by PG and PL digestion, Fig 35 and 36 have been redrawn as Fig. 41 and 42 and the respected values for both enzymatic digestions for all pectins are pointed out in each figure.

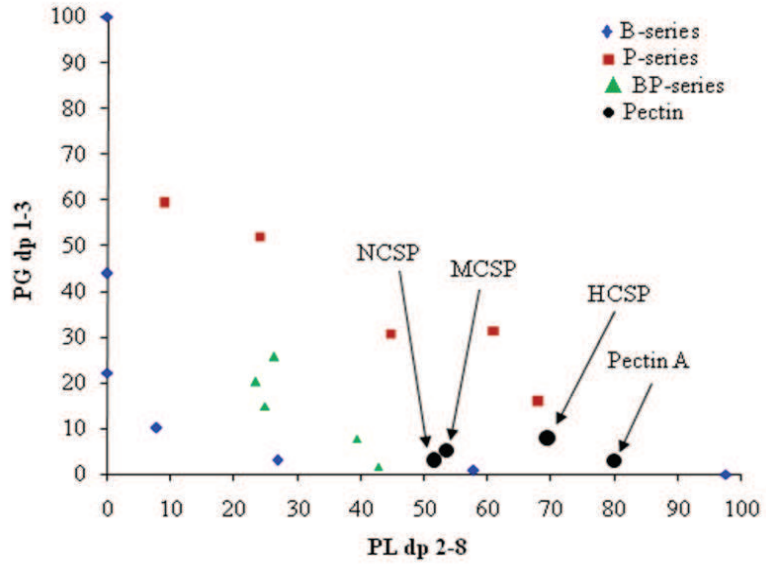

Fig. 41. Concentration of PG dp 1-3 versus PL dp 2-8 for all model HGs and pectins

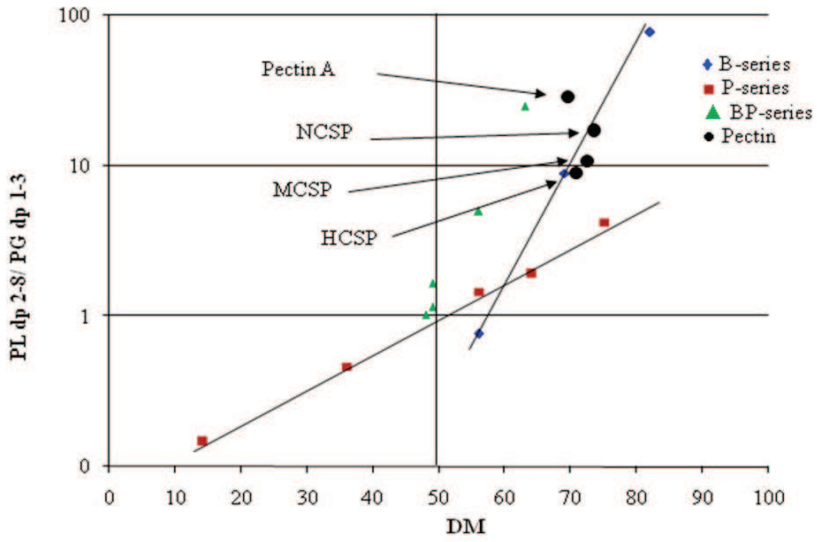

Fig. 42. Concentration of PL dp 2-8/PG dp1-3 ratio versus DM for all HGs and pectins

In Fig. 41, definite hydrolysable parts by PG and PL are presented in one figure for all samples. As observed, NCSP and Pectin A showed a behavior close to B-series, indicating very short blocks of non-methylated GalAs. HCSP exhibited the same behavior as HG-B76-P56, showing that few moderately long blocks of non-methylated GalAs could exist in pectin, confirming AEC results.

In Fig. 42, the ratio of PL digestion dp 2-8/ PG digestion dp 1-3 is plotted versus DM values of samples in logaritmic scale to magnify differences in both enzymatic digestions. Commercial pectins showed values close to B-series. NCSP and HCSP are almost the same according to the value of PL dp 2-8/PG dp 1-3. Pectin A exhibited a value similar to that of HG-B77-P63, evindencing that short blocks of non-methylated GalAs, enable to stick to PL subsites, could be scattered over HG domains.

This finding can explain the reason of homogeneity of all pectin in calcium sensitivity, blockiness and functionality.

4.5. Conclusion

Although there are in pectins a lot of PL degradable parts indicating numerous very highly methylated stretches over HG domains, the distribution pattern of methylesters cannot be defined as a random pattern. The structure homogeneity in pectins could explain their similar functionality. Since milk solid non-fat (MSNF) of Doogh is not high, it may be hypothesized that calcium sensitivity was not crucial in our stabilizing process.

GENERAL DISCUSSION, CONCLUSION AND PERSPECTIVES

1. Discussion

Fermented milk products are among the most widely consumed foods in the world today, enjoyed by peoples of nearly all cultures and backgrounds. Yogurt is perhaps the most well-known fermented milk product, but other popular fermented milk products include kefir in central Asia, yakult in Japan and doogh in Iran.

The main ingredients consist of yogurt, salt, water and/or mineral water. It is possible to add CO_2 (less than 0.6 g/100mL) which is favoured by Iranians. The pH must be less than 4.5 (generally 3.7-4.0), fat percentage not less than 0.5, salt percentage not more than 1% and the milk solid non-fat must be more than 3.2% for all types of this drink (generally 4-6%) (ISIRI 2009, No. 10528).

While yogurt remains a very popular comestible item, there is also a growing demand for liquid yogurt drinks. These yogurt drinks have the advantage of being more portable, easier and more convenient to consume than yogurt. Yogurt drinks often make use of pectin as a stabilizer against sedimentation of the milk solids that are found in yogurt. Pectin is a stabilizing material extracted from plants such as fruits and vegetables. Pectin is a particularly good stabilizer at pHs of between 3.7 to 4.3, which are the most typical pHs for commercial yogurt drinks (Tuinier *et al*., 2002). Commercial pectin used for yogurt drinks originates from citrus peels or occasionally from apple peels. The stabilizing function of negatively charged polysaccharides such as pectin arises from the fact that they adsorb onto the casein micelles (Parker *et al*., 1994). Due to the specific distribution of ester groups in the molecule, pectins have regions of different affinity as regards the protein binding. Regions of high affinity cover the protein surface (electrostatic stabilisation), regions of lower affinity extend out from the surface into the liquid and contribute to a so-called steric stabilisation. In the case of high methylester pectins the distribution of the free acid groups over the polygalacturonic acid chain – which may be random or blockwise – has a significant effect on the functional properties, especially on the stabilizing properties of acidified protein-rich products. A block by block distribution of the free carboxyl (or acid) groups generates regions with a higher electric charge

density at the pectin molecule, i.e. regions with a stronger affinity to the protein molecule, which, under acid conditions, is positively charged.

A specific mouthfeel can also be obtained by adding pectin. An increase in viscosity also enhances the protein stabilization in acidified milk drinks. Increased viscosity achieved by adding pectin counteracts the sedimentation of protein agglomerates. High methylester apple pectins are especially suited for this stabilization mechanism. If a low-viscosity end product is required, high methylester citrus pectins are mainly used. Only high methylester pectins within a very specific range of esterification are suited for the stabilization of acidified milk products. For optimum stabilization, pectins require a high molecular weight and defined calcium reactivity. High methylester citrus pectins fulfil these requirements (Herbstreith and Fox KG Corporate Group, 2003).

In order to have a new insight over pectins suitable for the stabilization of yogurt drink, three citrus pectins were kindly provided by Cargill. These pectins were claimed to be different in calcium sensitivity as non-calcium sensitive, medium-calcium sensitive and high calcium sensitive pectin. After characterization of commercial pectins, different acid-extraction methods were used to extract pectins from peels of "*Citrus aurantifolia*". Citrus peels was dried in oven or air-dried. Resulted pectins were characterized and subsequently, extraction by 0.03M HCl, at 85˚C for 3 × 20 min was chosen as the main extraction method.

Extracted HM-pectin with DM% 69.9 fairly matched with commercial pectins of DM 71-73%. M_w of extracted pectin was higher than that of commercial pectins but intrinsic viscosity (η) value of extracted pectin was similar to the values observed for commercial ones. Neutral sugar content was higher in the extracted pectin, indicating that the latter is richer in RG regions.

Functionality of pectins in Doogh showed that all types of pectins were quite good for stabilization at concentration ≥ 0.3%, which may offer another approval for homogeneity in DB and γCa^{2+} values. Pectin is more effective at pH=4, which confirms Herbstreith and Fox KG Corporate Group (2003) findings.

Several methods exist to appraise the repartition of methyl groups onto pectin as DB, DB_{abs} and calcium activity, referring to endo-PG digestion and direct calcium activity measurements.

DB and DB_{abs} values were subtlely different in pectin samples. Those values are however difficult to connect to precise patterns of methyl esterification as real standard pectins with different blockiness and calcium activity to compare with are lacking.

In order to simplify the complexity of pectin molecules to probe into distribution patterns of methylesters over HG stretches and pave the way to have its relation toward calcium sensitivity, model homogalacturonans with different charge density distribution were generated. HG was isolated from deesterified citrus pectin through mild acidic condition. Demethylated HG was chemically methylated under controlled condition in the presence of methyl iodide (Renard and Jarvis, 1999). Blockwise arrangement of carboxyl groups was generated by p-PME and random pattern generated by alkali treatment of almost fully methylated HG. In order to potentially generate shorter demethylated blocks, p-PME was applied to selected samples from the B-series (DM 76, 77 and 82), since the non-methylated GalA amount of the mother sample can control the p-PME action to some extent (van Alebeek *et al*., 2002). To summarize, three HG series (B, P and BP) potentially differing in their methylation pattern were generated.

For DM values > 15, whatever the experimental approach used, there is a very clear differentiation between alkali-deesterified samples, for which we know that the methyl distribution is purely random, and p-PME deesterified samples from the P-series that were generated from a very highly methylated mother HG. P-series samples exhibit high values of DB and DB_{abs} together with low values of γCa^{2+} and $\gamma_{exp}/_{theo}$. Those results are consistent with the presence of long demethylated stretches in HGs from the P-series, whatever the DM.

For those samples generated from HG-B~80, whatever the approach, two groups were identified. The first group comprised samples, that cannot or only hardly be differentiated from alkali-treated HGs of corresponding DM, and the second comprised samples that exhibit intermediate behaviour between random and fully blocky HGs of corresponding DM. The first group comprised the HG-BP of low values of [(DMi-DMf)/DMi] and the second group was with high values of [(DMi-DMf)/DMi].

Discriminate analysis of calcium sensitive samples is presented in Fig. 43. Two clusters have been identified for calcium sensitivity. The last cluster consists of three

samples, HG-P14, HG-P36 and HG-B20, which are the most calcium sensitive samples. The first cluster has been sub grouped. P-series are the most calcium sensitive samples even at high DM. For B-series a drop in γCa^{2+} is showed when DM decreases to < 40, which is consistent with previous reports (Ralet *et al*., 2001) and reconfirmed by Fig. 43. Tests of Equality of Group Means reassured us that values for DB and $\gamma Ca^{2+}{}_{exp}/_{theo}$ are not identical factors in the differentiation of sensitivity level ($p > 0.05$) while DB_{abs} , γCa^{2+} and DM are the main factors for ranking calcium sensitivity.

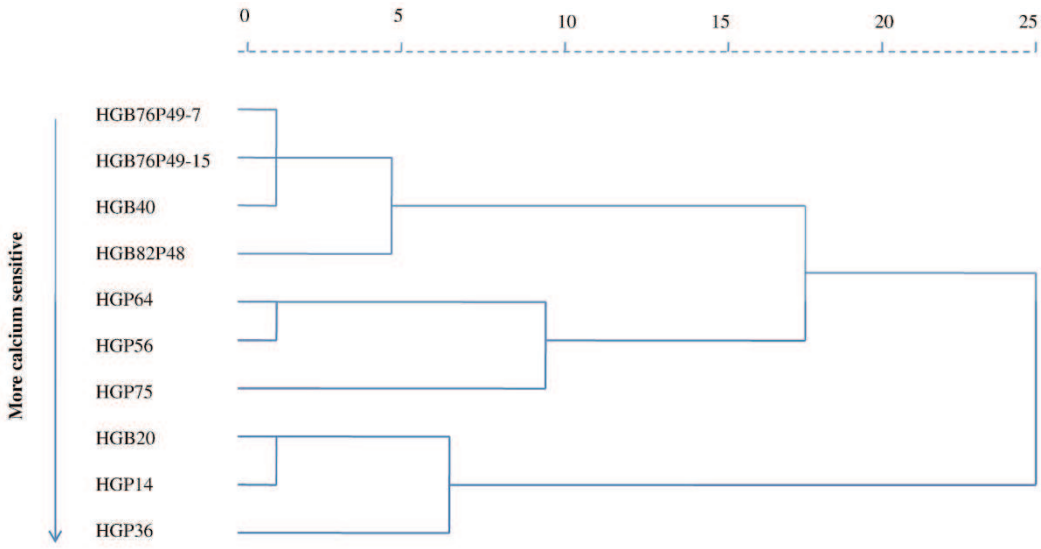

Fig. 43. Dendrogram of calcium sensitive samples.

Standardized Canonical Discriminate Function Coefficients shows that DB_{abs} is the most effective factor to estimate calcium sensitivity level followed by γCa^{2+} and $\gamma Ca^{2+}{}_{exp}/_{theo}$, while DM and DB are introduced as seducing factor for comparing the level of calcium sensitivity. It was previously reported by Ralet *et al*. (2001, 2003) and Ström *et al*. (2007) that $\gamma_{exp}/_{theo}$ and DB_{abs} are the most important factors correlated to calcium sensitivity.

Dendrogram of all samples is shown in Fig. 44. Two clusters have been identified. The first one has categorized non-calcium sensitive samples and the second one has sub-grouped the calcium sensitive samples from the highest to lowest value for sensitivity in each group. Tests of Equality of Group Means indicate that all factors (DM, DB, DB_{abs}, γCa^{2+} and $\gamma_{exp}/_{theo}$) are identical for calcium sensitivity (p

>0.05). It is worthwhile to mention that according to the classification results of discriminate analysis, 100% of original grouped cases were correctly classified.

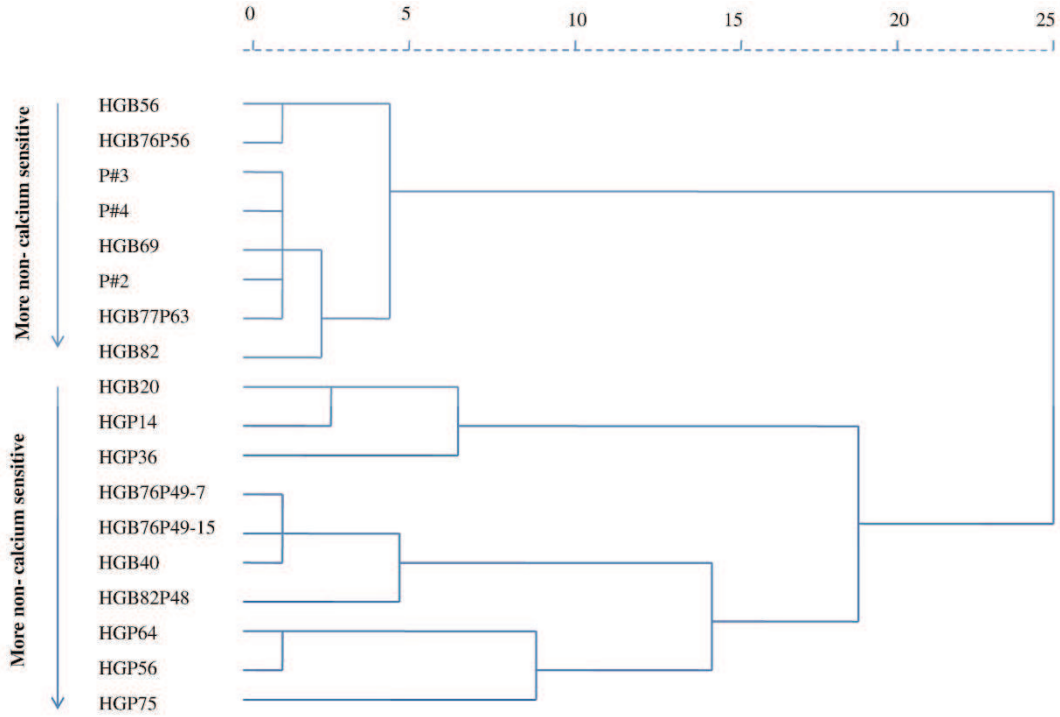

Fig. 44. Dendogram of all samples

Final cluster centers of dendrogram of all samples are represented in Table 17. This table shows statistically, the center points in each group. It means that, the values over the noted values for DM, γCa^{2+} and $\gamma Ca^{2+}{}_{exp}$ $/_{theo}$ and below the values noted for DB and DB_{abs} are considered as the most non-calcium sensitive samples in each group.

Table 17. Final cluster centers

Factor	Cluster	
	1	2
DM	63	45
DB	16	63
DB_{abs}	8	34
γCa^{2+}	0.56	0.17
$\gamma_{exp.} / _{theo}$	1.12	0.43

Fig. 45, offers more information about the DB and DB_{abs} values of pectins in comparison with model HGs.

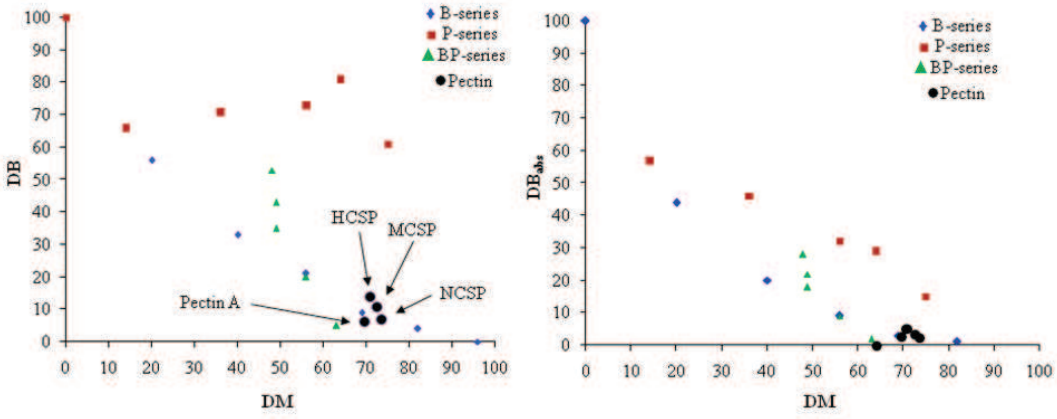

Fig. 45. DB and DB_{abs} of HGs and pectins

As observed, it is hard to differentiate pectins from B-series according to the DB and DB_{abs} values. In order to have a more precise information, samples (HGs and pectins) were hydrolyzed by PL and endo-PG and the products were analyzed to have a new insight over differences in distribution pattern of methylesters. In this regard, it was necessary to purify PL with specific mode of action. Purified PL had a specific activity of 3600 (nkat/mg), which is higher than the previous report by Limberg *et al*. (2000) from Pektolase^{TM}. Mode of action of purified PL is very

similar to the mode of action reported for the enzyme purified by Limberg *et al*. (2000), van Alebeek *et al*. (2001, 2002) and Körner *et al*. (1998).

A lot of work has been done on pectin digestion by endo-PG II by Daas, Guillotin and others who offered the major concepts of DB and DB_{abs}. The other parts of digests, which has been introduced as partially hydrolysed oligomers of dp 4-12 and non-hydrolysed part as oligomers dp > 12 in this present work has been studied for the first time in great details.

A few studies have also been performed on PL digestion of pectic molecules by van Alebeek, Körner and Limberg. In those studies however, PL digestion was not connected to PG hydrolysis to have an overview on methylated and non-methylated stretches at the same time. Since pectin structure is too complex to have all these interpretations, model HGs simplified these structural complexities to focus on specific molecular properties of HG pectin domains.

Despite of all these efforts, the structural complexity of pectins hampered the drawing of definite conclusions. Since all pectins had high DM values and quite similar intramolecular properties, all the estimations became as a general overview. We could find that pectins do not have a random distribution pattern of methylesters over HG domains but cannot be explained properly by methylester distribution patterns of P- or BP-series. Since many types of deesterification modes exist between totally random and blocky deesterification process, further work is needed to elucidate these differences at intramolecular level.

2. Conclusions & Perspectives

It is noteworthy to say that there could be many ways to distribute methylesters over HG domains and we explored only three of them. What happens in fact for the pectin in reality is that endogeneous plant PME could work on pectin in cell walls to make short but blocky type of non-methylated distribution patterns of GalAs and then extraction with acid could generate a kind of random deesterification process. What would be interesting to find, is the intermolecular heterogeneity and the probable differences with BP-series to get more idea about charge repartition on HG stretches.

Another interesting idea is to find out which part of pectin fractions is more involved in stabilization process of ADD and stick to the casein particles, either the

highly methylated fraction or the lowly methylated one or both with a defined ratio. Enzymatic fingerprinting on these sections can be the other matter of interest to expand the knowledge over DM and DB values of each fraction. It would be also worthwhile to compare the dangling properties of pectin by exo-PG to compare the overview of pectins in ADD. Choosing the proper pectins as standards for expressing as NCSP, MCSP and HCSP seems crucial for better comparison with model HGs.

RÉSUMÉ DES TRAVAUX DE THÈSE

Ce chapitre résume, en français, les travaux présentés dans le manuscrit. Il est divisé en cinq sous-chapitres:

- **Une introduction bibliographique**, qui permet de situer le contexte des travaux de la thèse. Cette introduction reprend les points essentiels de la bibliographie pertinente dans le cadre de ce travail. Sont en particulier décrits: la structure chimique des pectines et les structures macromoléculaires qui en découlent; les procédés utilisés industriellement pour extraire les pectines et les modifier; les applications des pectines dans l'industrie agroalimentaire en tant qu'agents gélifiants et en tant que stabilisants des boissons lactées acides; la réglementation et le marché de ces hydrocolloïdes.

- **Une partie "objectifs de la thèse"**, qui permet de comprendre les approches expérimentales choisies et mises en œuvre pour appréhender certains ressorts structuraux responsables de la capacité de certaines pectines de stabiliser les boissons lactées acides.

- **Une partie "Résultats et discussion"**, dans laquelle les résultats du travail de thèse sont exposés et discutés. Cette partie "Résultats et discussion" est elle-même divisée en cinq sous-chapitres: (i) Caractérisation de pectines d'agrumes et étude de leur capacité à stabiliser le "Doogh"; (ii) Production de domaines 'homogalacturonane' modèles; (iii) Purification d'une pectine lyase; (iv) Dégradation enzymatique des homogalacturonanes modèles; et (v) Dégradation enzymatique des pectines capables de stabiliser le "Doogh". Certaines méthodes clefs ont été expliquées de façon détaillée et apparaissent en *italique* dans le corps du texte.

- **Une discussion générale** qui permet de replacer l'ensemble des résultats obtenus dans le contexte actuel des connaissances dans le domaine.

- **Une conclusion**, qui permet de faire le point sur l'ensemble des résultats obtenus et d'en dégager quelques perspectives de recherche.

1. Introduction bibliographique

Les pectines ont été isolées pour la première fois d'extraits végétaux par Braconnot au XIX^{ème} siècle (Braconnot, 1825 a, b). Il trouva cet "acide" dans de nombreuses plantes et, suite à l'observation des propriétés gélifiantes de celui-ci,

nomma cette molécule "acide pectique" du grec "pektos" signifiant "ferme, rigide". Les pectines sont des hétéropolysaccharides complexes de la paroi des cellules végétales des dicotylédones et de la plupart des monocotylédones, à l'exception des graines de graminées. Elles représentent de l'ordre de 40% en poids sec des parois cellulaires primaires des dicotylédones où elles contribuent à de nombreuses fonctions de la paroi. Les lamelles moyennes sont particulièrement riches en pectines qui jouent un rôle majeur dans l'adhésion inter-cellulaire et sont ainsi des composants structuraux capitaux de l'intégrité tissulaire (McCann et Roberts, 1996). Les pectines ont également un rôle majeur dans le transport des ions, les phénomènes de rétention d'eau et la porosité de la paroi cellulaire. Elles sont également impliquées dans les mécanismes de défense des plantes à l'encontre de certains agents pathogènes. Les fonctions spécifiques des pectines sont fortement influencées par leur structure fine, elle-même sous le contrôle de divers processus chimiques et enzymatiques de remodelage.

Les pectines intéressent les industries alimentaires pour deux raisons principales:

- Elles interviennent dans l'évolution des produits végétaux en jouant un rôle essentiel dans les modifications de structure des parois au cours de la maturation, du stockage et de la transformation des fruits et légumes.
- Extraites à partir de co-produits végétaux spécifiques, leurs propriétés stabilisantes, épaississantes et gélifiantes en font des additifs alimentaires largement utilisés.

1.1. Structure des pectines

Les pectines sont des polymères complexes constitués de différents domaines structuraux liés entre eux de façon covalente. Les principaux domaines constitutifs sont les homogalacturonanes et leurs dérivés et les rhamnogalacturonanes de type I.

Les **homogalacturonanes** sont des polymères linéaires formés d'un enchaînement de résidus acide-α-D-galacturonique liés en 1-4. La fonction carboxylique en C-6 des résidus acide galacturonique est partiellement estérifiée par du méthanol (Voragen *et al.*, 1995). Le degré de méthylation (DM) est défini comme le pourcentage en nombre de résidus d'acides galacturoniques estérifiés par des groupements méthoxyles. Le DM est à la base de la classification des substances

pectiques. Ainsi, les pectines de DM > 50 sont appelées pectines hautement méthoxylées (ou méthylées) (HM: High Methoxy), les pectines de DM compris entre 5 et 50 sont dites faiblement méthoxylées (ou méthylées) (LM: Low Methoxy) et les pectines de DM < 5 sont appelées acides pectiques. Les pectines natives des plantes supérieures sont généralement hautement méthoxylées.

La distribution des esters méthyliques est extrêmement complexe au niveau intramoléculaire comme au niveau intermoléculaire (Daas *et al.*, 1998; 1999). Au niveau intramoléculaire, donc au sein d'une unique macromolécule, la distribution peut être aléatoire ou "en blocs". Dans ce dernier cas, des segments non méthylés plus ou moins longs alternent avec des segments pratiquement totalement méthylestérifiés (Voragen *et al.*, 1995; Daas *et al.*, 2000 a, b). La distribution intramoléculaire des acides galacturoniques non méthylés sur les pectines influence très fortement la fonctionnalité de ces macromolécules, en particulier leur capacité de dimérisation *via* les ions calcium. Différentes méthodes visant à décrire la distribution des groupements méthyles sur les pectines, en relation avec leurs propriétés fonctionnelles, ont été développées ces dix dernières années. Les concepts de "degré de blockiness, DB" et de "degré de blockiness absolu, DB_{abs}" se sont imposés ces dernières années pour quantifier la distribution des groupements méthyles sur les pectines. Ces concepts sont basés sur la quantification des produits de dégradation générés par l'action d'endo-polygalacturonases sur les pectines. Ces enzymes hydrolysent la liaison α-(1,4) entre les acides galacturoniques des homogalacturonanes. Elles préfèrent les substrats non-méthylestérifiés et leur activité décroît avec une augmentation du DM des homogalacturonanes. L'endo-polygalacturonase II de *Kluyveromyces fragilis* a particulièrement été étudiée. Cette enzyme hydrolyse le squelette homogalacturonane uniquement dans les zones comprenant plus de quatre acides galacturoniques non méthylestérifiés adjacents. Ainsi, la quantité de produits finaux de dégradation non méthylés de degré de polymérisation 1, 2 et 3 est représentative de la quantité de blocs non méthylestérifiés. Ces monomères, dimères et trimères de l'acide galacturonique peuvent être quantifiés par chromatographie d'échange d'anions haute performance (HPAEC) (Daas *et al.*, 2000) ou par électrophorère capillaire (CE) (Ström *et al.*, 2007). Le DB est calculé comme la quantité de monomères (1^0), dimères (2^0) et trimères (3^0) non méthylés produite quand la pectine est incubée en présence d'endo-

polygalacturonase II, divisée par la quantité d'acide galacturonique non méthylestérifié initialement présente dans l'échantillon pectique (Daas *et al.*, 1999; 2000). Le DB_{abs} est basé sur la même idée mais, au lieu de relier la quantité de monomères (1^0), dimères (2^0) et trimères (3^0) non méthylés produite à la quantité d'acide galacturonique non méthylestérifié initialement présente dans l'échantillon pectique, la quantité de monomères (1^0), dimères (2^0) et trimères (3^0) non méthylés produite est reliée à la quantité totale d'acides galacturoniques (méthylestérifiés et non méthylestérifiés) initialement présente dans l'échantillon pectique (Guillotin *et al.*, 2005).

Chez certains végétaux, les fonctions alcools secondaires des résidus acides galacturoniques peuvent être estérifiées par de l'acide acétique. Ainsi, le degré d'acétylation (DA) est défini comme le pourcentage en nombre des résidus acides galacturoniques estérifiés par des groupements acétyles.

La longueur des zones homogalacturonanes a été estimée à 100-200 résidus d'acide galacturonique (Thibault *et al.*, 1993; Bonnin *et al.*, 2002; Hellìn *et al.*, 2005; Yapo *et al.*, 2007). De récentes études suggèrent cependant que ces domaines pourraient avoir une longueur plus importante, de l'ordre de 320 résidus (Round *et al.*, 2010).

D'autres domaines pectiques sont constitués d'un squelette principal homogalacturonane substitué par des résidus ou des chaînes osidiques. On peut citer les xylogalacturonanes, les apiogalacturonanes et les **rhamnogalacturonanes de type II**. Le rhamnogalacturonane de type II est un domaine très fortement conservé dans le règne végétal. Il s'agit d'un méga-oligosaccharide de 5-10 kDa comprenant un court squelette principal de type homogalacturonane substitué par quatre chaînes latérales comprenant de nombreux oses rares (Schols et Voragen, 2002; Matsunaga *et al.*, 2004). Douze motifs glucidiques différents composent le rhamnogalacturonane de type II (Ridley *et al.*, 2001). Ce domaine structural, bien que pondéralement mineur, a un rôle capital dans l'architecture pariétale du fait de sa capacité à se dimériser *via* le bore (Ishii *et al.*, 1999; Ishii et Matsunaga, 2001). La structure du rhamnogalacturonane de type II de citron a récemment pu être étudiée par spectrométrie de masse après hydrolyse acide ménagée (Sévéno *et al.*, 2009).

Les **rhamnogalacturonanes de type I**, hétéropolysaccharides ramifiés, constituent le second type principal de domaine pectique. Le squelette des

rhamnogalacturonanes de type I est formé de répétitions du motif dimérique [-4) acide-α-D-galacturonique (1-2)-α-L-rhamnose (1] (McNeill *et al.*, 1984; Schols *et al.*, 1990; Albersheim *et al.*, 1996). Des chaînes latérales d'oses neutres sont généralement associées aux rhamnogalacturonanes de type I. De degré de polymérisation variable, elles sont branchées sur les unités rhamnosyles en position O-3 et/ou O4 (Voragen *et al.*, 1995). Le taux d'unités rhamnosyles substituées varie en fonction de l'espèce végétale. Le β-D-galactopyranose et le α-L-arabinofuranose sont les deux oses neutres majoritaires des chaînes latérales. Ils s'organisent selon des structures plus ou moins complexes de degré de polymérisation parfois élevé. Des domaines rhamnogalacturonanes de type I ont récemment été isolés à partir de pectines extraites d'écorces d'agrumes. Les pectines, après désestérification, ont été extensivement dégradées par des enzymes actives sur les domaines homogalacturonanes. L'utilisation subséquente d'enzymes de dégradation des chaînes latérales (arabinanase et galactanase) a permis d'isoler des polymères représentatifs du squelette [-4) acide-α-D-galacturonique (1-2)-α-L-rhamnose $(1]_n$. Ces polymères étaient de masse molaire élevée, avec un degré de polymérisation moyen estimé à 80 (Yapo *et al.*, 2007).

Bien que la structure globale des différents domaines pectiques soit maintenant bien connue, la façon dont ces différents domaines s'agencent pour former la macromolécule pectique constitue un sujet de controverse. Différents modèles ont ainsi été proposés afin de décrire l'agencement des motifs les uns par rapport aux autres. Pendant longtemps, la structure des pectines a été idéalisée comme une alternance stricte des deux zones principales (homogalacturonanes et rhamnogalacturonanes de type I) associées de manière covalente (Schols et Voragen, 1996). Un autre modèle a ensuite été proposé dans lequel la chaîne principale serait constituée du domaine rhamnogalacturonane de type I, ce squelette étant substitué à la fois par les chaînes latérales d'oses neutres et par les domaines homogalacturonanes (Vincken *et al.*, 2003).

1.2. Extraction industrielle et applications des pectines

Les sources industrielles de pectines se limitent aux marcs de pomme et aux écorces d'agrumes, résidus de l'industrie des jus de fruits. L'extraction industrielle des pectines est réalisée, après inactivation des enzymes endogènes par traitement

thermique, par voie acide (pH 1,5-2,5) et à chaud (60-85°C) (May, 1990). L'acide nitrique est le plus communément utilisé. À la fin de l'extraction, les phases solide et liquide sont séparées par centrifugation et/ou filtration. Cette étape est assez délicate car la séparation du liquide visqueux des particules solides est souvent malaisée, surtout pour les matières sujettes à la désintégration. Des aides à la filtration de type diatomées sont ainsi souvent utilisées (Rolin *et al.*, 1998; Joye et Luzio, 2000). Les extraits sont ensuite rapidement portés à pH 3-4 et la température abaissée pour éviter les phénomènes d'hydrolyse (dépolymérisation ou déméthylation). Les extraits pectiques sont ensuite clarifiés et concentrés. Les pectines sont récupérées par précipitation, soit dans l'alcool (isopropanol) soit dans le chlorure d'aluminium, ce dernier étant de moins en moins utilisé du fait de la capacité polluante très élevée des effluents générés. Les précipités sont récupérés par centrifugation et filtration puis graduellement purifiés par plusieurs lavages à l'alcool. Les pectines sont finalement séchées, broyées et conditionnées. Une étape d'échange d'ions permettant d'obtenir des pectates est souvent incluse (Joye et Luzio, 2000). Cette méthode d'extraction permet un rendement d'extraction élevé et préserve les zones homogalacturonanes et en grande partie les esters méthyliques. Les pectines obtenues sont de type HM avec un DM compris entre 55 et 75-80 selon les conditions d'extraction (temps, température).

Les pectines LM sont obtenues par désestérification chimique contrôlée en milieu hydroalcoolique des pectines HM. Lorsque la désestérification s'effectue en milieu ammoniacal, environ 20% des groupements méthoxyles sont remplacés par des groupements amides, conduisant à la production de pectines LM dites amidées. Une large gamme de pectines, amidées ou non et présentant des DM variables, est disponible sur le marché.

Les pectines sont principalement utilisées pour leurs propriétés gélifiantes, mais trouvent également des applications dans la stabilisation des boissons lactées acides (acid dairy drinks, ADD).

La capacité des pectines à former des gels dépend directement de leur DM. Ainsi, les pectines HM et LM ne gélifient pas dans les mêmes conditions.

La gélification des pectines HM se produit en milieu acide ($2,5 < pH < 4$) en présence d'un co-soluté, en général le saccharose. En solution aqueuse diluée et à pH neutre, les pectines sont fortement hydratées et chargées négativement du fait de la

dissociation des fonctions carboxyliques. Pour que les molécules puissent se rapprocher et former un gel, il est nécessaire à la fois de diminuer l'activité de l'eau en ajoutant un co-soluté et de réduire la répulsion intermoléculaire entre groupes ioniques en abaissant le pH. Ainsi, les interactions chaîne-chaîne entre domaines homogalacturonanes sont favorisées. La stabilité des zones de jonction serait assurée par la combinaison de liaisons de type hydrogène impliquant les acides galacturoniques et d'interactions hydrophobes entre groupements méthoxyles. Les zones rhamnogalacturonanes de type I limiteraient l'extension des zones de jonction et assureraient ainsi la formation d'un réseau tridimensionnel.

En présence de calcium, les pectines LM forment des gels ioniques rigides et thermoréversibles. Il est généralement admis que le calcium interviendrait en pontant les groupements carboxyliques entre les domaines homogalacturonanes de deux chaînes pectiques, cette dimérisation étant d'autant plus importante que le nombre de fonctions carboxyliques non méthoxylées contiguës est élevé. Ainsi, non seulement le DM mais aussi la répartition des groupements méthoxyles le long des domaines homogalacturonanes influence fortement l'aptitude à la gélification des pectines *via* les ions calcium. Une répartition en bloc des groupements carboxyles libres favorise la formation de zones de jonction. Une telle répartition peut être obtenue par l'utilisation d'enzymes de déméthylation spécifiques: les pectine méthylestérases de plantes (p-PME) (Thibault et Rinaudo, 1986; Ralet *et al.*, 2001). Au contraire, les déméthylations chimiques ainsi que les déméthylations enzymatiques par des pectine méthylestérases fongiques (f-PME) induisent un processus de déméthylation aléatoire.

Les pectines sont également utilisées pour la stabilisation de boissons lactées acides. Ces boissons peuvent être décrites comme un système protéique acidifié liquide d'une stabilité et d'une viscosité similaires à celle du lait. En l'absence d'agent stabilisant, les caséines du lait s'agrègent au cours de l'acidification. Les caséines dans le lait (pH 6,6) sont sous la forme de particules sub-microniques stables appelées micelles. À pH 6,6, les caséines sont chargées négativement, se repoussent mutuellement et ne s'agrègent pas. Au cours de l'acidification, la répulsion électrostatique diminue et les particules de caséines tendent à floculer. Au-dessous de pH 5, l'agrégation des caséines est irréversible (Laurent et Boulenguer, 2003). Les pectines, en formant des interactions associatives avec les caséines, permettent de

prévenir la floculation. En effet, à pH > 3,5, les pectines sont chargées négativement du fait de la dissociation des groupements carboxyliques. Ainsi, à pH 6,6, les deux polymères sont chargés négativement. Au cours de l'acidification, les caséines deviennent globalement positivement chargées et interagissent électrostatiquement avec les pectines, chargées négativement. Seules les pectines HM de DM 68-72% permettent une bonne stabilisation des boissons lactées acides. La répartition des groupements méthyles le long des domaines homogalacturonanes aurait également une influence sur les capacités de stabilisation des pectines (Laurent et Boulenguer, 2003; Willats *et al.*, 2006). Une relation claire entre la structure fine des pectines, en particulier la répartition des charges, et les propriétés stabilisantes n'a cependant pas été clairement établie à ce jour, d'autant plus que de nombreux paramètres relatifs à la formulation et au procédé industriels interviennent également:

- le pH de la boisson;
- la teneur en protéines et la taille des particules;
- les conditions de fermentation durant la préparation du yaourt (température, temps, souche bactérienne utilisée);
- les conditions d'acidification directe par le biais de jus de fruit ou d'acide;
- le traitement thermique appliqué durant le procédé de production;
- l'homogénéisation;
- le traitement thermique appliqué au produit fini;
- l'ajout éventuel de calcium exogène.

(Ye, 2007; Boulenguer et Laurent, 2003; Herbstrith and Fox KG Corporate Group, 2003; Janhoj *et al.*, 2008; Lucey, 2004; Sedlmeyer, 2004; Tholstrup *et al.*, 2007; Syrbe *et al.*, 1998; Tromp *et al.*, 2004).

1.3. Réglementation et marché

L'industrie des pectines est dominée par de grands groupes multinationaux qui ont connu des changements majeurs ces cinq dernières années. Les leaders mondiaux sont CP Kelco (racheté par I.M. Huber Corporation en 2005), Danisco, Cargill et Herbstreith and Fox (USDA Foreign Agricultural Service, 2007). Environ 35 000 tonnes de pectines sont produites et utilisées annuellement dans le monde

(USDA Foreign Agricultural Service, 2007). CP Kelco détient environ 40% du marché (35-40% du marché mondial, 35-45% du marché européen) suivi par Danisco (20-30% du marché mondial, 15-25% du marché européen), Cargill (10-20% du marché mondial, 15-25% du marché européen) et Herbstreith and Fox (5-15% du marché mondial, 10-20% du marché européen). Le marché des pectines croît de 3-5% par an depuis 2001. Les prix actuels sont stables aux alentours de 16 000 dollars par tonne depuis 2006. La législation européenne classe les pectines comme additifs alimentaires sous le numéro codique E440 en différenciant les pectines non-amidées (E440 (i)) et les pectines amidées (E440 (ii)).

2. Objectifs de la thèse

L'objectif principal de la thèse était de tester différents types de pectines pour leurs propriétés stabilisantes vis-à-vis d'une boisson lactée acide iranienne traditionnelle, le "Doogh". Différentes pectines d'agrumes commerciales ainsi qu'une pectine extraite d'écorces de *Citrus aurantifolia* ont été caractérisées chimiquement et leur capacité de stabilisation du "Doogh" a été étudiée. Afin de mieux comprendre le rôle de la répartition des groupements méthoxyles sur le procédé de stabilisation, différents homogalacturonanes modèles ont été produits et caractérisés. Deux enzymes de dégradation des domaines homogalacturonanes (endo-polygalacturonase II et pectine lyase, la purification de cette dernière ayant été réalisée au cours de la thèse) ont été utilisées et les produits de dégradation des homogalacturonanes modèles ont été identifiés et quantifiés. Ces approches avaient pour but de permettre de discriminer des homogalacturonanes hautement méthoxylés présentant des différences subtiles de répartition des groupements méthoxyles. Les approches enzymatiques développées ont été appliquées aux pectines d'intérêt dans le but de mieux comprendre les relations "structure-propriétés de stabilisation" des pectines.

3. Résultats et discussion

3.1. Caractérisation de pectines d'agrumes et étude de leur capacité à stabiliser le "Doogh"

Les boissons lactées acides constituent une classe de boissons incluant les yaourts à boire, les boissons contenant à la fois du lait et des jus de fruits, et les boissons contenant des matières d'origine laitière comme ingrédient. Le "Doogh" est une boisson traditionnelle iranienne à base de lait fermenté. Les ingrédients principaux du "Doogh" sont du yaourt, du sel et de l'eau. Il est possible d'y ajouter du dioxyde de carbone (en quantité inférieure à 6 g/L). Le pH doit être inférieur à 4,5 (généralement 3,7-4,0), le pourcentage de matières grasses ne doit pas être inférieur à 0,5%, le pourcentage de sel ne doit pas excéder 1% et la matière sèche hors matière grasse doit être supérieure à 3,2% (généralement 4-6%) (ISIRI 2009, n° 10528). Au cours du stockage, l'apparence visuelle du produit est clairement affectée par des phénomènes de séparation du petit-lait et de sédimentation. La texture est également altérée au cours du stockage et devient rapidement à la fois granuleuse et visqueuse.

Les pectines HM sont largement utilisées pour stabiliser les boissons lactées acides. Elles permettent en effet de prévenir les problèmes de sédimentation et de séparation du petit-lait causés par la floculation des protéines du lait (Glahn et Rolin, 1996; Nakamura *et al.*, 2003; Janhoj *et al.*, 2008). Les pectines utilisées pour cette application sont extraites d'écorces d'agrumes, en particulier des écorces de citron et de citron vert (Albersheim *et al.*, 1996; Willats *et al.*, 2001).

La stabilité des boissons lactées acides dépend principalement de la concentration et du type de pectines utilisées, de la concentration en caséines, de la force ionique et du pH. Les traitements thermiques et les traitements d'homogénéisation au cours du procédé ont également une influence sur la stabilité de ces boissons (Glahn et Rolin, 1996). À un niveau moléculaire, le procédé de stabilisation par les pectines peut être amélioré par (i) l'utilisation de pectines de DM élevé et de haute masse molaire; (ii) une distribution en bloc des segments d'acides galacturoniques non méthylés sur les homogalacturonanes; (iii) une adsorption importante des extrémités de la macromolécule avec peu d'affinité pour le solvant; et (iv) des parties non adsorbées (dites "pendantes") volumineuses et présentant au contraire une forte affinité pour le solvant (Boulenguer et Laurent, 2003; Herbstreith

and Fox KG Corporate Group, 2003; Janhoj *et al.,* 2008; Sedlmeyer *et al.,* 2004; Tholstrup Sejersen *et al.,* 2007; Syrbe *et al.,* 1998; Tromp *et al.,* 2004; Ye, 2007). L'importance de la distribution des groupements méthyles le long des zones homogalacturonane a été particulièrement mise en lumière (Ström *et al.*, 2007).

Selon la FAO (2007), l'Iran est le neuvième producteur mondial de citron et de citron vert. *Citrus aurantifolia* est la variété de citron vert la plus cultivée avec une production annuelle de 615 000 tonnes. Environ 30% de la production sont transformés dans l'industrie des jus de fruits. Si l'on considère que 50% en poids de ce tonnage sont constitués de résidus solides correspondant aux écorces, 100 000 tonnes de pectines seraient susceptibles d'être produites annuellement en Iran et pourraient être utilisées industriellement pour la stabilisation du "Doogh".

Au cours de la première partie de ce travail, différentes pectines, commerciales et extraites à partir d'écorces de *Citrus aurantifolia*, ont été caractérisées. La distribution des groupements méthyles le long des zones homogalacturonane a en particulier été étudiée. La capacité à stabiliser le "Doogh" de ces pectines a ensuite été évaluée.

Trois pectines d'agrumes HM commerciales de DM élevé (i) hautement sensible au calcium (HCS, high calcium sensitive), (ii) moyennement sensible au calcium (MCS, medium calcium sensitive) et (iii) non sensible au calcium (NCS, non calcium sensitive), classiquement utilisées pour stabiliser les boissons lactées acides, nous ont été gracieusement fournies par M. Patrick Boulenguer (Cargill). Parallèlement, des pectines ont été extraites au laboratoire à partir d'écorces de *Citrus aurantifolia* préalablement séchées, à l'air libre ou dans un four, puis traitées à l'éthanol. Différentes conditions d'extraction ont été appliquées (eau à température ambiante; HCl 0,025, 0,03 ou 0,05 M à 85°C) et des rendements d'extraction élevés ont été obtenus pour toutes les conditions acides testées (350-420 mg/g de matériel insoluble dans l'alcool), en accord avec les données de la littérature rapportées pour d'autres variétés d'agrumes (Koubala *et al.*, 2008 a, b). Les trois pectines commerciales et les pectines extraites par voie acide à partir des écorces de *Citrus aurantifolia* se sont avérées riches en acide galacturonique, de DM élevé (60-69) et de masses molaires assez élevées (176-287 kg/mol).

La répartition des groupements méthoxyles sur les domaines homogalacturonanes a été évaluée par mesure du "degré de blockiness" (DB). Cette

approche, mise au point par Daas et collaborateurs (1999) permet, grâce à la quantification des oligomères d'acide galacturonique de degré de polymérisation 1, 2 et 3 libérés par l'action d'une endo-polygalacturonase, de discriminer les pectines selon la répartition des groupements méthoxyles. En effet, les endo-polygalacturonases sont des enzymes hydrolytiques spécifiques des segments non méthylés des homogalacturonanes. Ainsi, plus le DB est élevé, plus la répartition des acides galacturoniques non méthylés est considérée comme étant "en blocs". Cette approche ne permet cependant pas de discriminer les pectines selon le nombre et la longueur des blocs non méthylés; une pectine possédant beaucoup de blocs courts ne peut pas être différenciée d'une pectine possédant peu de blocs plus longs (Guillotin *et al.*, 2005).

18 µL d'endo-polygalacturonase II ont été ajoutés à 2 mL de solution de pectines (~ 1 mg/mL) dans du tampon acétate de sodium 50 mM pH 4. L'enzyme a été purifiée à partir d'une préparation liquide d'Aspergillus niger (Novozyme) comme décrit par Sakamoto et collaborateurs (2003). L'hydrolyse a été effectuée à 40°C pendant 3 jours, le même volume d'enzyme ayant été ajouté au bout de 24 et de 48h. Les hydrolysats ont ensuite été filtrés (0,45 µm, Millipore) puis injectés sur un système chromatographique d'échange d'anions haute performance. En conditions basiques (pH 13), les groupements hydroxyles des oses réducteurs sont sous forme anionique et les oligosaccharides peuvent ainsi être séparés par chromatographie d'échange d'anions. 20 µL des solutions d'oligosaccharides issues des dégradations enzymatiques sont injectés sur une résine échangeuse d'anions CarboPac PA1. La chaîne chromatographique utilisée est composée d'une pompe Waters 626 équipée d'un contrôleur Waters 600S, d'un auto-injecteur Waters 717 et d'un détecteur ampérométrique (EC 2000, Thermo Separation Products). L'élution a été effectuée à 0,25 mL/min par une solution de soude (100 mM) et un gradient d'acétate de sodium. Les conditions d'élution étaient les suivantes:

Temps (minutes)	*NaOAc 1M (%)*	*NaOH 0,5M (%)*	*H_2O (%)*
0-20	*25-50*	*20*	*55-30*
20-30	*50-60*	*20*	*30-20*
30-31	*60-25*	*20*	*20-55*
31-60	*25*	*20*	*55*

Les monomères, dimères et trimères de l'acide galacturonique ont été utilisés comme standards (Bonnin et al., 2002). Les facteurs de réponse (frp) ont été calculés comme suit:

frp = aire du pic/concentration

Le "degré de blockiness" (DB) et le "degré de blockiness absolu" (DB_{abs}) ont été calculés comme suit:

$$DB = \frac{[(1\times 1^0)+(2\times 2^0)+(3\times 3^0)]M_w^{GalA}}{(1-DM/100)m_{HG}(m_{GalA}/m_{HG})}\times 100$$

$$DB_{abs} = \frac{[(1\times 1^0)+(2\times 2^0)+(3\times 3^0)]M_w^{GalA}}{m_{HG}(m_{GalA}/m_{HG})}\times 100$$

Les DB obtenus pour les pectines commerciales étaient en adéquation avec leur nomenclature, avec des valeurs de DB d'autant plus élevées que la pectine était annoncée comme sensible au calcium (HCS: 13,8; MCS: 10,5; NCS: 6,7). Les pectines extraites par voie acide à partir des écorces de *Citrus aurantifolia* présentaient des DB compris entre 9 et 16, proches des valeurs observées pour les pectines commerciales. Une extraction (HCl 0,03 M) à l'échelle semi-préparative a été effectuée directement à partir d'écorces de *Citrus aurantifolia* préalablement séchées à l'air libre. La pectine extraite et les trois pectines commerciales ont été utilisées pour la stabilisation de "Doogh". La mesure de la quantité de précipité formé après centrifugation et le suivi de la stabilité du produit ont permis de montrer que, utilisées à une concentration ≥ 0,3% du produit final, toutes les pectines permettaient une stabilisation du "Doogh" à pH 4, et ce pour toute la durée de vie du produit (21 jours). Pour des concentrations moindres, les pectines MCS et NCS se sont avérées sensiblement plus efficaces en terme de stabilisation que les pectines HCS et la pectine extraite.

Pour mieux comprendre les ressorts structuraux responsables la capacité de ces pectines à stabiliser le "Doogh", il était nécessaire de pouvoir étudier finement la répartition des groupements méthoxyles le long des domaines homogalacturonanes. Pour réaliser ce projet, différents homogalacturonanes modèles ont été préparés et caractérisés comme détaillé dans le chapitre suivant.

3.2. Production de domaines homogalacturonanes modèles

Les domaines homogalacturonanes peuvent être isolés sélectivement par voie chimique à partir de pectines natives (Thibault *et al.*, 1993). Une pectine d'agrume commerciale a été totalement désestérifiée par voie basique puis hydrolysée par HCl 0,1 M à 80°C pendant 72h. Dans ces conditions, les liaisons glycosidiques au sein des chaînes latérales d'oses neutres et entre résidu rhamnose et résidu acide galacturonique au sein du squelette rhamnogalacturonane de type I sont beaucoup plus labiles que les liaisons entre résidus acides galacturoniques non méthylés au sein des domaines homogalacturonanes. Les domaines homogalacturonanes précipitent en milieu acide et les domaines rhamnogalacturonanes de type I hydrolysés restent solubles. Après centrifugation, le précipité a été mis en suspension. Pour un aliquot de la suspension, les fonctions carboxyliques ont été mises sous forme sodium par ajout de soude (pH final ~ 6). La solution obtenue, contenant exclusivement des domaines homogalacturonanes non méthylés (HG0), a été lyophilisée. Pour un autre aliquot de la suspension, les fonctions carboxyliques ont été mises sous forme tétrabutylammonium par ajout d'hydroxyde de tétrabutylammonium. Après lyophylisation, HG0-TBA a été mis en solution dans le DMSO avant ajout d'iodure de méthyle (rapport molaire acide galacturonique/TBA = 1). Un homogalacturonane très hautement méthylé (HG96, DM96) a ainsi été obtenu.

HG96 a servi de substrat de départ pour la production de domaines homogalacturonanes de DM variés (~ 15-80). Une série de 5 homogalacturonanes de différents DM a été produite par traitement à la soude de HG96 (série B) et une série de 5 homogalacturonanes de différents DM a été produite par traitement par la PME de plante de HG96 (série P). Une série supplémentaire a été produite par traitement par la PME de plante d'un homogalacturonane choisi de la série B (HG-B ~ 80) (série BP).

Chaque homogalacturonane a été analysé (i) pour sa teneur en acide galacturonique par la méthode colorimétrique automatisée au méta-hydroxybiphényle (Thibault, 1979); (ii) pour sa teneur en oses neutres individuels par chromatographie en phase gazeuse après dérivatisation des oses monomériques en acétates d'alditols (Blakeney et al., 1983); (iii) pour son degré de méthylation par titrimétrie. Les homogalacturonanes ont été dissous dans de l'eau ultra pure avant percolation sur une résine échangeuse de cations forte (Amberlite IR 120). Les

solutions d'homogalacturonanes sous forme H^+ à ~ 1 milliéquivalent/L ont été préparées extemporanément. Les titrages par NaOH à 10 milliéquivalents/L ont été suivies par conductimétrie. La constante de cellule a été déterminée à l'aide de NaCl 0,05% avant chaque série de mesures. Les fonctions carboxyliques libres (non méthylestérifiées) ont ainsi été quantifiées comme correspondant au point de neutralisation de la titration. Les fonctions carboxyliques totales (non méthylestérifiées et méthylestérifiées) ont été déterminées par colorimétrie (Thibault, 1979) sur les mêmes solutions après saponification (Ralet et Thibault, 2002). Le DM a été calculé comme suit:

DM = 100 x (fonctions carboxyliques totales - fonctions carboxyliques libres)/fonctions carboxyliques totales

Les homogalacturonanes étaient constitués à plus de 98 mol% d'acide galacturonique (sur une base de 100 moles d'oses totaux). Des traces de rhamnose, xylose et galactose ont été détectées. Une nomenclature des homogalacturonanes basée sur (i) le degré de méthylation de l'homogalacturonane "parent" ayant servi de substrat de départ; (ii) les conditions de désestérification utilisées (B, base; P, PME de plante); et (iii) le DM final obtenu a été élaborée. La nomenclature utilisée, les conditions expérimentales mises en œuvre, le DM des homogalacturonanes ayant servi de substrat de départ et les DM finaux obtenus sont résumés dans le Tableau 18.

Tableau 18: Conditions chimiques et enzymatiques mises en œuvre et nomenclature des différents homogalacturonanes modèles générés

Nomenclature	Conditions chimiques et enzymatiques	DMi[a]	DMf[b]
Série B			
HG96-B82	0.82 mol NaOH/g HG, 18h, 4°C	96	82
HG96-B69	1.54 mol NaOH/g HG, 18h, 4°C	96	69
HG96-B56	2.26 mol NaOH/g HG, 18h, 4°C	96	56
HG96-B40	2.98 mol NaOH/g HG, 18h, 4°C	96	40
HG96-B20	3.70 mol NaOH/g HG, 18h, 4°C	96	20
Série P			
HG96-P75	353 unités *p*-PME/g HG, 25 min, 30°C	96	75
HG96-P64	353 unités *p*-PME/g HG, 70 min, 30°C	96	64
HG96-P56	1765 unités *p*-PME/g HG, 40 min, 30°C	96	56
HG96-P36	1765 unités *p*-PME/g HG, 120 min, 30°C	96	36
HG96-P14	1765 unités *p*-PME/g HG, 480 min, 30°C	96	14
Série BP			
HG-B77-P63	5.5 unités *p*-PME /g of HG, 5 min, 30°C	77	63
HG-B76-P56	27 unités *p*-PME /g of HG, 5 min, 30°C	76	56
HG-B76-P49a	109 unités *p*-PME /g of HG, 7 min, 30°C	76	49
HG-B76-P49b	109 unités *p*-PME /g of HG, 15 min, 30°C	76	49
HG-B82-P48	109 unités *p*-PME /g of HG, 15 min, 30°C	82	48

[a] DM_i : DM de l'homogalacturonane initial

[b] DM_f : DM final obtenu

Les différents homogalacturonanes ont ensuite été analysés pour leurs caractéristiques macromoléculaires. Un système de chromatographie d'exclusion stérique haute performance (HP-SEC) couplé à un détecteur laser de diffusion de la lumière à angles multiples (MALLS), à un détecteur de concentration et à un détecteur viscosimétrique a été utilisé. Les échantillons ont été solubilisés à une concentration d'environ 10 mg/mL en milieu $NaNO_3$ 50mM contenant du NaN_3 0,02% comme agent anti-microbien. Le système chromatographique comprend une pré-colonne (Shodex OH-SB-G) et deux colonnes analytiques montées en série

(Shodex-OH-Pack SB-804 HQ et Shodex-OH-Pack SB-805 HQ) (Showa Denko Co., Tokyo, Japon) dont les limites d'exclusion respectives pour les pullulanes sont de 1 x 10^6 et 4 x 10^6 g/mol. Cinquante microlitres d'échantillon sont injectés et l'élution est réalisée par $NaNO_3$ 50mM contenant du NaN_3 0,02% à température ambiante à un débit de 0,7 mL/min. Les macromolécules sont ainsi séparées selon leur volume hydrodynamique.

La détermination de la concentration le long du profil de fractionnement s'effectue par réfractométrie différentielle, un dn/dc de 0,146 étant utilisé.

La détermination des masses molaires le long du profil de fractionnement s'effectue grâce à un détecteur de diffusion de la lumière multi-angles (41°, 90° et 138°) (Mini-Dawn®, Wyatt Technology, Santa Barbara, CA, USA). Le diagramme de Debye consiste à porter ($\Delta R_\theta/KC$) en fonction de ($\sin^2(\theta/2) + kC$) (avec $\Delta R\theta$, le rapport de Rayleigh; K, une constante fonction de la longueur d'onde dans le vide de la radiation incidente, de l'indice de réfraction du solvant pur et de l'incrément d'indice de réfraction de la solution; C, la concentration; θ, l'angle de la lumière diffusée par rapport à l'angle incident; et k le module du vecteur de diffusion). L'intersection de la droite avec l'axe des ordonnées est égale à M_w, masse molaire moyenne en poids. Le traitement des données s'effectue par le logiciel Astra 1.4.

La détermination des viscosités intrinsèques le long du profil de fractionnement s'effectue grâce à un détecteur viscosimétrique (T-50A, Viscotek, Houston, TX, USA). Ce détecteur est composé d'un pont (pont de Wheatstone) formé de quatre capillaires calibrés de manière à obtenir une résistance à l'écoulement identique dans chacune des branches. Lorsqu'une solution de polymères est éluée à travers le pont, la colonne retard présente sur l'un des capillaires permet d'obtenir une différence de pression entre les branches (ΔP). Cette différence de pression est reliée à la viscosité spécifique (η_{sp}) de la solution selon l'équation:

$$\eta_{sp} = \frac{4\Delta P}{IP - 2\Delta P}$$

$$\eta_{sp} \approx \frac{4\Delta P}{IP}$$

$$[\eta]_i = \lim_{c \to 0} \frac{\eta_{spi}}{C_i} \approx \frac{\eta_{spi}}{C_i}$$

$$[\eta] = \sum_i [\eta]_i$$

avec $\Delta P = P2 - P1$; *IP = pression entre l'entrée et la sortie*

ΔP étant largement négligeable devant IP :

$$\eta_{sp} \approx \frac{4\Delta P}{IP}$$

Les concentrations Ci en chaque point du chromatogramme étant très faibles, il est possible d'estimer la viscosité intrinsèque de la fraction i par la relation:

$$[\eta]_i = \lim_{c \to 0} \frac{\eta_{spi}}{C_i} \approx \frac{\eta_{spi}}{C_i}$$

et de déduire la viscosité intrinsèque de l'échantillon par la somme de toutes les fractions:

$$[\eta] = \sum_i [\eta]_i$$

Le traitement des données s'effectue par le logiciel TriSEC version 3.0.

L'analyse par HP-SEC-MALLS a permis de montrer que HG0 avait une masse molaire moyenne en poids de l'ordre de 17 kg/mol, correspondant à un degré de polymérisation moyen en poids de l'ordre de 100, un indice de polydispersité de 1,1 et une viscosité intrinsèque de 78 mL/g, en accord avec les résultats de la littérature (Thibault *et al.*, 1993; Yapo *et al.*, 2007). La procédure de méthylation de HG0 a été effectuée selon la procédure de Matricardi *et al.* (1995) et de Renard et Jarvis (1999) avec quelques modifications. Un DM de 96 a pu être obtenu. Du fait de ce DM particulièrement élevé, HG96 était difficilement soluble dans l'eau. Une solubilisation "visuelle" a néanmoins pu être obtenue par traitement thermique des solutions de HG96 à 40°C pendant 15 min. Une masse molaire moyenne en poids de 23 kg/mol, correspondant à un degré de polymérisation moyen en poids de 121, a été estimée. Ces valeurs sont cependant probablement surestimées du fait de la présence d'agrégats résiduels. En effet, une valeur de viscosité intrinsèque (peu sensible à la

présence d'agrégats) de 80 mL/g, proche de la valeur observée pour HG0, a été déterminée. Les homogalacturonanes des séries B et P ont également été analysés pour leurs caractéristiques macromoléculaires. Les deux modes de désestérification appliqués (chimique et enzymatique) n'ont pas entraîné de dégradation des homogalacturonanes et des masses molaires moyennes en poids similaires à celles de HG0 (15 à 21 kg/mol) ont été observées.

Ainsi, trois séries d'homogalacturonanes de masses molaires similaires, couvrant une large gamme de DM et présentant potentiellement, du fait des modes de désestérification appliqués, des répartitions de groupements méthoxyles différentes ont été produites.

Deux approches ont été utilisées pour déterminer la répartition des groupements méthoxyles sur les homogalacturonanes produits: (i) la mesure du "degré de blockiness" (DB) et du "degré de blockiness absolu" (DB_{abs}) (voir § précédent) et (ii) la mesure du coefficient d'activité du calcium (Ralet *et al.*, 2001) (Figure 46).

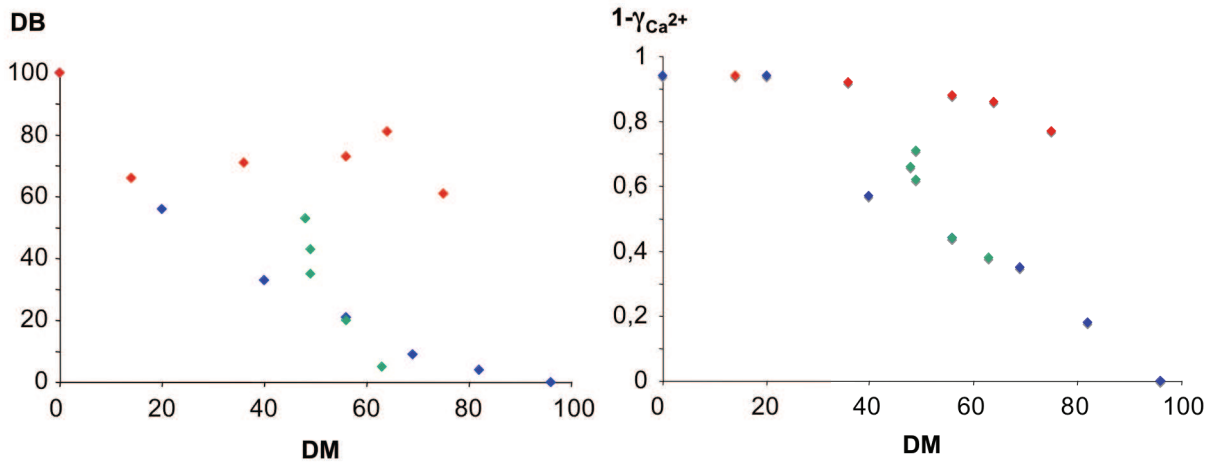

Figure 46 : Evolution du "degré de blockiness" et de la capacité de dimérisation *via* les ions calcium en fonction du degré de méthylation pour les différentes séries de HG (série P; série B; série BP)

Une relation polynomiale entre DB et DM a été observée pour les homogalacturonanes de la série B, les valeurs de DB diminuant rapidement avec une

augmentation du DM. Au contraire, les homogalacturonanes de la série P présentaient des valeurs de DB élevées et ce quel que soit le DM sur la plage de valeurs étudiée. Deux homogalacturonanes de la série BP n'ont pas pu être différenciés de ceux de la série B sur la base de leur DB, ces deux homogalacturonanes étant ceux pour lesquels la différence entre le DM de l'homogalacturonane ayant servi de substrat de départ et le DM final obtenu (DM_i - DM_f) était faible (HG-B77-P63 et HG-B76-P56). Les trois autres homogalacturonanes de la série BP présentaient des valeurs de DB intermédiaires entre les valeurs obtenues pour des homogalacturonanes de la série B et des homogalacturonanes de la série P de DM similaire. Le DM initial (DM_i) a donc été pris en considération pour tenter de différencier au mieux les séries d'homogalacturonanes produites. La représentation graphique DB en fonction de [(DM_i-DM_f)/DM_i] a permis une meilleure différenciation des séries d'homogalacturonanes à l'exception de l'homogalacturonane de la série BP HG-B77-P63 qui n'était toujours pas différenciable de son homologue de la série B de DM similaire. Certains auteurs ayant rapporté que les valeurs de DB_{abs} s'avèraient plus informatives que les valeurs de DB pour la détermination de la distribution des méthyles (Guillotin *et al.*, 2005; Ström *et al.*, 2007), les représentations graphiques DB_{abs} en fonction du DM et DB_{abs} en fonction de [(DM_i-DM_f)/DM_i] ont été tracées. La seconde représentation graphique a permis de distinguer légèrement l'homogalacturonane de la série BP HG-B77-P63 de son homologue de la série B.

La seconde approche expérimentale utilisée était la mesure du coefficient d'activité du calcium.

Les homogalacturonanes ont été dissous dans de l'eau ultra pure avant percolation sur une résine échangeuse de cations forte (Amberlite IR 120). Les solutions d'homogalacturonanes sous forme H^+ à ~ 1 milliéquivalent/L ont été préparées extemporanément. Les titrages par $Ca(OH)_2$ à 10 milliéquivalents/L ont été suivies par conductimétrie. La constante de cellule a été déterminée à l'aide de NaCl 0,05% avant chaque série de mesures. Le point de neutralisation a ainsi été déterminé. Le coefficient d'activité du calcium au point de neutralisation (γ_{Ca2+}) a ensuite été déterminé par la méthode spectrophotométrique de rapport d'absorbance. Cette méthode spectrophotométrique utilise un complexant coloré, le tétraméthylmurexide ou TMMX, comme témoin de l'activité des cations métalliques

en solution. Elle est fondée sur le fait que le spectre d'absorption du TMMX dans le visible se déplace lors de sa complexation avec certains cations métalliques et ceci proportionnellement à leur activité. L'absorbance A est mesurée aux longueurs d'onde correspondant aux maxima d'absorbance du TMMX seul, λ, et du complexe Me-TMMX, λ'. Une courbe étalon, établie en portant le rapport $\Phi = A_{\lambda'}/A_{\lambda}$ *en fonction de l'activité du cation, permet de retrouver son activité dans la solution à titrer. Cependant, la constante d'équilibre* $K_{éq}$ *de formation du complexe Me-TMMX doit avoir une valeur telle que, dans les conditions expérimentales, l'équilibre ne soit pas complètement déplacé ni dans un sens, ni dans l'autre. En pratique, cela veut dire que les valeurs de log* $K_{éq}$ *doivent être comprises entre 2,7 et 4. Dans le cas du calcium, la courbe étalon est effectuée pour des concentrations en* Ca^{2+} *de 6 x* 10^{-5} *à 4 x* 10^{-3} *mol/L et une concentration finale en TMMX de 2 x* 10^{-5} *mol/L. La courbe* $[Ca^{2+}] = f\ \Phi$ *(avec* $\Phi = A_{\lambda'}/A_{\lambda}$*;* $\lambda = 530$ *nm et* $\lambda' = 493$ *nm) est un polynôme du troisième ordre. Pour le dosage, à l'équilibre de formation du complexe Me-pectines (ici* Ca^{2+}*-homogalacturonane), on introduit 30 µL d'une solution préparée extemporanément de TMMX (concentration finale de TMMX = 2 x* 10^{-5} *mol/L). La mesure d'activité est réalisée directement dans le milieu réactionnel qui ne doit donc subir aucune variation, y compris de dilution. Aucune mesure d'activité n'est donc possible en cas de dépassement de la borne supérieure de la gamme de calibration, ce qui constitue un facteur limitant pour l'étude de la fixation à des concentrations en cations métalliques trop élevées. Cette méthode n'est donc adaptée qu'aux études en milieu dilué.*

Les valeurs de $(1- \gamma_{Ca2+})$ ont été portées en fonction du DM (Figure 46). Pour les homogalacturonanes de la série B, la capacité de fixation du calcium augmente progressivement avec une diminution du DM, en accord avec les résultats de la littérature (Ralet *et al.*, 2001). Seul HG96-B20 présente une capacité de fixation du calcium maximale. Les homogalacturonanes de la série P présentent au contraire une capacité de fixation du calcium maximale quel que soit le DM, contrairement à ce qui avait été démontré précédemment (Ralet *et al.*, 2001). En effet pour des pectines obtenues par traitement par une PME de plante d'une pectine "parent" de DM 81, la capacité de fixation du calcium augmentait progressivement avec une diminution du DM pour atteindre une valeur maximale pour des DM inférieurs à 40. Cette différence de comportement peut être attribuée à la différence de DM des substrats

initiaux utilisés (81 dans les travaux de Ralet et collaborateurs (2001) et 96 dans ce travail). Cela met à nouveau en exergue l'importance capitale du substrat initial, la longueur moyenne des segments déméthylés générés par une PME de plante augmentant avec le DM du substrat initial. Deux homogalacturonanes de la série BP n'ont pas pu être différenciés de ceux de la série B sur la base de leur capacité à fixer le calcium, ces deux homogalacturonanes étant ceux pour lesquels la différence entre le DM de l'homogalacturonane ayant servi de substrat de départ et le DM final obtenu (DM_i - DM_f) était faible (HG-B77-P63 et HG-B76-P56). Les trois autres homogalacturonanes de la série BP présentaient des valeurs de capacité à fixer le calcium intermédiaires entre les valeurs obtenues pour des homogalacturonanes de la série B et des homogalacturonanes de la série P de DM similaire. Ces résultats sont parfaitement en accord avec ceux obtenus par la mesure du DB et du DB_{abs}.

Les propriétés physico-chimiques des polyélectrolytes en solution diluée et en absence de sel sont principalement déterminées par la densité de charge linéaire. Ainsi, les valeurs théoriques de coefficient d'activité du calcium peuvent être calculées grâce au paramètre de charge du polyélectrolyte considéré et à la valence du contre-ion utilisé. Les valeurs théoriques de coefficient d'activité du calcium ont été calculées grâce à la théorie de Manning (Manning, 1969, 1974, 1975):

$$\gamma_{Ca^{2+}} = e^{-\frac{|z|\bar{\xi}}{2}} \qquad quand\ z\bar{\xi} < 1$$

$$\gamma_{Ca^{2+}} = \frac{e^{-\frac{1}{2}}}{|z|\bar{\xi}} \qquad quand\ z\bar{\xi} \geq 1$$

avec $\bar{\xi}$ *la densité de charge structurale* $\bar{\xi} = 1.61 \times \frac{100 - DM}{100}$

et z *la charge du contre-ion.*

La théorie de Manning ne tient pas compte de la capacité des homogalacturonanes de "piéger" les ions calcium. Ainsi, en l'absence de dimérisation ou de polymérisation des chaînes homogalacturonane via les ions calcium, les valeurs expérimentales de γ_{Ca2+} *sont proches des valeurs théoriques (*$\gamma_{Ca2+}exp/\ \gamma_{Ca2+}thq \sim 1\text{-}1{,}4$*). Au contraire, en présence du phénomène de dimérisation ou de polymérisation des chaînes homogalacturonane, les valeurs expérimentales de* γ_{Ca2+} *sont beaucoup plus faibles que les valeurs théoriques (*$\gamma_{Ca2+}exp/\ \gamma_{Ca2+}thq \sim 0{,}2\text{-}0{,}4$*).*

Les valeurs du rapport γ_{Ca2+}exp/ γ_{Ca2+}thq étaient comprises entre 1,07 et 1,37 pour tous les homogalacturonanes de la série B de DM supérieur ou égal à 40 ainsi que pour les deux homogalacturonanes de la série BP pour lesquels la différence entre le DM de l'homogalacturonane ayant servi de substrat de départ et le DM final obtenu (DM_i - DM_f) était faible (HG-B77-P63 et HG-B76-P56). Les valeurs du rapport γ_{Ca2+}exp/ γ_{Ca2+}thq étaient comprises entre 0,26 et 0,34 pour tous les homogalacturonanes de la série P et pour HG96-B20. Les trois homogalacturonanes de la série BP pour lesquels la différence entre le DM de l'homogalacturonane ayant servi de substrat de départ et le DM final obtenu (DM_i - DM_f) était importante (HG-B76-P49a, HG-B76-P49b et HG-B82-P48) présentaient des valeurs γ_{Ca2+}exp/ γ_{Ca2+}thq intermédiaires entre ces deux extrêmes.

Les deux approches expérimentales (mesure du "degré de blockiness" et du coefficient d'activité du calcium) mises en œuvre ont permis de mettre en évidence une répartition parfaitement aléatoire des groupements méthoxyles sur les homogalacturonanes de la série B, en accord avec ce qui était attendu vu le mode de désestérification appliqué. Les homogalacturonanes de la série P, désestérifiés au moyen d'une PME de plante connue pour son mode d'action processif, présentaient un DB et une capacité d'interaction *via* les ions calcium particulièrement élevés, indépendamment du DM final obtenu. Ces résultats démontrent la création de quelques longs segments déméthylés dès le début de la désestérification, en accord avec le peu de points d'ancrage pour la PME du fait du DM très élevé de l'homogalacturonane "parent" (DM 96). Pour la série BP, générée par action d'une PME de plante sur un homogalacturonane de DM ~ 80 à répartition des méthyles aléatoire, un comportement global proche du type "répartition aléatoire" a été observé pour les homogalacturonanes de DM final élevé (63 et 56) et un comportement intermédiaire entre "répartition en blocs" et "répartition aléatoire" a été observé pour les homogalacturonanes de DM final plus faible (48-49). Ces résultats sont en accord avec la création de blocs déméthylés très courts en début de désestérification par la PME de plante, blocs trop courts pour pouvoir être hydrolysés en monomères, dimères et trimères de l'acide galacturonique par l'endo-polygalacturonase II ou pour promouvoir une dimérisation des homogalacturonanes *via* les ions calcium. Pour une désestérification par la PME de plante plus poussée, les blocs déméthylés générés sont progressivement plus longs et peuvent alors en

partie être hydrolysés en monomères, dimères et trimères de l'aide galacturonique par l'endo-polygalacturonase II et induire une dimérisation des homogalacturonanes *via* les ions calcium.

Des séries d'homogalacturonanes couvrant une large gamme de DM et présentant, du fait des modes de désestérification appliqués, des répartitions de groupements méthoxyles différentes ont été produites avec succès. Cependant, les approches classiques mises en œuvre pour étudier le mode de répartition des groupements méthoxyles n'ont pas permis de distinguer certains homogalacturonanes entre eux. En particulier, les homogalacturonanes de DM > 50-60 présentant des blocs déméthylés courts n'ont pas, ou difficilement, pu être distingués de ceux dont la répartition des méthyles est purement aléatoire. La purification d'une enzyme de dégradation des homogalacturonanes spécifique des zones hautement méthoxylées, et donc plus adaptée à notre problématique, a été envisagée.

3.3. Purification d'une pectine lyase

Nous avons entrepris, à partir d'un mélange enzymatique commercial, la purification d'une pectine lyase, enzyme de dégradation des homogalacturonanes spécifique des segments hautement méthylestérifiés. Les pectine lyases sont en effet les seules enzymes capables de dépolymériser les pectines hautement méthylestérifiées sans l'action préalable d'autres enzymes (Delgado *et al.*, 1993). Ces enzymes clivent les pectines par un mécanisme de β-élimination de la liaison α-(1, 4) entre deux résidus d'acide galacturonique et introduisent une double liaison entre le C-4 et le C-5 de l'extrémité non réductrice nouvellement formée. L'action de ces enzymes sur les pectines résulte ainsi en la formation d'oligomères d'acide galacturonique insaturés sur leur extrémité non-réductrice. L'affinité de ces enzymes pour leur substrat augmente avec le DM.

Trois étapes ont été nécessaires pour permettre la purification d'une pectine lyase dépourvue d'activités contaminantes pectate lyase et endo-polygalacturonase à partir d'une préparation commerciale (Peclyve, Lyven). La première étape a consisté en une précipitation fractionnée au sulfate d'ammonium.

La précipitation fractionnée au sulfate d'ammonium est une première étape courante des protocoles de purification de protéines car elle se prête au traitement

de volumes importants. Cette technique utilise la solubilité différentielle des protéines due à la répartition des résidus polaires et apolaires à leur surface. La protéine est insolubilisée par augmentation de la force ionique du milieu, ici par une augmentation graduelle de la concentration en sulfate d'ammonium. Plus la surface hydrophobe est grande, plus la protéine aura tendance à précipiter. Dans un extrait brut, il y a co-agrégation de certaines protéines, mais chaque espèce précipite dans une zone de concentration en sel assez étroite. Cette précipitation est réversible et le sulfate d'ammonium est généralement un bon stabilisateur car il protège les protéines d'une éventuelle protéolyse.

La précipitation a été réalisée pour des saturations en sulfate d'ammonium de 50, 60, 80 et 100%. Seules les saturations en sulfate d'ammonium de 60, 80 et 100% ont permis d'obtenir un précipité. Les activités pectine lyase, endo-polygalacturonase et galactanase ont été mesurées dans chacune de ces fractions. Un rendement particulièrement élevé a été obtenu pour l'activité pectine lyase (96%). Plus la concentration en sulfate d'ammonium était élevée, plus l'activité pectine lyase était importante. Ainsi, 47% de l'activité a été retrouvée dans la fraction 100% de sulfate d'ammonium, 32% dans la fraction 80% de sulfate d'ammonium et 15% dans la fraction 60% de sulfate d'ammonium. L'activité contaminante principale était l'activité endo-polygalacturonase; il y avait en fait dans le mélange commercial initial dix fois plus d'activité endo-polygalacturonase que d'activité pectine lyase. Seuls 44% de l'activité endo-polygalacturonase initiale est retrouvée dans les différentes fractions, parmi lesquels 39% dans la fraction 100% de sulfate d'ammonium. Ainsi, le rapport d'activité pectine lyase/endo-polygalacturonase était de 0,09 dans le mélange commercial de départ, 2,58 dans la fraction 60% de sulfate d'ammonium, 0,57 dans la fraction 80% de sulfate d'ammonium et 0,10 dans la fraction 100% de sulfate d'ammonium. La fraction 60% de sulfate d'ammonium, bien que n'étant pas la meilleure en terme d'activité pectine lyase totale, a été choisie pour les étapes de purification ultérieures sur le critère "rapport d'activité pectine lyase/endo-polygalacturonase".

La deuxième étape de purification a consisté en une étape de chromatographie d'interaction hydrophobe. La fraction 60% de sulfate d'ammonium, après avoir été conditionnée en tampon phosphate de sodium 50 mM pH 6 + sulfate d'ammonium

1,5 M, a été fractionnée sur une colonne Resource Ether (GE Healthcare, Amersham Bioscience) à un débit de 1 mL/min. Le gradient utilisé était le suivant:

- 0-7 mL: 100% B
- 7-25 mL: 100 à 0% B
- 25-30 mL: 0% B (100% A)

avec A = tampon phosphate 50 mM pH 6 et B = tampon phosphate 50 mM pH 6 + sulfate d'ammonium 1,5 M.

Quatre fractions ont été rassemblées en fonction des pics protéiques repérés sur le profil d'absorbance à 280 nm. Ces fractions ont été analysées pour leurs activités endo-polygalacturonase, pectine lyase, pectate lyase et galactanase. La fraction A, non retenue sur la colonne, a été choisie pour les étapes de purification ultérieures sur différents critères:

- L'activité pectine lyase totale correcte.
- L'absence d'activité pectate lyase.
- La très faible activité galactanase (rapport d'activité pectine lyase/galactanase = 88).
- La relativement faible activité endo-polygalacturonase (rapport d'activité pectine lyase/endo-polygalacturonase = 1,5).

La fraction A60, après avoir été conditionnée en tampon acétate de sodium 50 mM pH 5, a été fractionnée sur une colonne échangeuse d'anions (MonoQ HR5/5) à un débit de 1 mL/min. Le gradient utilisé était le suivant:

- 0-6 mL: 100% A
- 6-26 mL: 100 à 50% A (0 à 50% B)
- 26-37 mL: 50 à 0% A (50 à 100% B)

avec A = tampon acétate 50 mM pH 5 et B = tampon phosphate 50 mM pH 6 + NaCl 1 M.

Quatre fractions ont été rassemblées en fonction des pics protéiques repérés sur le profil d'absorbance à 280 nm. Ces fractions ont été analysées pour leurs activités endo-polygalacturonase, pectine lyase et galactanase. La fraction A60c,

éluée pour 0,3 M NaCl, a été sélectionnée comme la fraction d'intérêt. Cette fraction était en effet riche en activité pectine lyase et totalement dépourvue d'activités endo-polygalacturonase et galactanase contaminantes.

La Figure 47 résume les trois étapes de purification mises en œuvre (précipitation au sulfate d'ammonium, chromatographie d'interaction hydrophobe et chromatographie d'échange d'anions). À l'issue du protocole de purification, une protéine de 38 kDa a été isolée. L'activité spécifique était de 3600 nkat/mg.

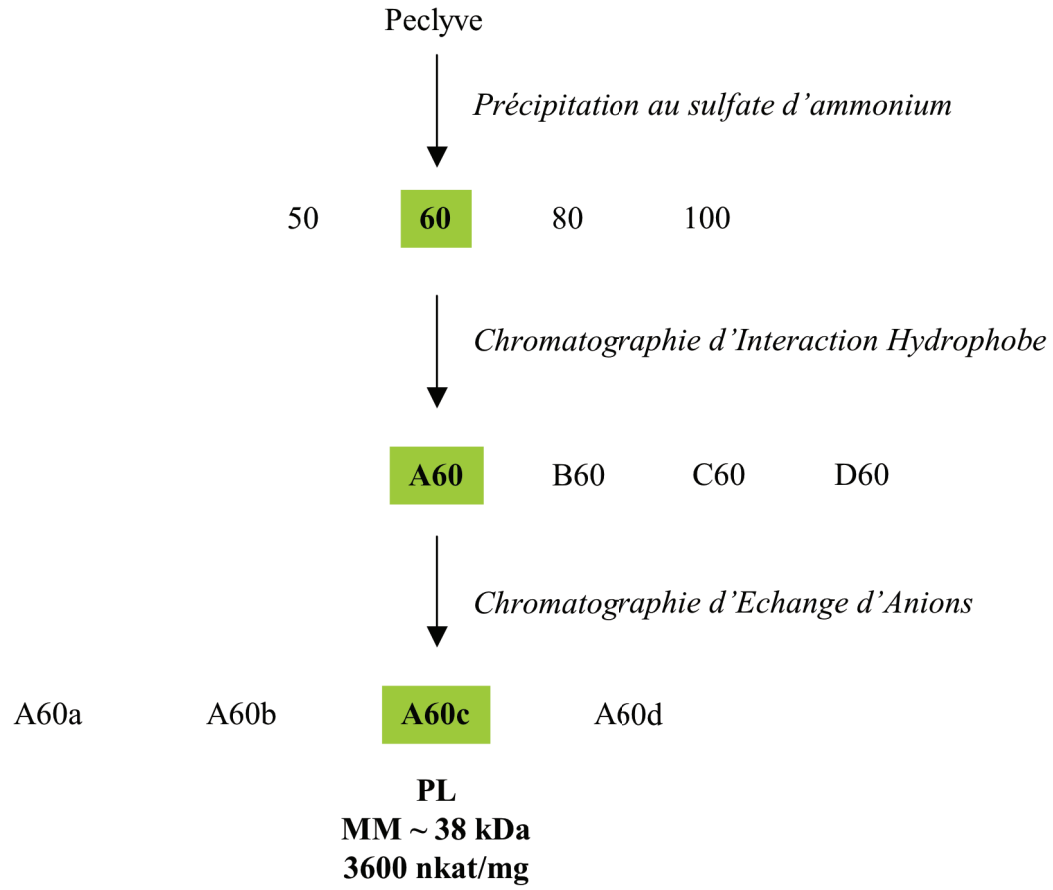

Figure 47: Schéma du protocole de purification de la pectine lyase

Quatre homogalacturonanes modèles (HG96-B40, HG96-B69, HG96-P36 et HG96-P64) ont été dégradés par la pectine lyase purifiée. Les produits de dégradation ont été séparés par chromatographie d'exclusion stérique basse pression

sur un système constitué de deux colonnes (Biogel P4 fine et Biogel P6 ultrafine) montées en série (Volume total ~ 870 mL).

L'élution a été réalisée par du tampon acétate de sodium 100 mM pH 3,6 à un débit de 10 mL/h. Les fractions collectées (~ 5 mL) ont été analysées pour leur teneur en acide galacturonique par la méthode colorimétrique automatisée au méta-hydroxybiphényle (Thibault, 1979). Les tubes d'intérêt ont ensuite été sélectionnés et rassemblés en fractions d'intérêt. Les fractions oligomériques ont été concentrées avant dessalage par chromatographie d'exclusion stérique sur Séphadex-G10. Les fractions oligomériques dessalées ont ensuite été concentrées de façon à avoir une concentration finale en acide galacturonique de l'ordre de 150 µg/mL. Les fractions polymériques ont été dialysées puis concentrées de façon à avoir une concentration finale en acide galacturonique de l'ordre de 1-2 mg/mL. Chaque fraction a été analysée pour sa teneur en acide galacturonique (Thibault, 1979) et pour son DM par la méthode colorimétrique au méthylbenzothiazolinone-2-hydrazone (MBTH) et à l'alcool oxidase (Anthon et Barrett, 2004; Slavov et al., 2009).

Une fraction polymérique, éluée au volume mort du système chromatographique et représentant des zones peu ou pas dégradées, a été observée pour tous les homogalacturonanes. Par ailleurs, les profils d'élution des produits de dégradation par la pectine lyase des homogalacturonanes de la série B et de la série P se sont révélés radicalement différents.

Pour la série P, et ce pour les deux DM considérés, les profils d'élution montraient la présence d'une fraction polymérique et la présence de 4 ou 5 fractions oligomériques bien résolues. L'analyse chimique de ces différentes fractions a montré que la fraction polymérique était de DM très faible (< 10) alors que toutes les fractions oligomériques étaient de DM très élevé (> 95). Seules les proportions relatives "fraction polymérique/fractions oligomériques" différaient: 75/25 pour HG96-P34 et 43/57 pour HG96-P64. Ces résultats démontrent à nouveau la création de quelques longs segments déméthylés (et donc le maintien de quelques longs segments totalement méthylés) dès le début de la désestérification, en accord avec le peu de points d'ancrage pour la PME de plante du fait du DM très élevé de l'homogalacturonane "parent" (96).

Pour la série B, le profil chromatographique obtenu a montré que HG96-B40 ne constituait pas un substrat pour la pectine lyase. En effet, aucun produit de

dégradation oligomérique n'a été obtenu. Le profil d'élution observé pour les produits de dégradation de HG96-B69 a montré la présence d'une large gamme de produits de degré de polymérisation varié. L'analyse chimique des différentes fractions collectées a montré que les deux premières fractions, considérées comme polymériques, étaient de DM un peu plus faible (~ 60) que le DM initial (69) alors que les fractions oligomériques étaient de DM plus élevé (66-87).

La sensibilité très élevée, les temps d'analyse courts et la possibilité d'analyser des mélanges d'oligosaccharides offerts par les spectromètres de masse de nouvelle génération ont entraîné une réémergence de l'utilisation de la spectrométrie de masse pour l'analyse des oligosaccharides ces dix dernières années. Ainsi, l'utilisation conjointe d'enzymes de dégradation très spécifiques des polysaccharides et de la spectrométrie de masse ont contribué grandement à la caractérisation structurale des polysaccharides en général et de pectines en particulier (Mutenda et al., 2002; Ralet et al., 2009). Ainsi, de nombreuses techniques de spectrométrie de masse (différant par le type d'ionisation aussi bien que par le type d'analyseur) ont été utilisées pour la caractérisation structurale d'oligomères d'acide galacturonique, saturés ou non, partiellement méthylés et/ou partiellement acétylés (Ralet et al., 2009). Dans le cas particulier des oligomères insaturés de l'acide galacturonique, il a été montré que les analyses par spectrométrie de masse en mode positif étaient plus informatives que celles effectuées en mode négatif. En effet, l'analyse par spectrométrie de masse en mode positif permet une détection de tous les ions parents et fragments, qu'ils soient partiellement ou totalement méthylestérifiés tandis que l'analyse en mode négatif ne permet la détection que des ions partiellement méthylestérifiés (Mutenda et al., 2002). Cependant, les spectres obtenus en mode négatif ont un rapport signal sur bruit bien meilleur que celui obtenu en mode positif. De plus, l'interprétation des spectres obtenus en mode positif est beaucoup plus complexe que celle des spectres obtenus en mode négatif (Mutenda et al., 2002).

L'analyse par spectrométrie de masse (MALDI-TOF-MS) des différentes fractions collectées a été effectuée en mode positif. Une matrice DMA/DHB (N,N-dimethylaniline/2,5-dihydroxybenzoic acid) a été utilisée. Le mélange DMA/DHB a été préparé en dissolvant 100 mg de DHB dans 1 mL de H_2O/ACN (1:1). 20 µL de DMA ont alors été ajoutés à la solution de DHB. 1 µL d'échantillon a été mélangé à

1 µL de solution de matrice directement sur la surface en acier du plateau. Les acquisitions de spectres ont été réalisées en mode positif sur un spectromètre de masse Autoflex III MALDI TOF/TOF (Bruker Daltonics, Bremen, Germany) équipé d'un laser Smartbeam (355 nm). La puissance du laser a été adaptée à chaque échantillon. Les spectres de masses ont été traités automatiquement par FlexAnalysis (Bruker Daltonics, Bremen, Germany).

Les fractions oligomériques issues des fractionnements par chromatographie d'exclusion stérique des produits de dégradation par la pectine lyase de HG96-B69 et de HG96-P36 ont été analysées par spectrométrie de masse. Le degré de polymérisation des oligomères de l'acide galacturonique contenus dans les différentes fractions a pu être déterminé, de même que leur DM. Quelle que soit la fraction considérée, les spectres de masse étaient caractérisés par la prédominance des ions correspondant aux adduits sodium des oligomères insaturés plus ou moins méthylés de l'acide galacturonique. La sodiation des fonctions carboxyliques libres a été observée de façon systématique. Enfin, les adduits sodium de certains oligomères saturés de l'acide galacturonique ont été détectés. Ces derniers étaient de façon systématique totalement méthylés et ne pouvaient donc pas correspondre à des produits de dégradation issus d'une activité endo-polygalacturonase résiduelle. Ces oligomères saturés hautement méthylés sont probablement représentatifs des extrémités non réductrices des homogalacturonanes, comme montré par van Alebeek et collaborateurs (2002).

Il a pu être démontré que la dégradation par la pectine lyase des homogalacturonanes de la série P générait des oligomères insaturés de degré de polymérisation (dp) 3 à 7 ainsi que des oligomères saturés représentatifs des extrémités non réductrices des homogalacturonanes. Seuls deux types d'oligomères ont été détectés : des oligogalacturonates totalement méthylés (n^n, dp^{DM}), qui sont largement majoritaires, et des oligogalacturonates (n^{n-1}). Les produits de dégradation par la pectine lyase de HG96-B69 étaient beaucoup plus complexes. Des oligogalacturonates de degré de polymérisation compris entre 3 et 31 ont pu être observés. Le DM des différents oligomères a pu être déterminé pour les oligogalacturonates de degré de polymérisation compris entre 3 et 16. Une large gamme de DM a pu être mise en évidence pour tous les oligogalacturonates avec, par exemple, la présence d'oligogalacturonates insaturés de type n^n, n^{n-1}, n^{n-2} et n^{n-3}

(dp^{DM}) pour les degrés de polymérisation 5 et 6. Pour les oligomères de degré de polymérisation supérieur à 6, les formes totalement méthylées n^n n'étaient plus détectables et les oligomères les plus faiblement méthylés détectés avaient un DM ~ 50.

Ainsi, une enzyme de dégradation des homogalacturonanes, spécifique des zones hautement méthylées, a été purifiée et caractérisée. La dégradation d'homogalacturonanes hautement méthylés (DM 64-69) par cette enzyme (pectine lyase) a permis de différencier clairement les deux homogalacturonanes de répartition des groupements méthoxyles distincte sur la base de la nature de leurs produits de dégradation. Cet outil enzymatique a ensuite été testé sur l'ensemble des homogalacturonanes produits.

3.4. Dégradation enzymatique des homogalacturonanes modèles

La compréhension de la distribution des groupements méthoxyles le long des segments homogalacturonane des pectines a toujours été la source de nombreux développements méthodologiques dans la mesure où cette distribution est directement à l'origine de la fonctionnalité des pectines (Hellìn *et al.*, 2005; Guillotin *et al.*, 2005). Des techniques d'empreinte enzymatique par des enzymes de dégradation des homogalacturonanes pures ont été développées en parallèle avec des techniques puissantes de séparation des oligomères produits, elles-mêmes alliées à des outils de détection sensibles (Hotchkiss *et al.*, 2002; Cameron *et al.*, 2008). L'analyse extensive de la distribution intramoléculaire des groupements méthoxyles a été initiée grâce à l'utilisation d'endo-polygalacturonase et/ou de pectine lyase et/ou d'exo-polygalacturonase par Limberg et collaborateurs (2000 a, b) et par Daas et collaborateurs (1998, 1999, 2000). Ces auteurs ont utilisé la chromatographie d'échange d'anions haute performance à pH 5 ou 6 (et non, comme communément, à pH 13) ce qui a permis de séparer les différents oligogalacturonates générés en fonction de leur degré de polymérisation et de leur DM. L'analyse par spectrométrie de masse des oligogalacturonates purifiés a ensuite permis de qualifier les différents oligogalacturonates séparés.

Dans le but de pouvoir quantifier les différents produits de dégradation par la pectine lyase et par l'endo-polygalacturonase II des homogalacturonanes modèles par

chromatographie d'échange d'anions haute performance, des standards d'oligogalacturonates saturés et insaturés ont été produits et purifiés.

Un mélange d'oligogalacturonates saturés a été généré par autolyse de l'acide polygalacturonique. L'acide polygalacturonique (4,4 g dans 440 mL d'eau distillée) a été autolysé à 100°C pendant 25 h sous agitation magnétique. Après centrifugation (20 000g, 15 min, 20°C), le surnageant contenant les oligomères d'acide galacturonique a été prélevé et deux volumes d'éthanol absolu ont été ajoutés. Le mélange a été laissé une nuit à 4°C pour permettre la précipitation des oligomères de degré de polymérisation élevé. Après centrifugation (10 000 g, 15 min, 4°C), la fraction éthanolo-soluble a été concentrée à 60 mL et dialysée deux fois (40 puis 70 minutes) contre de l'eau distillée de façon à éliminer les oligomères de degré de polymérisation 1, 2 et 3. Le dialysat a ensuite été lyophilisé. Le lyophilisat (1,11 g) a été solubilisé dans 10 mL d'eau distillée sous agitation magnétique pendant une nuit. La solution a ensuite été concentrée (volume final inférieur à 1 mL). Les produits de dégradation ont été séparés par chromatographie d'exclusion stérique basse pression sur un système constitué de deux colonnes (Biogel P4 fine et Biogel P6 ultrafine) montées en série (Volume total ~ 870 mL). L'élution a été réalisée par du tampon acétate de sodium 100 mM pH 3,6 à un débit de 10 mL/h. Les fractions collectées (~ 5 mL) ont été analysées pour leur teneur en acide galacturonique par la méthode colorimétrique automatisée au méta-hydroxybiphényle (Thibault, 1979). Les tubes d'intérêt ont ensuite été sélectionnés et rassemblés en deux fractions, une fraction correspondant aux degrés de polymérisation pairs, et une fraction correspondant aux degrés de polymérisation impairs. Les deux fractions oligomériques ont été concentrées avant dessalage par chromatographie d'exclusion stérique sur Séphadex-G10. Chaque fraction a été chromatographiée à nouveau sur le même système chromatographique (Biogel P4 fine et Biogel P6 ultrafine). Chaque pic correspondant à un degré de polymérisation donné a été concentré avant dessalage par chromatographie d'exclusion stérique sur Séphadex-G10 et concentration par évaporation rotative à 40°C sous vide. Chaque oligomère a ensuite été purifié par chromatographie d'échange d'anions haute performance semi-préparative. Une colonne Dionex-PA100 éluée à un débit de 3 mL/min a été utilisée. Chaque oligomère purifié élué a ensuite été neutralisé par HCl 1M et concentré avant dessalage par chromatographie d'exclusion stérique sur Séphadex-G10.

Chaque solution d'oligomère saturé de l'acide galacturonique a été analysée pour sa concentration en acide galacturonique. Des standards d'oligogalacturonates saturés de dp 4 à 14, de concentration connue et de degré de pureté supérieur à 98% ont ainsi été produits.

Un mélange d'oligogalacturonates insaturés a été généré par action de la pectine lyase sur HG96-P64 (voir § précédent). Les produits de dégradation ont été séparés par chromatographie d'exclusion stérique basse pression sur un système constitué de deux colonnes (Biogel P4 fine et Biogel P6 ultrafine) montées en série (Volume total ~ 870 mL). L'élution a été réalisée par du tampon acétate de sodium 100 mM pH 3,6 à un débit de 10 mL/h. Les fractions collectées (~ 5 mL) ont été analysées pour leur teneur en acide galacturonique par la méthode colorimétrique automatisée au méta-hydroxybiphényle (Thibault, 1979). Les tubes d'intérêt ont ensuite été sélectionnés et rassemblés. Chaque fraction récoltée a été purifiée par chromatographie d'échange d'anions haute performance semi-préparative. . Une colonne Dionex-PA200 éluée à un débit de 0,4 mL/min a été utilisée. Chaque oligomère purifié élué a ensuite été neutralisé par HCl 1M et concentré avant dessalage par chromatographie d'exclusion stérique sur Séphadex-G10. Chaque solution d'oligomère saturé de l'acide galacturonique a été analysée pour sa concentration en acide galacturonique. Des standards d'oligogalacturonates insaturés de dp 2 à 8, de concentration connue et de degré de pureté supérieur à 98% ont ainsi été produits.

Ces différents standards, dont la concentration exacte en acide galacturonique a été déterminée, ont été injectés sur le système de chromatographie d'échange d'anions haute performance analytique (colonne CarboPac PA1). Ceci a permis d'établir des courbes de réponse "aire du pic/concentration (µg/mL) (i.e. frp) = f (dp)" pour les oligogalacturonates saturés et pour les oligogalacturonates insaturés. Les fonctions suivantes ont été déterminées:

Oligogalacturonates saturés: $frp = 0,41042 \times dp^{-1,4646}$ ($R^2 > 0,99$)

Oligogalacturonates insaturés: $frp = 0,30731 \times dp^{-1,292}$ ($R^2 > 0,98$)

Les frp utilisés par la suite ont été calculés sur la base des équations précédentes.

Les différents homogalacturonanes modèles ont été dégradés par l'endo-polygalacturonase II ou la pectine lyase dans les conditions explicitées ci-après. Les homogalacturonanes ont été solubilisés à une concentration d'environ 1 mg/mL dans du tampon acétate de sodium 50 mM (pH 4 pour l'endo-polygalacturonase et pH 5 pour la pectine lyase). 2 mL de solution d'homogalacturonanes (pH5) ont été prélevés auxquels ont été ajoutés 24 µL de pectine lyase (28,7 nkat/mL). La dégradation enzymatique a été effectuée pendant 24 h à 40°C. 2 mL de solution d'homogalacturonanes (pH4) ont été prélevés auxquels ont été ajoutés 12 µL d'endo-polygalacturonase (35,5 nkat/mL). La dégradation enzymatique a été effectuée pendant 72 h à 30°C, 12 µL d'enzyme ayant été ajoutés aux temps 24h et 48h. Les hydrolysats ont ensuite été filtrés (0,45 µm, Millipore) puis injectés sur un système chromatographique analytique d'échange d'anions haute performance. La teneur en acide galacturonique de chaque solution d'homogalacturonane a été déterminée, après saponification, par la méthode colorimétrique automatisée au méta-hydroxybiphényle (Thibault, 1979).

Les différents produits de dégradation par la pectine lyase et par l'endo-polygalacturonase II des homogalacturonanes modèles ont pu être quantifiés par chromatographie d'échange d'anions haute performance analytique (colonne CarboPac PA1) *via* l'étalonnage externe mis en place comme suit:

$concentration_{dp(x)}$ (µg/mL) = aire du $pic_{dp(x)}/frp_{dp(x)}$

Pour chaque degré de polymérisation, le rendement de production a été calculé comme suit:

rendement = $concentration_{dp(x)}$ x 100 / concentration totale en acide galacturonique;

la concentration totale en acide galacturonique ayant été déterminée par la méthode colorimétrique automatisée au méta-hydroxybiphényle (Thibault, 1979).

3.4.1. Dégradation enzymatique par la pectine lyase

La dégradation enzymatique d'un substrat "idéal" pour la pectine lyase, à savoir l'homogalacturonane très hautement méthylé HG96, a montré que les oligogalacturonates insaturés de degré de polymérisation compris entre 3 et 7 constituaient les produits de dégradation majoritaires tandis que les oligogalacturonates insaturés de degré de polymérisation 2 et 8 constituaient des produits de dégradation minoritaires. Le rendement total de production

d'oligogalacturonates insaturés, calculé en utilisant les facteurs de réponse obtenus en parallèle, a montré que 97,5% de l'acide galacturonique initialement présent étaient retrouvés sous forme d'oligogalacturonates insaturés de degré de polymérisation compris entre 2 et 8. Ces résultats montrent la fiabilité de l'approche et permettent de considérer que la somme des teneurs en oligogalacturonates insaturés de degré de polymérisation compris entre 2 et 8 peut être considérée comme représentative de la partie des homogalacturonanes dégradable par la pectine lyase.

Comme illustré sur la Figure 48, les homogalacturonanes de la série P, constitués de longs segments méthylés alternant avec de longs segments non-méthylés, se sont avérés des substrats quasi idéaux pour la pectine lyase. Ainsi, le rendement en oligogalacturonates insaturés de degré de polymérisation compris entre 2 et 8 était à l'image du DM avec une relation proche de "dp < 9 = DM". Ainsi les segments méthylés complètement dégradables par la pectine lyase alternent avec les segments non méthylés parfaitement non-dégradables par la pectine lyase.

Les homogalacturonanes de la série B se sont comportés de façon très différente avec un rendement en oligogalacturonates insaturés de degré de polymérisation compris entre 2 et 8 qui n'était pas à l'image du DM. Une relation "dp < 9 = 0,43 DM + 57" a pu être mise en évidence pour les DM compris entre 65 et 100. En dessous de DM ~ 45, les homogalacturonanes de la série B ne sont plus substrats de l'enzyme. Comme précédemment évoqué par van Alebeek et collaborateurs (2002), lorsque les groupements méthyles sont distribués de façon aléatoire, l'effet délétère des acides galacturoniques non méthylestérifiés sur l'activité de la pectine lyase est important dans la mesure où les zones correspondant à des substrats idéaux pour la pectine lyase deviennent de plus en plus rares au fur et à mesure de la diminution du DM. En conséquence, l'activité de la pectine lyase est moins affectée lorsqu'une pectine de DM similaire mais de répartition des acides galacturoniques méthylestérifiés en blocs est utilisée. L'étude des homogalacturonanes modèles générés au cours de cette thèse a permis de montrer que, pour les DM compris entre 65 et 100, l'activité de la pectine lyase est 2,3 fois plus affectée par la diminution du DM lorsque les groupements méthoxyles sont distribués de façon aléatoire que lorsqu'ils sont distribués en blocs.

Les homogalacturonanes de la série BP présentaient un comportement globalement similaire à celui de la série P sur l'échelle de DM étudiée avec une droite

de régression "dp < 9 = f (DM)" parallèle à celle observée pour les homogalacturonanes de la série P . Une relation proche de "dp < 9 = DM + 20" a pu être mise en évidence pour les DM compris entre 48 et 63. Le rendement en oligogalacturonates insaturés de degré de polymérisation compris entre 2 et 8 était à l'image du DM, mais en prenant l'homogalacturonane "parent" de la série BP (HG96-B~80) comme point initial. Ainsi, le mode de désestérification appliqué à HG96-B~80 semblait préserver les segments hautement méthylés existants au sein de cet échantillon "parent" en générant des homogalacturonanes beaucoup plus dégradables par la pectine lyase qu'une désestérification aléatoire ne l'aurait fait.

3.4.2. Dégradation enzymatique par l'endo-polygalacturonase II

Les produits de dégradation des différents homogalacturonanes par l'endo-polygalacturonase II ont également été étudiés (Figure 48). La dégradation enzymatique d'un substrat "idéal" pour l'endo-polygalacturonase II, à savoir l'homogalacturonane non méthylé HG0, a montré que les oligogalacturonates saturés de degré de polymérisation compris entre 1 et 3 constituaient les seuls produits de dégradation, en accord avec les résultats de la littérature (Limberg *et al.*, 2000; Ström *et al.*, 2007). Le rendement total de production d'oligogalacturonates saturés, calculé en utilisant les facteurs de réponse obtenus en parallèle, a montré que 102% de l'acide galacturonique initialement présent étaient retrouvés sous forme d'oligogalacturonates saturés de degré de polymérisation compris entre 1 et 3. Ces résultats montrent la fiabilité de l'approche et permettent de considérer que la somme des teneurs en oligogalacturonates saturés de degré de polymérisation compris entre 1 et 3 peut être considérée comme représentative de la partie des homogalacturonanes hydrolysable par l'endo-polygalacturonase II.

Trois classes de produits de dégradation ont été définies : (i) les oligogalacturonates saturés de degré de polymérisation compris entre 1 et 3, produits finaux de dégradation du substrat idéal (HG0); (ii) les oligogalacturonates saturés de degré de polymérisation compris entre 4 et 12, produits de dégradation intermédiaires partiellement méthylés; et (iii) les polymères partiellement méthylés de degré de polymérisation supérieur à 12.

Des différences majeures de profil d'hydrolyse ont été observées entre les séries d'homogalacturonanes dans une fourchette de DM de 15 à 80 (Figure 48).

Pour les homogalacturonanes de la série P, des zones parfaitement hydrolysables par l'endo-polygalacturonase II, générant des oligogalacturonates saturés de degré de polymérisation compris entre 1 et 3, étaient présentes dès le début de la désestérification. Ces zones sont d'autant plus présentes que le DM est faible. Les oligogalacturonates saturés de degré de polymérisation compris entre 1 et 3, représentatifs des zones déméthylées parfaitement hydrolysables par l'endo-polygalacturonase sont complétées essentiellement par des zones non-hydrolysables de degré de polymérisation supérieur à 12. Peu d'oligogalacturonates saturés de degré de polymérisation intermédiaire, correspondant à des zones partiellement hydrolysables, ont été observés. Ces résultats sont à nouveau en accord avec la présence de longs segments déméthylés, parfaitement hydrolysables par l'endo-polygalacturonase II, alternant avec de longs segments méthylés, non-hydrolysables par l'enzyme. Les homogalacturonanes de la série P sont de bons substrats pour l'endo-polygalacturonase II avec davantage oligogalacturonates saturés de degré de polymérisation compris entre 1 et 3 produits que dans le cas de leurs homologues (en terme de DM) de la série B.

Une caractéristique de la série B est que la diminution de production d'oligogalacturonates saturés de degré de polymérisation compris entre 1 et 3 avec la diminution du DM est compensée à la fois par la production de polymères de degré de polymérisation supérieur à 12 et par la production d'oligogalacturonates saturés de degré de polymérisation intermédiaire. Pour des DM de 40-50, les oligogalacturonates saturés de degré de polymérisation compris entre 4 et 12 sont même les principaux produits d'hydrolyse. Ce profil de produits d'hydrolyse est, considérant le mode d'action de l'endo-polygalacturonase II, représentatif d'une répartition purement aléatoire des groupements méthoxyles.

Pour la série BP, comme mis en évidence dans le § 3.2. sur la base du rendement en oligogalacturonates saturés de degré de polymérisation compris entre 1 et 3, un comportement global de type "répartition aléatoire" a été observé pour les homogalacturonanes de DM final élevé (63 et 56) et un comportement intermédiaire entre "répartition en blocs" et "répartition aléatoire" a été observé pour les homogalacturonanes de DM final plus faible (48-49). Cependant, comme pour la série P, les zones parfaitement hydrolysables étaient complétées essentiellement par des zones non-hydrolysables de degré de polymérisation supérieur à 12. Peu

d'oligomères de degré de polymérisation intermédiaire, correspondant à des zones partiellement hydrolysables, ont été observés.

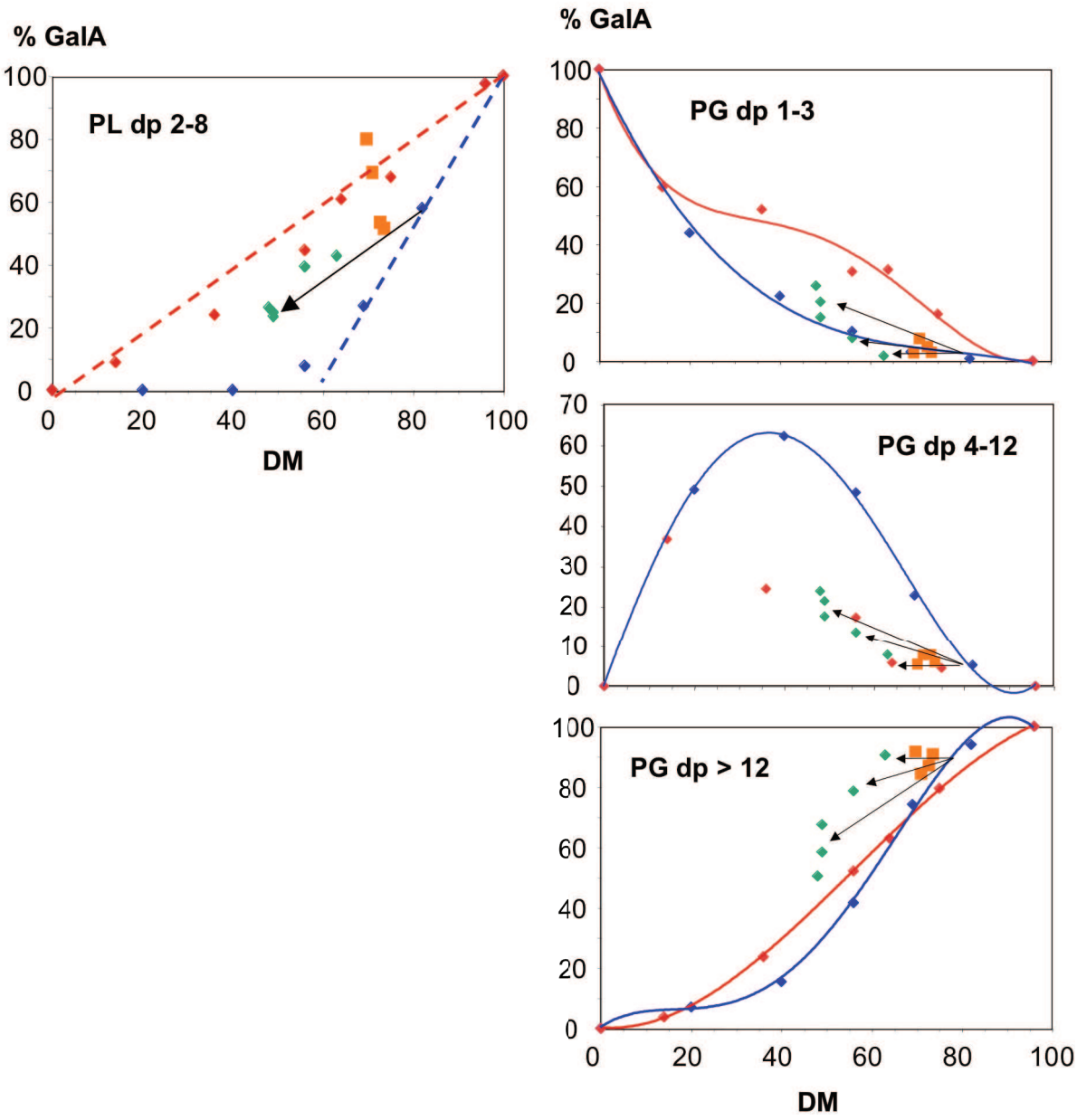

Figure 48 : Produits de dégradation des HG modèles et des pectines par la pectine lyase (PL) et l'endo-polygalacturonase II (PG). HG série B; HG série P; HG série BP; pectines.

Ainsi, les différentes séries d'homogalacturonanes [(i) B, répartition des groupements carboxyliques aléatoire; (ii) P, longs blocs non-méthylés alternant avec de longs blocs méthylés; et (iii) BP, courts blocs non-méthylés alternant avec de

longs blocs méthylés] ont pu être clairement différenciées grâce à l'étude, en parallèle, des produits de dégradation par l'endo-polygalacturonase II et par la pectine lyase. Pour une gamme de DM de 25 à 75, les séries B et P se différenciaient clairement par (i) le taux d'oligomères de degré de polymérisation compris entre 2 et 8 générés par la pectine lyase, (ii) le taux d'oligomères de degré de polymérisation compris entre 1 et 3 et compris entre 4 et 12 générés par l'endo-polygalacturonase II. Sur la gamme de DM étudiée (48-63), les séries B et BP se différenciaient par (i) le taux d'oligomères de degré de polymérisation compris entre 2 et 8 générés par la pectine lyase, (ii) le taux d'oligomères de degré de polymérisation compris entre 4 et 12 et de degré de polymérisation supérieur à 12 générés par l'endo-polygalacturonase II. Enfin, les séries BP et P se différenciaient par le taux d'oligomères de degré de polymérisation compris entre 1 et 3 et de degré de polymérisation supérieur à 12 générés par l'endo-polygalacturonase II.

Les profils de rendements en oligogalacturonates saturés et insaturés produits pour des homogalacturonanes de DM compris entre 25 et 75 peuvent se résumer comme suit:

Degré de polymérisation	Pectine lyase (oligogalacturonates insaturés)	Endopolygalacturonase II (oligogalacturonates saturés)
2 à 8	P > BP >> B	
1 à 3		P > BP ≈ B
4 à 12		B >> BP ≈ P
> 12		BP > P ≥ B

Le rapport (taux d'oligomères de degré de polymérisation compris entre 2 et 8 générés par la pectine lyase/taux d'oligomères de degré de polymérisation compris entre 1 et 3 générés par l'endopolygalacturonase) a été tracé en fonction du DM (Figure 49).

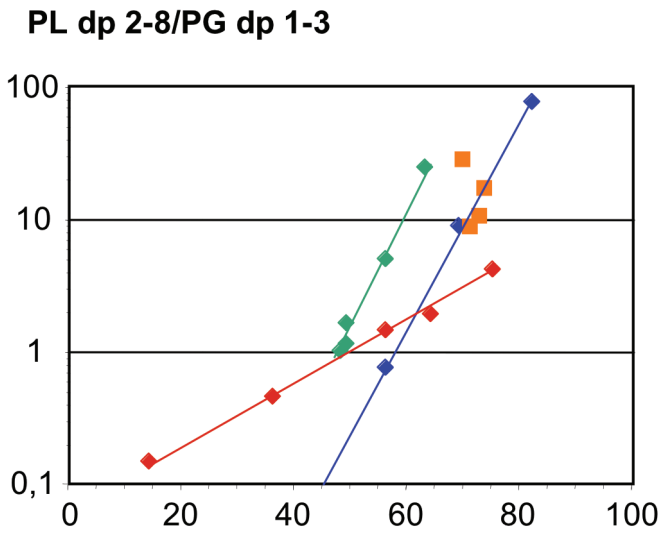

Figure 49: Rapport "taux d'oligomères de degré de polymérisation compris entre 2 et 8 générés par la pectine lyase/taux d'oligomères de degré de polymérisation compris entre 1 et 3 générés par l'endopolygalacturonase II" pour les homogalacturonanes modèles et les pectines en fonction du DM. HG série B; HG série P; HG série BP; pectines.

Il apparaît que les homogalacturonanes de la série BP de DM élevé, constitués de courts blocs non-méthylés alternant avec de longs blocs méthylés, se différencient clairement de leurs homologues des séries B et P. Les seuls critères de "degré de blockiness" et de "degré de blockiness absolu" ne permettent pas de différencier des homogalacturonanes de DM > 50-60 présentant des blocs déméthylés courts de ceux dont la répartition des méthyles est purement aléatoire. Nos travaux montrent que la prise en considération du taux d'oligomères de degré de polymérisation compris entre 2 et 8 générés par la pectine lyase et/ou du taux d'oligomères de degré de polymérisation compris entre 4 et 12 générés par l'endo-polygalacturonase II, permet de distinguer ces deux types d'homogalacturonanes.

3.5. Dégradation enzymatique des pectines capables de stabiliser le "Doogh"

Les pectines utilisées pour la stabilisation du "Doogh" (voir § 3.1.) ont été dégradées par l'endo-polygalacturonase II et la pectine lyase et leurs profils de dégradation ont été comparés aux résultats obtenus pour les homogalacturonanes

modèles (voir § 3.4.). Dans le cas des pectines, une partie de l'acide galacturonique total dosé est contenue dans les domaines rhamnogalacturonane I. Le pourcentage d'acide galacturonique présent dans les domaines homogalacturonanes a été estimé à 91,2 (+/-3)% par Ralet et collaborateur (2007) et à ~ 95% par Yapo et collaborateurs (2007). Une valeur de moyenne de 93% a été prise en compte dans les calculs de taux d'oligomères formés (g $GalA_{oligomères}$/100 g $GalA_{HG}$; avec $GalA_{HG} = GalA_{pectine}$ x 0,93). Les groupements méthyles n'étant présents que sur les domaines homogalacturonaness, le DM réel des homogalacturonanes a également été calculé comme suit: $DM_{HG} = DM_{pectine}/0,93$.

Le rendement en oligogalacturonates insaturés de degré de polymérisation compris entre 2 et 8 générés par la pectine lyase était significativement plus élevé que le rendement obtenu pour des homogalacturonanes de répartition de charges aléatoire (Figure 48). Deux pectines (HCS et pectine d'extraction) présentaient même un comportement proche de celui observé pour les homogalacturonanes de la série P. Sur la base des résultats de dégradation par la pectine lyase, une répartition en longs blocs d'acides galacturoniques méthoxylés alternant avec de plus ou moins longs blocs d'acides galacturoniques non méthoxylés pouvait être envisagée. L'étude des produits de dégradation générés par l'endo-polygalacturonase II a permis de nuancer ce propos. En effet, dans la gamme de DM des pectines étudiées (69,9-73,6), les homogalacturonanes de la série B ou BP et ceux de la série P sont nettement différenciés avec un rendement en oligogalacturonates saturés de degré de polymérisation compris entre 1 et 3 au moins cinq fois plus important pour les homogalacturonanes de la série P (Figure 48). Les pectines étudiées présentaient un rendement en oligogalacturonates saturés de degré de polymérisation compris entre 1 et 3 proche de celui observé pour les homogalacturonanes de la série B de même DM, ce qui permet de conclure que ces pectines ne possèdent pas majoritairement de longs blocs d'acides galacturoniques non méthoxylés. L'étude du rendement en oligogalacturonates saturés de degré de polymérisation compris entre 4 et 12 et supérieur à 12 montrent que ces pectines ont un comportement proche de celui observé pour les homogalacturonanes de la série BP, à savoir peu oligogalacturonates saturés de degré de polymérisation compris entre 4 et 12 et une quantité assez élevée d'oligogalacturonates saturés de degré de polymérisation supérieur à 12.

Ainsi, les pectines utilisées pour la stabilisation du "Doogh" se différencient nettement des homogalacturonanes de la série B selon trois critères: (i) le taux d'oligomères de degré de polymérisation compris entre 2 et 8 générés par la pectine lyase, (ii) le taux d'oligomères de degré de polymérisation compris entre 4 et 12 générés par l'endo-polygalacturonase II; et (iii) le taux d'oligomères de degré de polymérisation supérieur à 12 générés par l'endo-polygalacturonase II. Ces résultats laissent présumer une répartition des charges non-aléatoire sur les segments homogalacturonane de ces pectines. La répartition des charges diffère cependant de celle obtenue pour les homogalacturonanes modèles de la série P. La présence de longs blocs hautement méthylés entrecoupés de zones de DM plus faible à répartition de charges aléatoire et/ou comprenant de courts blocs déméthylés peut être envisagée.

4. Discussion générale

Les produits laitiers fermentés font partie des aliments les plus consommés au monde et sont appréciés de populations culturellement très diverses. Le yaourt est probablement le produit laitier fermenté le plus connu et des produits liquides dérivés du yaourt comme le "kefir" en Asie centrale, le "yakult" au Japon ou le "doogh" en Iran sont extrêmement populaires. De même, la demande pour les "yaourts à boire", plus pratiques à consommer en dehors du foyer, connaît une forte croissance, en particulier en Europe. Les pectines sont largement utilisées pour la stabilisation des "yaourts à boire", dans la mesure où elles permettent d'éviter la sédimentation des protéines du lait. Les pectines sont particulièrement adaptées à la stabilisation des produits dont le pH se situe entre 3,7 et 4,3, gamme de pH typique des "yaourts à boire" (Tuinier *et al.*, 2002). La capacité des pectines, macromolécules anioniques, à stabiliser ces boissons provient du fait qu'elles ont la capacité de s'adsorber à la surface des micelles de caséine. Du fait de la distribution spécifique des groupements anioniques, représentés par les fonctions carboxyliques non méthylestérifiées présentes au sein des macromolécules pectiques, les pectines comportent des régions présentant différentes capacités en terme d'adsorption sur les protéines. Les régions de forte affinité s'adsorbent à la surface des micelles de caséine par un mécanisme électrostatique tandis que les régions d'affinité moindre s'étendent à partir de la

surface et contribuent à une stabilisation dite "stérique". Ainsi, la distribution des fonctions carboxyliques non méthylestérifiées le long des chaînes homogalacturonane, constituants majeurs des pectines, a un impact significatif sur les propriétés fonctionnelles des pectines en général, et sur leur capacité à stabiliser les boissons lactées acides en particulier.

L'objectif principal de cette thèse était de tester différents types de pectines pour leurs propriétés stabilisantes vis-à-vis d'une boisson lactée acide iranienne traditionnelle, le "Doogh" et de mieux comprendre le rôle de la répartition des groupements méthoxyles sur le mécanisme de stabilisation.

Les pectines utilisées étaient des pectines de citron hautement méthylestérifiées qui se sont toutes avérées capables, à des concentrations ≥ 0,3%, de stabiliser le "Doogh". Différentes méthodes permettent d'évaluer la répartition des fonctions carboxyliques non méthylestérifiées le long des chaînes homogalacturonanes des pectines. Les concepts de "degré de blockiness" (Daas *et al.*, 1998, 1999, 2000) et de "degré de blockiness absolu" (Guillotin *et al.*, 2005), faisant tous deux référence à la digestibilité enzymatique des pectines par une endo-polygalacturonase, ont été particulièrement utilisés depuis leur création (Ström *et al.*, 2007; Slavov *et al.*, 2009). De même, la mesure directe du coefficient d'activité du calcium et la mesure des caractéristiques viscoélastiques des gels pectiques formés en présence de calcium, ont été largement utilisées (Ralet *et al.*, 2001; Ström *et al.*, 2007; Cameron *et al.*, 2008; Luzio et Cameron, 2008; Slavov *et al.*, 2009). Ces méthodes ont permis de différencier nettement des échantillons présentant des répartitions des fonctions carboxyliques non méthylestérifiées extrêmes (aléatoire et en blocs longs). Les valeurs obtenues pour des DM élevés et/ou pour des répartitions plus subtiles (par exemple de plus ou moins nombreux blocs courts) sont toutefois difficiles à associer à des répartitions des fonctions carboxyliques non méthylestérifiées précises dans la mesure où il n'existe pas de pectines standard dont la répartition des charges est parfaitement connue et maîtrisée.

Ainsi, nous nous sommes attaché à produire des séries d'homogalacturonanes modèles couvrant une large gamme de DM et présentant, du fait des modes de désestérification appliqués, des répartitions de groupements méthoxyles différentes. Il s'agissait d'une part de réduire la complexité structurale des pectines en focalisant sur le domaine structural d'intérêt, en l'occurrence le domaine homogalacturonane, et

d'autre part de disposer d'échantillons de référence dont la répartition des charges était parfaitement maîtrisée. Renard et Jarvis (1999) ont mis en évidence que des homogalacturones isolés par voie acide à partir de pectines d'agrumes désestérifiées pouvaient être re-méthylés chimiquement. Un DM de 89 (94 par RMN) avait pu être obtenu en utilisant un rapport molaire CH_3I/acide galacturonique de 1. Nous avons mis en œuvre une approche expérimentale similaire qui a permis l'obtention d'un homogalacturonane très hautement méthylé (DM96). Cet homogalacturonane a servi de substrat de départ pour la préparation, par voie basique, d'une série d'homogalacturonanes à répartition de charges aléatoire (série B) et pour la préparation, par voie enzymatique, d'une série d'homogalacturonanes à répartition de charges en blocs (série P). Une troisième série (série BP) a été produite par action d'une pectine méthyle estérase de plante (voie enzymatique) sur un homogalacturonane préalablement partiellement désestérifié par voie basique. Une répartition de type "courts blocs non-méthylés alternant avec de longs blocs méthylés" était attendue pour cette série d'homogalacturonanes.

Les mesures directes de "degré de blockiness" et de "degré de blockiness absolu" et la mesure directe du coefficient d'activité du calcium ont permis de différencier clairement les séries B et P. Les valeurs obtenues pour les homogalacturonanes de la série B à répartition de charges aléatoires étaient en accord avec les valeurs de la littérature pour des pectines de même type de répartition de charges (Ralet *et al.*, 2001; Ström *et al.*, 2007). Les valeurs obtenues pour les homogalacturonanes de la série P ont mis en évidence la création de quelques longs segments déméthylés dès le début de la désestérification, en accord avec le peu de points d'ancrage pour l'enzyme du fait du DM très élevé de l'homogalacturonane "parent" (DM 96). Ainsi, les méthodes classiques mises en œuvre ont permis, comme attendu sur la base de la littérature, de différencier nettement des homogalacturonanes présentant des répartitions des fonctions carboxyliques non méthylestérifiées extrêmes (aléatoire et en blocs longs). Cependant, ces mêmes approches n'ont pas permis de différencier certains homogalacturonanes entre eux. En particulier, les homogalacturonanes de DM > 50-60 présentant des blocs déméthylés courts n'ont pas, ou difficilement, pu être distingués de ceux dont la répartition des méthyles est purement aléatoire.

Les produits de dégradation des homogalacturonanes générés par une pectine lyase purifiée au cours de cette thèse ont été étudiés. Cette enzyme dégradant les zones hautement méthylées des homogalacturonanes (Limberg *et al.*, 2000; van Alebeek *et al.*, 2002; Mutenda *et al.*, 2002) semblait en effet plus adaptée à notre problématique. La série BP se différenciait très nettement de la série B, ces résultats allant à l'encontre des résultats obtenus suite à l'étude des produits de dégradation de degré de polymérisation 1, 2 et 3 générés par l'action de l'endo-polygalacturonase (i.e. "degré de blockiness" et "degré de blockiness absolu"). Les produits de dégradation de degré de polymérisation plus élevé générés par l'action de l'endo-polygalacturonase ont alors été pris en considération. Comme dit précédemment, les seuls critères de "degré de blockiness" et de "degré de blockiness absolu" ne permettent pas de différencier des homogalacturonanes de DM > 50-60 présentant des blocs déméthylés courts de ceux dont la répartition des méthyles est purement aléatoire. Nos travaux ont montré que la prise en considération du taux d'oligomères de degré de polymérisation compris entre 2 et 8 générés par la pectine lyase et/ou du taux d'oligomères de degré de polymérisation compris entre 4 et 12 générés par l'endo-polygalacturonase II, permet de distinguer ces deux types d'homogalacturonanes. Si l'étude des produits de dégradation générés par l'action d'une pectine lyase ou d'une endo-polygalacturonase à partir de séries de pectines de répartition des charges différentes a été rapportée dans la littérature (Limberg *et al.*, 2000a, b), les résultats obtenus pour chacune des deux enzymes n'ont, à notre connaissance, jamais été mis en regard les uns des autres. L'étude systématique d'homogalacturonanes modèles couvrant une large gamme de DM et présentant, du fait des modes de désestérification appliqués, des répartitions de groupements méthoxyles différentes et maîtrisées s'est avérée capitale pour déterminer les degrés de polymérisation des oligogalacturonates produits à prendre en considération lors de l'étude de la dégradation enzymatique des pectines.

Ces nouveaux critères ont été appliqués aux pectines d'intérêt pour ce travail, à savoir un ensemble de pectines de citron hautement méthylées capables de stabiliser efficacement le "Doogh". Les modes de répartition des charges purement aléatoire ou en longs blocs ont pu être écartés. La répartition des charges sur ces pectines se rapproche du modèle "courts blocs non-méthylés alternant avec de longs

blocs méthylés" mais ne peut néanmoins pas être parfaitement expliquée par ce modèle.

5. Conclusion et perspectives

Trois séries d'homogalacturonanes couvrant une large gamme de DM et présentant, du fait des modes de désestérification appliqués, des répartitions de groupements méthoxyles différentes ont été produites avec succès. Ces différentes séries d'homogalacturonanes [(i) B, répartition des groupements carboxyliques aléatoire; (ii) P, longs blocs non-méthylés alternant avec de longs blocs méthylés; et (iii) BP, courts blocs non-méthylés alternant avec de longs blocs méthylés] ont pu être clairement différenciées grâce à l'étude des produits de dégradation par l'endo-polygalacturonase II et par une pectine lyase purifiée et caractérisée au cours de la thèse. Ces dégradations enzymatiques ont été mises en œuvre sur différentes pectines capables de stabiliser le "Doogh". La répartition des charges sur ces pectines s'est avérée assez proche du modèle " courts blocs non-méthylés alternant avec de longs blocs méthylés".

Il existe une infinité de façon de répartir résidus acides galacturoniques méthylés et résidus acides galacturoniques non méthylés le long d'une chaîne homogalacturonane. Au cours de ce travail, nous avons exploré trois modes de répartition distincts, aucun de ces modes ne permettant de rendre parfaitement compte de la répartition des deux types de résidus acides galacturoniques sur les pectines étudiées. Une autre série d'homogalacturonanes modèles pourrait être générée en tâchant de s'approcher des modes de désestérification attendus pour ces pectines, à savoir: (i) l'action limitée d'une pectine méthyle estérase endogène sur les pectines biosynthétisées sous forme hautement méthylée suivie (ii) de la désestéérification chimique due à l'extraction des pectines par voie acide. Il convient également de garder en mémoire que les pectines présentent une hétérogénéité de densité de charge intermoléculaire assez importante. Ainsi, ce travail pourrait être complété par l'isolement et l'étude approfondie des différentes populations de macromolécules. Enfin, il est possible que seules certaines populations de pectines soient impliquées dans la stabilisation du "Doogh". Les pectines non adsorbées pourraient être isolées et leurs caractéristiques, en particulier leur dégradabilité par la pectine lyase et l'endo-polygalacturonase, étudiées.

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Citrus Pectin: Structure and Application in Acid Dairy Drinks

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ABSTRACT

Pectin, a plant cell wall polysaccharide, is mainly used in food industries for its gelling and stabilizing properties. In industrial applications, pectin is usually widely extracted from citrus peels, and in some intances, apple pomace is also used. Lime and lemon are the preferred citrus species used in the extraction of pectin, while orange and grapefruit are used less often. In the food industry, pectin is widely employed in the production of jams and jellies, confectionary products and bakery fillings. The fine structure of pectin is affected by many parameters, such as the origin of raw material and extraction conditions. This structural variability impacts greatly on pectin functional properties. The other major use of pectin concerns the stabilization of acidified milk drinks and yogurts. With their refreshing natural taste and high nutritional value, acidified milk drinks enjoy great popularity. A large selection of different sour milk drinks, which vary according to the manufacturing process, ingredients and consistency, is available to meet the needs of every consumer. In all cases, protein flocculation and whey separation occur in the absence of stabilizers in acidified milk drinks. To prevent this behaviour and to stabilize milk drinks, citrus pectin can be added as a protecting colloid. This review presents the structure of citrus pectin and functionality, with a special emphasis on acid dairy drinks.

Keywords: gelation, polysaccharide stabilisation
Abbreviations: **ADD**, acid dairy drink; **ADI**, acceptable daily intake; **AFM**, atomic force microscopy; **Ara**, arabinose; **CCAFC**, codex committee on food additives and contaminants; **CSP**, Ca^{2+} sensitive pectin; **DA**, degree of acetylation; **DAm**, degree of amidation; **DB**, degree of blockiness; **DE**, degree of esterification; **DM**, degree of methoxylation; **EEA**, European economic area; ***endo*-PG**, *endo*-polygalacturonase; **EU**, European Union; **FAO**, food and agriculture organization of the United Nation; **Gal**, galactose; **GalA**, galacturonic acid; **GRAS**, generally recognized as safe; **HG**, homogalacturonan; **HM-pectin**, high methoxyl pectin; **INS**, international numbering system; **LMA**, low methyl-esterified amidated; **LM-pectin**, low methoxyl pectin; **MALDI-TOF MS**, matrix-assisted laser adsorption/ionization time of flight mass spectrometry; **MSNF**, milk solid non-fat; **MT**, metric ton; **NCSP**, non-Ca^{2+} sensitive pectin; **NMR**, nuclear magnetic resonance; **PME**, pectin methyl esterase; **RG-*I***, rhamnogalacturonan *I*; **RG-*II***, rhamnogalacturonan *II*; **Rha**, rhamnose; **USDA**, United States department of agriculture; **WHO**, world health organization; **XGA**, xylogalacturonan; **Xyl**, xylose

CONTENTS

Received: 27 June, 2008. Accepted: 25 November, 2008.

Invited Review

INTRODUCTION

Pectin is a natural constituent of all terrestrial plants. It is one of the major plant cell wall components and is probably the most complex macromolecule in nature (Ridley *et al.* 2001; Coenen 2007). Pectin is a heterogeneous complex polysaccharide. Its composition and fine structure vary depending on the plant source and the extraction conditions applied. The fine structure of pectin deeply affects its functionality and applicability. Although most plant tissues contain pectin, only a few plant sources are currently used for the commercial pectin extraction. Commercial pectin is produced almost exclusively from citrus peel or apple pomace (Rolin 2002). The major constituent of pectin consists of linear sequences of (1→4)-linked α–D–galactopyranosyluronic acid (GalA) with some of the carboxyl groups esterified with methanol (Voragen *et al.* 1995). The proportion of GalA units that is methyl–esterified is one of the key parameters that determines the functional properties of pectin (Rolin *et al.* 1998; Ralet *et al.* 2001). Generally, commercial pectin preparations are divided into low-methoxyl (LM) (degree of methylation (DM) < 50%) and high-methoxyl (HM) (DM > 50%) pectin. The major outlet for pectin is through food applications, i.e., as a thickener and gelling agent in jams and jellies, bakery fillings, glazing, fruit and milk beverages (Voragen *et al.* 1995; May 2000; Rolin 2002). Recently, pectin utilization for healthcare applications – as drug delivery, gene repair and tissue repair – has offered food ingredient manufacturers as much as $ 890 million in sales to the healthcare industry (Kalomara information 2007). This review presents the structure of citrus pectin and functionality, with a special emphasis on acid dairy drinks.

CITRUS AND CITRUS PROCESSING

Citrus is the largest fruit crop worldwide. World citrus production in selected major producing countries in 2005/06 is estimated at 72.8 million MT (USDA Foreign Agriculture Service). Citrus is grown in two belts on both sides of the equator about 20 to 40 degrees of latitude. All citrus is thought to originate from the Himalayan region of south western China and northern India. Columbus brought citrus seeds to the western hemisphere in 1493 and planted them first on the island of Hispaniola, now called Haiti (FAO 2003). Citrus became commercialized in the Americas in the late 1800s. In the early to mid 1900s, the principal producing states were Florida, Texas and California, USA (Bates *et al.* 2001; FAO 2003). Following the devastating freeze in Florida in 1962; a group of Florida businessmen began to establish citrus groves, and later citrus industry, around São Paulo, Brazil (FAO 2003). In 2005/06, citrus production in Brazil exceeded 18 million tons followed by China and USA with around 15 million tons and 10 million tons, respectively (USDA Foreign Agriculture Service). In this regard around 14 million tons and 7 million tons of Brazil and USA production, respectively, went under processing last year. Presently, more than 55% of citrus production in developed countries and 22% of production in developing countries go under processing each year. About 75% of the processing belongs to citrus juice industry, 13% citrus canning industry and 7% as dried products (Bates *et al.* 2001; FAO 2003; Boriss 2006).

There is a great variety of citrus species and citrus fruits can be roughly classified as follows:

Orange-fruit types
- sweet orange
- bitter orange
- mandarin

Yellow-fruit types
- lemon
- lime, limetta
- grapefruit

Orange is the most important citrus product in the citrus industry, followed by grapefruits, lemons and mandarins (Schottler *et al.* 2002).

ECONOMICALLY FEASIBLE BY-PRODUCTS FROM CITRUS

Worldwide, industrial citrus waste is estimated at more than 15×10^6 tons per year, as the amount of residue obtained from the fruits accounts for 50% of the original whole fruit mass (Marin *et al.* 2007). By-products (sometimes called specialty products) are those saleable products made from citrus fruits besides juice. One of the opportunities of starting a new citrus processing operation is the unique opportunity to tailor the citrus processing plant's production of specialty products to the customer's needs. This needs to be done early in the design phase so that plants can be specially designed for the production of multiple products. Over 400 specialty-products can be made from citrus, in addition to juice. Many of these products are only research realities that lack either the backing or timing to be made profitable. It is vital to make plans on how to economically dispose of the peel and other solid wastes from operations before engaging in a fruit juice operation. There are presently 6 to 12 products that have established markets (Bates *et al.* 2001):

- Pectin
- Pectin pomace and dietary fibre
- Dried citrus peel
- Pulp wash
- Juice sacks and whole juice vesicles
- Beverage base and clouding agents
- Healthful, nutraceutical citrus beverages
- Fractionated citrus oils and D-limonene
- Citrus molasses and beverage alcohol base
- Flavonoids and limonin.

PECTIN STRUCTURE

Historical outline

The scientist Vauquelin discovered the existence of pectin in fruit juices some 200 years ago (Vauquelin 1790). At that time, however, it was not yet called by that name. The name pectin was first used in 1825, when Braconnot continued the research started by Vauquelin. He referred to the gel-forming substance as "pectic acid", from the Greek word *pektikos*, which means congealed, or solidified. Smolenski, in 1923, was the first to describe pectin as a polymer of GalA. In 1930, Meyer and Mark discovered the chain formation of the pectin molecule and Schneider and Bock, in 1937, established the first formula of the pectin molecule (as cited by Kertesz, 1951). The work of Kertesz (1951) defined pectin as a hetero-polysaccharide containing mainly GalA residues that are partly methyl-esterified. A certain amount of neutral sugars were detected, but the manner in which they were integrated in the pectin molecule was not determined. The work of De Vries and co-workers (De Vries *et al.* 1981, 1982) was instrumental in showing the existence of the two main pectic regions: "smooth" homogalacturonic regions and "hairy" rhamnogalacturonic regions encompassing neutral sugars side chains. From then, several other pectic domains have been described.

By 1965, pectin had been established in the industry essentially as a gelling agent in jams or jellies, though thereafter other application areas were identified. Presently, pectin is used in food systems as a fat substitute, dietary fibre and as stabilizer in acidified milk systems (Ralet *et al.* 2002).

Chemical structure of pectic domains

The term "pectin" is somewhat misleading since it implies the existence of a single well-defined macromolecule (Willats *et al.* 2006). In fact, pectin is currently strongly believed to contain different structural elements, the amount and fine structure of each varies widely with respect to plant origin (Schols and Voragen 1996), between different cell types, at different stages of cellular development, and even within the thickness of a given wall (Jauneau *et al.* 1998; Willats *et al.* 2001; Scheller *et al.* 2007).

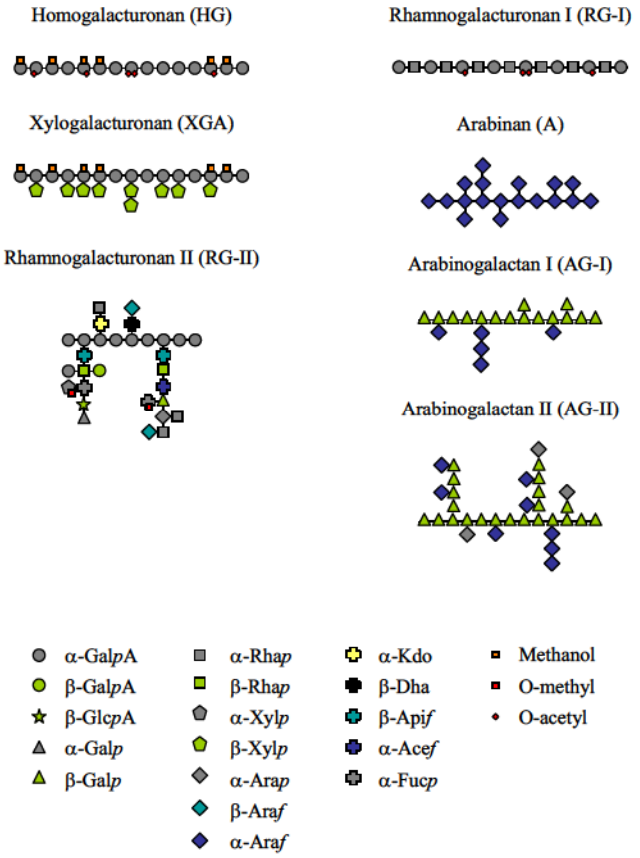

Fig. 1 Schematic representation of the structural domains of pectin. Structures are drawn with the reducing *terminus* to the right. Homogalacturonan and homogalacturonan-derived domains are shown in the first column; rhamnogalacturonan I and attached neutral sugars side-chains are shown in the second column. The symbols of the building units are shown in the accompanying legend and the predominant linkages are explained in the text. Adapted from Vincken *et al.* (2003).

The most commonly found structural elements are: (i) homogalacturonan, (ii) xylogalacturonan, (iii) rhamnogalacturonan I backbone, (iv) rhamnogalacturonan II, (v) arabinan, (vi) arabinogalactan I, and (vii) arabinogalactan II (Schols and Voragen 2002; Coenen 2007) (**Fig. 1**).

Homogalacturonan

Homogalacturonan (HG) is the simplest and most abundant pectic structural domain. It consists of a linear backbone of (1→4)-linked α-D-GalA residues (Ridley *et al.* 2001). The possible presence of single rhamnose (Rha) residues within HG regions was convincingly argued against by Zhan *et al.* (1998). The minimum estimated length of this domain is ~100 GalA residues (Thibault *et al.* 1993). For citrus, chemical as well as enzymatic approaches have led to the isolation of HG domains of narrow molar mass distribution with a degree of polymerization of 100-120 GalA residues (Thibault *et al.* 1993; Hellìn *et al.* 2005; Yapo *et al.* 2007). GalA residues are commonly partly methyl-esterified at C-6 (Voragen *et al.* 1995) and, in some plant species, partly acetyl-esterified at *O*-2 or *O*-3 (Ralet *et al.* 2005, 2008a). Both the degree of methyl-esterification (DM) (i.e. the number of methyl-esterified GalA residues for 100 total GalA residues) and degree of acetylation (DA) (i.e. the number of acetyl-esterified GalA residues for 100 total GalA residues) have a profound impact on functional properties. Lime pectin is particularly rich in HG, which accounts for ~80-90% of raw pectin mass (Ralet and Thibault 1994; Hellín *et al.* 2005; Yapo *et al.* 2007). Native citrus pectin, and its constitutive HG domains, is highly methyl-esterified and lowly acetyl-esterified (Ralet and Thibault 1994; Ros *et al.* 1996, 1998).

Xylogalacturonan

Xylogalacturonan (XGA) consists of a HG backbone with mostly single-unit substituents of β-D-xylopyranose (Xyl) linked at *O*-3 of GalA residues. In lemon albedo pectin, the presence of very limited amounts of XGA has been reported (Ros *et al.* 1998).

Rhamnogalacturonan I backbone and neutral sugars side-chains

The rhamnogalacturonan I (RG-I) backbone consists of [→2)-α-L-Rha*p*-(1→4)-α-D-Gal*p*A-(1→] repeats (McNeill *et al.* 1980, 1984; Albersheim *et al.* 1996). RG-I domains isolated from several plant species, including citrus, were shown to be highly acetylated at *O*-2 and/or *O*-3 of GalA units (Komavilas and Mort 1989; Ralet and Thibault 1994; Schols and Voragen 1994; Ros *et al.* 1996; Ralet *et al.* 2005). So far, no evidence has been published that GalA units in RG-I domains are methyl-esterified. The Rha residues of RG-I backbone are substituted, mainly at *O*-4, with α-L-arabinofuranose (Ara)- and β-D-galactopyranose (Gal)-containing side-chains (Voragen *et al.* 1995). Lemon pectins were shown to contain arabinan structures with a central core of (1→5)-linked Ara*f* residues carrying essentially single Ara*f* substituents at C-3 (Ralet and Thibault 1994; Ros *et al.* 1996). Both types of galactans: (1→4)-linked type I (arabino)-galactans and (1→3),(1→6)-linked type II arabinogalactans were also detected (Ralet and Thibault 1994; Ros *et al.* 1996) as well as single D-Gal*p*-(1→4) substitutions (Ros *et al.* 1996, 1998). Rhamnogalacturonans I was recently recovered from citrus peels pectins after extensive degradation by homogalacturonan-degrading enzymes (Yapo *et al.* 2007). Further use of side-chains-degrading enzymes allowed the recovery of a high molar mass RG-I backbone (Yapo *et al.* 2007).

Rhamnogalacturonan II

Rhamnogalacturonan II (RG-II) is a highly conserved structure in the plant kingdom. It is a low molar mass (5-10 kDa) highly complex macromolecule with a short HG-like backbone substituted by four different side-chains encompassing several unusual sugar residues (Schols and Voragen 2002). This structural element, although present in very limited amounts, plays a key role as it is strongly believed to be involved in the cross-linking of two pectin molecules within the cell wall through a borate di-ester (Ishii *et al.* 1999; Ishii and Matsunaga 2001). RG-II was recently isolated from citrus peels pectin after extensive degradation by homogalacturonan-degrading enzymes (Yapo *et al.* 2007).

Pectin models

Although the structure of the different pectic domains is now quite well known, the way they are connected one with another to form a macromolecular structure is still a matter of debate. Two pectic models are considered nowadays: the "smooth and hairy regions" model (Schols and Voragen 1996) (**Fig. 2**) and the "RG-I backbone" model (Vincken *et al.* 2003) (**Fig. 3**). In the first one, "smooth regions" (HG) alternate with "hairy regions" (XGA and RG-I encompassing neutral sugars side chains) to form the pectin molecule backbone, RG-II being an integral part of some HG domains. In the second one, HG, XGA, arabinan and galactan would occur as side-chains of RG-I backbone, forming a "*molecular brush*" (Vincken *et al.* 2003), RG-II being again an integral part of some HG domains.

Macromolecular features

The macromolecular characteristics of pectin mainly include its molar mass and conformation. These are determinants of pectin industrial applications as the strength of pectin gels is positively correlated with apparent molar mass

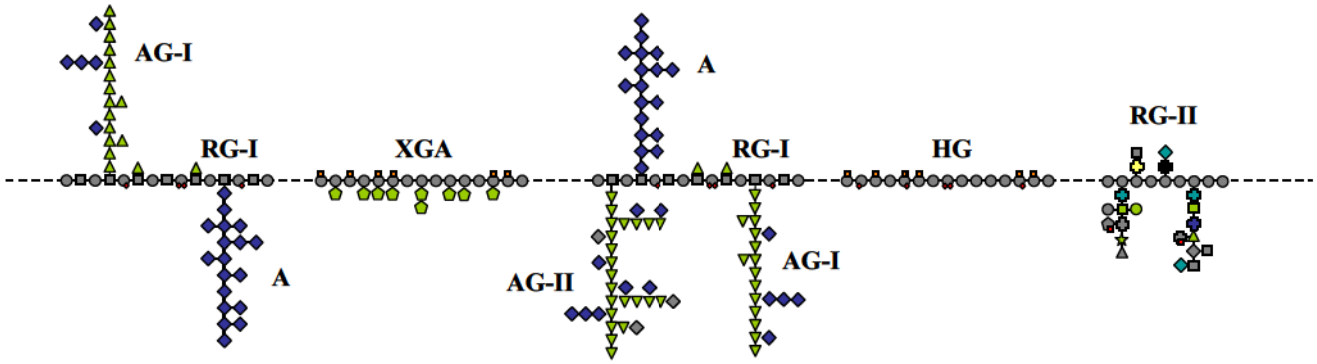

Fig. 2 Schematic representation of the smooth and hairy model of pectin. In this "smooth and hairy regions" model (Schols and Voragen 1996) the pectic backbone consists of alternating homogalacturonan (HG) (smooth regions), xylogalacturonan and rhamnogalacturonan-I (RG-I). Rhamnogalacturonan-II (RG-II) is considered an integral part of HG domains. Arabinan (A) and arabinogalactan I and II (AG-I, AG-II) make up the "hairy" part of the macromolecule.

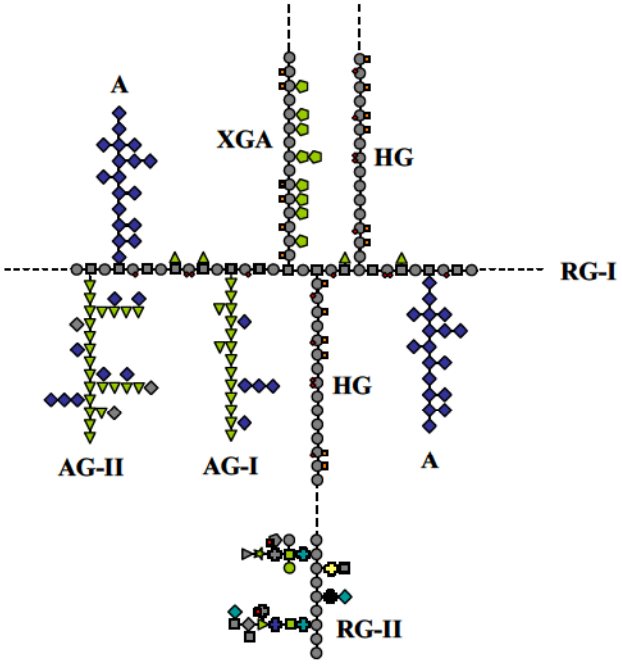

Fig. 3 Schematic representation of the RG-backbone model. In this "RG-I backbone" model (Vincken *et al.* 2003), the RG-I backbone is decorated, not only with arabinan and arabinogalactan I and II side chains, but also with homogalacturonan (HG) and xylogalacturonan (XGA) domains. Rhamnogalacturonan-II (RG-II) is considered an integral part of HG domains.

(Rolin 2002). Pectin molar mass vary with plant source, raw material stage of ripening and extraction conditions. It should be emphasized that a large number of factors, such as charge density, neutral sugars content and solvent quality can affect pectin aggregation and molecular state in solution. It was also shown that pectin molecules of similar molar masses may exhibit different hydrodynamic properties due to differences in DM, branching and neutral sugars content which make their solution behaviour even more complex (Rolin *et al.* 1998). Molar mass determination is still a challenge due to problems of heterogeneity and aggregation in addition to the usual broad molar mass distribution. Developments in high-performance size-exclusion chromatography coupled with laser light scattering and/or viscometric detection, led to an improvement in pectin characterization, although aggregation and poly-dispersity can disturb light-scattering data (Ralet *et al.* 2002).

Pectin conformation is a matter of complexity. As the individual sugar rings are essentially rigid, the overall conformation of the chain is primarily determined by the bridge angle and the relative orientations of the component sugars as defined by the rotational angles. The linkages between monomers in HG are axial-axial, giving the polymer an intrinsic stiffness due to the severe conformational constraints imposed by this type of linkage (Burton and Brant 1983). A rather extended conformation with a persistence length of 4.5 to 13 nm was determined on isolated HG domains or HG-rich citrus pectin (Cros *et al.* 1996; Morris *et al.* 2008; Ralet *et al.* 2008b). Some contradictory conclusions have been reported concerning the influence of DM on the conformation of the pectin macromolecules. Molecular modelling, nuclear magnetic resonance and small angle neutron scattering have shown that methoxyl groups have no significant influence on the flexibility of the linkages between GalA residues (Cros *et al.* 1992, 1996). However, other studies have shown a general decrease in the hydrodynamic volume of the pectin molecules and an increase in chain stiffness with decreasing DM, with both steric and electrostatic interactions playing an important role in conformational changes (Morris *et al.* 2000). Using another experimental approach combining intrinsic viscosity, sedimentation coefficient and weight-average molar mass determinations, the same authors recently concluded that all citrus pectin molecules tested were of similar conformation, whatever their DM (Morris *et al.* 2008). Isolated RG-I domains or RG-I-rich pectin appear very flexible (Axelos and Thibault 1991; Hourdet and Muller 1991; Ralet *et al.* 2008b). A complex set of different factors may influence the conformation of the pectin molecules and may partly explain some of the discrepancies found in literature about this subject.

INDUSTRIAL PECTIN PRODUCTION

The citrus industry produces three intermediate products (percentage values in relation to the raw product mass):

Juice/pulp	45-55%
Peel	45-55%
Essential oil	0.2-0.5% (Schottler *et al.* 2002)

Apple pomace, that contains 15-20% pectin, and citrus peel, which contain 30-35% pectin, are currently the only raw materials of importance to the manufacture of pectin. Historically, the major part of the pectin industry developed from utilization of apples (Rolin 2002; Mesbahi *et al.* 2005), but today, dried citrus peel is the largest source. Among the citrus, lime and lemon are preferred and orange and grapefruit are less often used. Indeed, lime and lemon yield pectin of higher specific viscosity and lower Ca^{2+} sensitivity than orange and grapefruit (Rolin 2002).

Pectin has been manufactured from citrus peel for more than 50 years. Pectin manufacture involves leaching, acid extraction, precipitation, purification and standardization (**Fig. 4**).

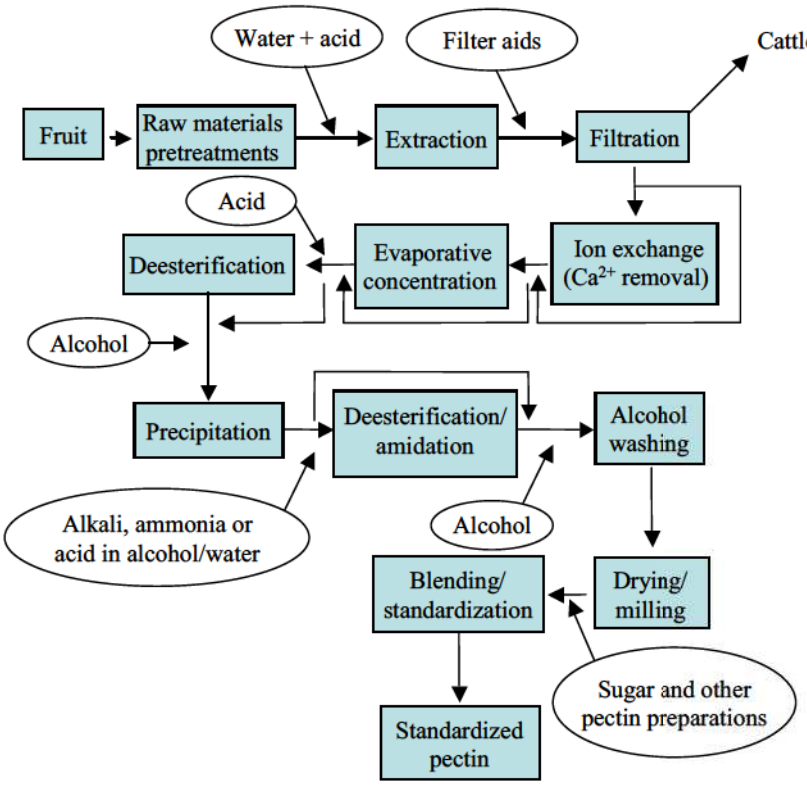

Fig. 4 Industrial pectin production.

Process

Pre-treatment and extraction

Most pectin extraction in industry is done in Europe using lime peels imported from Mexico and South America. Before industrial extraction, the raw materials are generally subjected to a pre-treatment (blanching, washing, drying) to inactivate enzymes that otherwise would rapidly degrade the pectin molecules and to increase the product stability during transportation. Washing in water, in order to leach out sugars, is necessary prior to drying so as to minimize caramelisation. Washing may even be preferred when the raw material can be used without drying, because the leachable material has to be separated from the pectin at a later stage to reach a pure product. Citrus peel is extensively leached with water and this leach water has the potential for large pollution problems.

Extraction of pectins from raw materials is usually performed by acid treatment (pH 1 to 3) at high temperature (50-90°C), with nitric acid for 3-12 hours (Rolin 2002). The extraction conditions (pH, temperature, time) must be optimized to provide good yields of pectin that also has the desired DM (typically 55-80). Acid extraction has at least two pitfalls: (i) it may degrade pectin structure, and (ii) it does not meet environmental safety standards. Acid-extracted pectins often contain a mixture of both Ca^{2+}-sensitive (CSP) and non-Ca^{2+}-sensitive (NCSP) molecules. Separation, from extracted juice, of these two types of pectin by selective precipitation has been described by Glahn (1995a, 1995b). Selective precipitation is achieved by a solution of water and alcohol that contains a dissolved polyvalent cation such as Ca^{2+} (usually nitrate salt), which causes the CSP to gel. It is also possible to separate CSP and NCSP directly *in-situ* by fractional extraction at differential pH. At pH around 3, NCSP is separated and at pH 2 (typical of normal pH for pectin extraction) extracted pectin turns out to be CSP.

Extraction technology is being continually studied and extraction processes using steam injection under pressure, microwave heating, enzymatic and microbial tools have been proposed (Fishman *et al.* 2000, 2003, 2006; Panouillé *et al.* 2006). It has been shown that flash extracted pectin from orange albedo by microwave heating under pressure exhibited increased molar mass, size and intrinsic viscosity compared to pectin extracted by conventional heating techniques (Fishman *et al.* 2000). Moreover, gel forming properties of orange and lime pectin prepared by rapid microwave heating were better than those of commercial citrus pectin (Fishman *et al.* 2003). The USA is one of the largest producers of oranges in the world and 90% of this huge production is used to manufacture juice. Adapted extraction processes could result in the growth of pectin extraction industry in the USA using source-orange-peels that now go to waste (Walker 2003).

An enzyme-hydrolytic technology would be environmentally safer and potentially more effective in terms of pectin yields (Panouillé *et al.* 2006). Analysis of the scientific and patent literature shows that a number of research centers have been conducting studies to develop a biotechnological method for pectin extraction, but these studies are of exploratory nature only and their results are still far from industrial application (Ptichkina *et al.* 2008).

Filtration and purification

The pectin raw extract is separated from the plant residue by filtration and/or centrifugation processes. The solid: liquid ratio has to be well defined for an efficient liquid/solid separation. Ratios of 1:17 for apple and 1:35 for citrus are often used. Efficient filtration requires reasonably low viscosity, but the more water added to the process, the more energy is needed to remove it. Currently, rotary drum vacuum filtration is commonly used in the industry. Insoluble filter aids, such as wood cellulose and perlite, may be used to facilitate this process. Extracts are rapidly brought to pH 3-4, whereupon the temperature is lowered to avoid pectin demethylation and depolymerisation. Weak bases, like sodium carbonate or ammonia are used to minimize β-elimination reactions. The clarified extract may be passed through a column with cation-exchange resin to remove Ca^{2+} ions and generate the sodium form of pectin, which is better adapted for food application. Next, the solution is concentrated to 3-4% dry material by evaporation or membrane filtration. Pectin is then precipitated by pouring the extract into an appropriate alcohol (usually iso-propanol). The precipitate obtained is pressed, washed in a fresh bath of alcohol, pressed and finally dried and milled to a desired particle size. To recover HM-pectin, an alternative to alco-

hol precipitation is precipitation by adding appropriate metal salts to the extract. Pectin forms insoluble salts with, for example, Cu^{2+} and Al^{3+}. Removal of metal ions from the precipitated pectin is done by washing in acidified aqueous alcohol. Pectin suspended in alcohol is in a very suitable form for further modification. The LM-pectins are generally obtained by controlled acid de-esterification or by alkali de-esterification. The temperature should not exceed 50°C to avoid depolymerisation reactions. This treatment can yield HM-pectin with DM values in the range of 55-75% or LM-pectin in the DM range of 20-45%. Ammonia may convert methyl-esterified carboxylate groups of pectin to primary amides. This is done industrially by suspending precipitated pectin in a mixture of alcohol and water with dissolved ammonia. By choosing proper conditions with respect to ammonia concentration, pectin with various proportions of amidated, methyl-esterified and free carboxylate groups can be produced. Regulation requires that the degree of amidation (DAm) does not exceed 25% (Voragen *et al.* 1995; Rolin *et al.* 1998; May 2000).

Standardization

Standardization is a current industrial practice to produce pectin samples with consistent properties. Diversity of natural raw material, climatic conditions to which the plants have been exposed, ripeness and peel pre-treatment processes may lead to large differences in pectin properties and functionalities. HM-pectin is therefore diluted and standardized with sucrose, dextrose, glucose or lactose to a given gelling power defined as degree sag. The standard of 150° sag means that 1 g of pectin is able to gel 150 g of sucrose under defined conditions of pH and temperature. Due to their large application range, LM-pectins are not necessarily standardized. For pharmaceutical purposes, pectin without admixed sugar is also available from the major manufacturers (Voragen *et al.* 1995; Rolin *et al.* 1998; May 2000). So, when using pectin, it is obviously important to choose a type, which has been standardized in a way that reasonably corresponds to the intended use.

World market and regulations

Pectin extraction is an intensive energy utilizing process that requires sophisticated operations and control. Tropical developing countries may have a locally owned pectin manufacturing operation, but it is typically hard pressed to compete with imported pectin unless the native operation is given governmental protection. Typically, pectin operations are co-located with large-scale juice operations that run at least 300,000 MT of fruit per year (Bates *et al.* 2001).

The pectin industry is dominated by large multinational firms, which have undergone major changes in the past five years. The world leaders include CP Kelco (owned by I.M. Huber Corporation since 2005), Danisco sugar (Denmark), Cargill (acquired Citrico and Degussa Food Ingredients) and Herbstreith and Fox (Germany) (USDA Foreign Agricultural Service 2007). Approximately 35000 tons of pectin are produced and used each year in the world (Daniells 2007; USDA Foreign Agricultural Service 2007;). CP Kelco has around 40% of the market (35-40% of the world market and 35-45% of European economic area (EEA) market) followed by Danisco (20-30% of the world market and 15-25% of EEA market), Degussa Food Ingredients (10-20% of the world market and 15-25% of the EEA market), Herbsteith and Fox (5-15% of the world market and 10-20 of EEA market), Cargill and Obipektin (10% of the world and EEA market, separately). The remaining 5% is held by small processors (USDA Foreign Agricultural Service 2007).

Pectin is an essential additive in many food applications, such as beverages, protein drinks, yogurts, jams, jellies and desserts. In addition to these general applications, pectin may also hold applications as a prebiotic, potential source of soluble fiber and fat replacer in functional food and nutraceutical applications. Recent *in vitro* tests have shown that pectin acts as a prebiotic, by preventing pathogens from binding to the intestine and increasing the growth of probiotic bacteria in the large intestine (Iisakka 2003). In regard to fiber additives, Functional Food and Nutraceuticals estimates that besides oat bran, psyllium and soya fiber, pectin will fare the best in nutraceutical application based on a combination of cost, quality, performance and versatility advantages. In addition, a physically modified version of pectin is in the market as a fat replacer. Pectin also holds medical applications for colon specific drug delivery, which may face a potential $1 billion market in the medical field (Kalorama Information 2007). The market of pectin has been estimated to be growing at a rate of 3-5% annually since 2001. The average price of HM and LM-pectins are approximately $16000 per ton since 2006 (USDA Foreign Agricultural Service 2007).

Although the FAO/WHO joint Expert Committee on Food Additives and the EU, have not established a numerical acceptable daily intake (ADI) and consider pectin as safe, some national regulations may limit the amount of pectin added in some applications. In the US, pectin is GRAS-Generally Recognized as Safe. In most food it can be used according to good manufacturing practice in the levels needed for its application *quantum satis*. In the International Numbering System (INS), that provides an agreed International numerical system for identifying food additives, created by the Codex Committee on Food Additives and Contaminants (CCFAC), pectin has the number 440. In Europe, it is differentiated into E440(i) for non-amidated pectin and E440(ii) for amidated one (USDA Foreign Agricultural Service 2007).

PECTIN GELLING PROPERTIES AND APPLICATIONS

In many food products, gelation of polysaccharides is critical to the formation of structures with the desired texture. On a molecular level, an aqueous gel consists of three elements:

- Junction zones where polymer molecules are joined together;
- Inter-junction segments of polymers that are relatively mobile;
- Water entrapped in the polymer network.

The properties of pectin gels are strongly influenced by several factors. Extrinsic factors, such as pH, ionic strength, co-solute concentrations and temperature, influence the strength, texture and general viscoelastic properties of pectin gels (Rao and Lopes da Silva 2006). Intrinsic variables of the pectin macromolecules, including the molar mass, sequence of sugars along the chain, DM and distribution of the methyl groups along the chain, greatly impact on gelling properties of this polymer. Among them, DM plays the most critical role (Rolin *et al.* 1998; Cardoso *et al.* 2003).

Pectin with a high degree of polymerization is more viscous in solution than otherwise comparable pectin of lower degree of polymerization. The dependence of gel strength on the molar mass is more pronounced with breaking strength method than with non-destructive test methods. It seems highly plausible that non-homogalacturonan portion kinks the molecules and prevents aligning as well as the formation of a precipitate (Rolin *et al.* 1998).

Native citrus pectin is highly methylated (HM-pectin). LM pectin is generally obtained by controlled acid or alkali de-esterification but other means, namely enzymes and ammonia, can be used. Treatment of pectin with acid, alkali or microbial (*Aspergillus niger*, *Aspergillus japonicus*, *Aspergillus foetidus*) pectin methyl esterase (PME) leads to pectin with a random distribution of free carboxyl group, whereas the action of alkaline PMEs from higher plants (tomato, orange, alfalfa, apple) and from fungi (*Trichoderma reesei*) results in a blockwise arrangement of carboxyl groups on the pectin molecule (Kohn *et al.* 1983; Thibault and Rinaudo 1985; Denes *et al.* 2000). The distribution pattern of free and esterified carboxyl groups has a profound

effect on gelling properties (Ralet *et al.* 2001). A random distribution of methyl esters gives a low Ca^{2+} sensivity, while a blockwise distribution generates a local charge concentration, which may hold Ca^{2+} ions in place in gel structure. CSP can gel in the presence of Ca^{2+} ions without sugars and are therefore useful for low-fat or sugarless, acidic food stuff formulations (Joye and Luzio 2000). Several methods, such as conductimetry and NMR, allow the determination, to a certain extent, of the distribution of methyl-esters along HG domains (Kohn *et al.* 1983; Thibault and Rinaudo 1985; Grasladen *et al.* 1988). More recently, an enzymatic method was developed (Daas *et al.* 1998, 1999, 2000; Limberg *et al.* 2000a, 2000b; Daas *et al.* 2001a, 2001b). Pure pectolytic enzymes were used and the generated oligosaccharides were identified and quantified by a combination of mass spectrometry and high-performance anion-exchange chromatography at pH 5. From their work, Daas and co-workers introduced the concept of "degree of blockiness" (DB) in pectin. The DB increases when the GalA residues are distributed in a more blockwise way over the pectin molecule.

The deesterification method using ammonia produces a different type of LM pectin in which some carboxylic group have been amidated. Amidated pectin has been claimed to have a blockwise distribution pattern of the amide groups and a random distribution of the free carboxyl groups (Racapé *et al.* 1989). For amidated pectin, it is possible to identify the distribution of methyl esters and amide groups by using off-line coupled high-performance anion-exchange chromatography at pH 5 and mass spectrometry (Guillotin *et al.* 2006).

HM-pectin

Jam manufacture is the main user of industrially extracted pectin, utilizing the ability of HM pectin to form a gel with sugar and acid – the so-called low water-activity gels or sugar-acid-pectin gels. Such a gel is considered a three-dimensional network of pectin molecules in which the solvent (water) encompassing the co-solutes (sugar 55-75% w/w, and acid pH 2.5-3.5) is immobilized, resulting in a system resisting deformation and showing a stress/strain relationship for small deformations. The high sugar concentration creates conditions of low water activity, which in turn promote chain-chain rather than chain-solvent interactions; whereas the acid lessens the negative charges on the carboxyl groups, thus diminishing electrostatic chain repulsion. The fact that sucrose can be replaced by other polyols, that less acid is necessary for pectin with higher DM and that a completely methoxylated pectin will gel without any acid, are considered as proof of these functions (Voragen *et al.* 1995). Junction zones formation is made possible through the smooth regions of pectin. To avoid turbidity, syneresis and precipitation, there must be junction-zone – terminating structural elements present in the chain, and "hairy regions" are thought to play such a role (Ralet *et al.* 2002). Gels formed under these conditions are stabilized by aggregated helices supported by hydrogen bonds and grouping of methyl-ester groups through hydrophobic interactions within a cage of water molecules. Recently, global structures of HM pectin in gels were visualized by atomic force microscopy (AFM) (Fishman *et al.* 2004, 2007). Rods, segmented rods, rings, branched molecules, and dense circular areas of pectin were visible.

Many factors influence the conditions of gel formation and strength, among them DM is a key factor. DM controls the rate of gelation and the gelling temperature. In general, increasing the DM leads to a faster gelation and higher gelling temperature. Moreover, at constant pH and co-solute concentrations, the final gel strength increases with increasing DM. As the DM of HM-pectin decreases, a lower pH is required for gelation, although the apparent pk_a increases due the decrease of DM (Rao and Lopes da Silva 2006). DM correlates with the gel setting rate and gel texture under otherwise similar conditions, which means that very highly esterified pectin gels quicker at higher temperature than HM pectin of lower DM. Very highly methylated pectin will also form a more elastic and brittle gel texture compared to pectin of lower DM (Herbstreith and Fox KG Corporate Group 2003).

HM-pectin is also used in the confectionary industry for making fruit jellies and jelly centers, in fruit juices and fruit drink concentrates as a stabilizer and/or to provide mouthfeel. It is also used in fermented and directly acidified dairy drinks (Ralet *et al.* 2002).

LM-pectin

Ionic-mediated gelation through divalent cations, of which the most relevant is Ca^{2+}, is the classical mechanism of LM-pectin gelation. The mechanism of Ca^{2+} binding to the ionized carboxyl groups on the pectin chains is similar to the egg-box proposed for alginate. The Ca^{2+} ions occupy the electronegative cavities in a two-fold buckled ribbon structure of the GalA residues (Rao and Lopes da Silva 2006).

Because of the electrostatic nature of the bonds, LM-pectin is very sensitive to intrinsic parameters that can modify the environment of the carboxyl groups, such as the nature, distribution and amounts of substituents along the galacturonic backbone. Thus, the gel forming ability increases with decreasing DM. Furthermore, LM-pectin with a blockwise distribution of free carboxyl groups are very sensitive to low Ca^{2+} levels (Kohn *et al.* 1983; Thibault and Rinaudo 1985; Ralet *et al.* 2001). For LM blockwise pectin at low level of Ca^{2+}, the gel structure will be less elastic, rather pasty with lower breaking strength compared to a gel prepared with an optimum level of Ca^{2+} (Herbstreith and Fox KG Corporate Group 2003; Dixon 2008). An overdose of Ca^{2+} will lead to pectin precipitation, also called pre-gelling. This is reversible only to a limited extent, even when the gel is once more heated above its setting temperature and cooled down without destruction (Herbstreith and Fox KG Corporate Group 2003). The traditional application of LM-pectin is in jams with soluble solids below 55% (low-calorie jams, jelly preserves and conserves). This is the limit for HM-pectin. The heat reversibility of LM-pectin gels may be utilized in bakery jams and jellies for glazing purpose. LM-pectin also finds application in the production of fruit preparations for yogurt and fruit/milk desserts (Ralet *et al.* 2002).

Amidated LM-pectin can gel under the same conditions as LM-pectin. It has a lower gelling temperature than non-amidated pectin of the same DE. Amidated LM-pectin accounts for the major part of today's use of LM-pectin. Compared to non-amidated LM-pectin, the gels are less prone to syneresis and their texture is easier to control due to functional saturation with Ca^{2+} ions in most applications (Rolin 2002). Amidated pectin needs less Ca^{2+} to gel and is less prone to precipitation at high Ca^{2+} levels, suggesting that its gelation cannot be fully explained by the "egg-box model". Indeed, blocks of amide groups along the chain promote association through hydrogen bonding (Racapé *et al.* 1989; Alonso-Mougan *et al.* 2002; Rolin 2002).

System parameters

At a pH of roughly 3.6 (depending on pectin type), around half of the carboxyl groups of pectin are dissociated. Repulsion between the charged groups contributes to solubility and decreases the tendency for gelation. At low pH, fewer charges are present and solubility declines, whereas at a high pH, more charges are present and solubility increases. If Ca^{2+} salt is added, Ca^{2+} ions may form bridges between molecules (LM-pectin mechanism) and this increases the viscosity or causes gelation. Protons compete with Ca^{2+} especially at low pH. In the absence of Ca^{2+}, the viscosity is inversely related to pH. That is why pectin of very low DM can gel at pH values below 2.5 (Löfgren *et al.* 2005).

In the presence of monovalent salts, such as NaCl, less Ca^{2+} is needed for pectin to pass sol-gel transition points.

More Ca^{2+} ions are indeed able to establish junction zones, while the charge of the polymer is increasingly screened by monovalent ions. The combined effect of pH and sugars promotes gelation at lower Ca^{2+} levels. pH and co-solute effects on chain-solvent interactions counterbalance the decrease in the number of carboxyl groups available for Ca^{2+} binding and chain-chain interactions are promoted (Voragen *et al.* 1995).

In the pH range where gelation of HM-pectin usually occurs (~2.0-3.7), gel strength, setting temperature and setting rate increase with the reduction in pH, when other conditions, such as total solids content and ionic strength, remain unaltered. This is due to the enhancement of macromolecular interaction resulting from the reduction of the pectin charges. Generally, the upper limit of pH for HM-pectin gelation is raised if, either the DM, total solid concentration or pectin concentration, increases. pH is a factor that is not so critical in the development of LM-pectin gels (2.0-6.0). However, it has a significant role in the final properties of the gels. A decrease in pH leads to different kinetics of gel formation and to a decrease in shear modulus, due to the decrease in the number of ionized carboxyl groups necessary for ionic complexation and gel formation. At a low pH, more Ca^{2+} is needed to induce gelation than at a neutral pH. Compared to a salt-free solution, less Ca^{2+} is necessary to form gels when the ionic strength increases. However, gels in salt-free solution are formed more rapidly, but the final modulus is lower (Rao and Lopes da Silva 2006). When pH is below 3.5, there is the predominance of non-dissociated acid groups, which leads to more hydrogen binding in the gel network. This gives rise to a more rigid, non-shear-reversible gel network. When pH is above 3.5, there is a predominance of ionized acid groups, which favours Ca^{2+} cross-linking, leading to the formation of a more spreadable, shear reversible gel network. With LM-pectin, as the soluble solids increase, the requirement for Ca^{2+} decreases and the Ca^{2+} bandwidth decreases (Ralet *et al.* 2002).

The affinity of pectin for Ca^{2+} increases with decreasing average DM and increasing length of unsubstituted galacturonan stretches. The affinity of HM-pectin for Ca^{2+} is generally not high enough to lead to sufficient chain association for gelation to occur. Generally, increasing the concentration of sugar increases the gel strength, gelling temperature and gelling rate. The minimum concentration of sugar or polyol required for gelation depends on its ability to sufficiently stabilize hydrophobic interaction. For LM-pectin, the presence of sugar or other co-solutes is not necessary, but there is an increase in gelling temperature and gel strength and a decrease in syneresis as the total soluble solids increase (Rao and Lopes da Silva 2006).

ACID-DAIRY-DRINKS – A GROWTH SECTOR WITH A SIGNIFICANT POTENTIAL

Acid dairy drinks (ADD) are worldwide products existing in many variations, e.g. fruit milk drinks, yoghurt drinks, soy milk, butter-milk, whey drinks, kefir among others. These beverages can be described as an acidified protein liquid system with stability and viscosity similar to natural milk. Such drinks are usually composed of an acid dairy phase (fermented base) or a neutral base (milk, soy milk) with an acidic medium (fruit phase) (Laurent and Boulenguer 2003).

According to DMI Dairy Management Inc™ (2007), drinkable yoghurt stands out as the fastest growing seller, not just in dairy, but in the food and beverage industry as a whole. China topped the list of producers with a whopping 49% year-over-year growth rate. Annual sales of drinkable yoghurt grew by 18.4% to $7.76 billion from mid-2005 to mid-2006 (DMI Dairy Management Inc™ 2007). Numerous opportunities for functional ingredients, such as Omega-3, phytosterols and probiotics in drinkable yoghurt, have made the products an attractive area for innovation.

Stabilizing properties (pectin-protein interactions at interfaces)

A substantial portion of pectin today is used for the stabilization of low-pH dairy drinks, including fermented drinks and mixture of fruit juice and milk. The drinks may be heat-treated in order to increase their shelf life. Low viscosity and homogenous appearance are preferred characteristics. Casein, however, is prone to aggregation at low pH, particularly when subjected to heat treatment. Thus, in the absence of a stabilizer, high viscosity, whey exudation and sandy mouth feel are likely quality defects in these types of drinks (Rolin *et al.* 1998). In the late 1950s, it was proven that addition of HM-pectin to acidified milk drinks prevented the formation of sediment. Casein in milk, at its natural pH of approximately 6.6, is in the form of stable sub-micron particles, often called micelles (Ye 2007). At this pH, caseins are negatively charged. They are mutually repellent, which prevents precipitation. During acidification, electrostatic repulsion decreases and casein particles tend to aggregate. At the isoelectric point (pH 4.6), casein particles are uncharged and exhibit the weakest hydration. Below the isoelectric point, casein particles exhibit a net positive charge.

The detailed changes of the micelles above the isoelectric point can be summarized as followed:

- Between pH 5.8 and 5.5, the zeta potential decreases, leading the micelles to form clusters (particle size change from 180 to 1300 nm at pH 5.5).

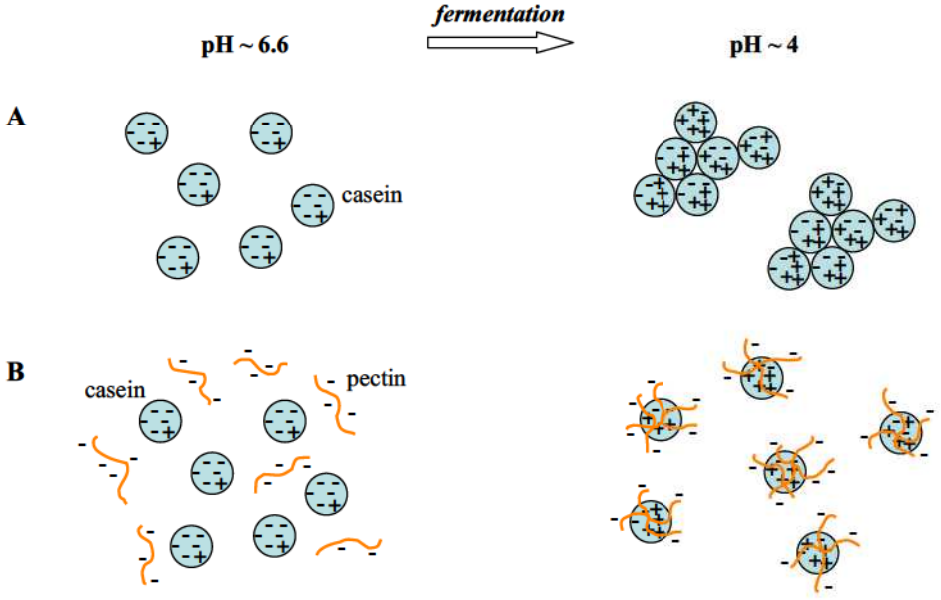

Fig. 5 Schematic representation of pectin stabilizing properties in acidified dairy drinks. (**A**) Casein in milk at its natural pH (~ 6.6) is in the form of stable sub-micron particles that are globally negatively charged. During fermentation, acidification occurs and casein particles become globally positively charged. Below pH 5, aggregation of the casein is irreversible. (**B**) At pH > 3.5, pectin is negatively charged due to the presence of carboxylic functions. At pH 6.6, both polymers are negatively charged and repel each other. At pH ~ 4, pectin electrostatically stick to the positively charged areas of casein particles producing a highly hydrated coating, which prevents casein aggregation. Such pectin-stabilized particles are often depicted as "fuzzy golf balls".

- Between pH 5.5 and 5, a re-organization of the different kinds of caseins occurs in the micelle by solubilization of α and β casein. Micelles are then associated in multi-strands and are no longer spherical.
- Below pH 5, Ca^{2+} is completely soluble, the aggregation of casein is irreversible and a three-dimensional network is formed by the cluster of aggregated strands (Laurent and Boulenguer 2003).

A mechanism of stabilization by pectin, under acidic conditions, was proposed in which the adsorption of the pectin on the casein micelle surface throughout carboxylic-rich zones of HG domains is involved. The highly methylated (uncharged) pectin segments would form entropy-rich loops that extend into the solution. These loops result in the repulsive interaction between the micelles at low pH in the same way as κ-casein chains do at milk pH (Rao and Lopes da Silva 2006) (**Fig. 5**). The stabilization of casein with pectin is only effective in the pH interval 3.2-4.5. By extension, pectin is not sufficiently dissociated at a pH below 3.5. It then either does not efficiently anchor or there is weak repulsion between "hairs" in an interpenetration zone. At a pH above 4.5, casein does not possess enough positively charged areas and there is no longer an attraction between pectin and casein (Rolin *et al.* 1998). In more concentrated acidified milk systems, it was suggested that stability is associated with the existence of a network of pectin-coated casein micelles, but a large fraction of pectin does not interact directly with acidified milk gel (large casein micelles). It was shown that less than 20% of the pectin added directly interacts with casein particles (Syrbe *et al.* 1998). The remaining 80% is involved in a network with casein-pectin complexes, but plays no role in stabilizing the final product. This excess fraction seems however necessary to produce a stable system (Janhøj *et al.* 2008).

Recently, it was shown that (i) stabilization in ADD might be caused by a combination of depletion interaction between pectin coated casein micelles and a pectin network, (ii) that 50-90% of all pectin is bound to casein and (iii) that stability is not affected by the remaining non-bound fraction (Boulenguer and Laurent 2003; Tromp *et al.* 2004).

Pectin dosage is a determinant factor in acidified milk drink stabilization. Too little pectin destabilizes the product compared to no pectin addition. If a series of milk drinks is prepared with different pectin additions, the viscosity first increases until a certain concentration is reached. A further increase in pectin amount causes a sharp decline in viscosity. A point of minimum viscosity is then reached, beyond which further addition of pectin causes a new increase in viscosity. The tendency for sediment formation grossly follows viscosity until the point of minimum viscosity. It is believed that the de-stabilization at low pectin dosages is because the adsorbing pectin molecules tend to wrap around casein particles when the surface is not crowded with pectin. Electrostatic repulsion is at a minimum because pectin has balanced the initial casein charge rather than increased numeric net charge. At full stabilization, only those pectin molecular areas, which interact strongly with casein, are anchored at the casein particles surface, whereas those areas, which are less strongly attached, are forced away from the surface (Rolin *et al.* 1998). Viscosity overshoot and final viscosity reduction become particularly pronounced when the casein particle size is low. It is striking that, independent of casein particle size, the maximum of low shear viscosity occurs at about half the pectin concentration at which the viscosity minimum (and product stability) is observed. This could point to bridging by weakly adsorbed polymers, expected to be the most pronounced at about 50% surface coverage. In contrast to the results at low shear rates, the limiting high-shear viscosity increased steadily with pectin concentration. This was interpreted as an increase in particle volume by the absorbed pectin layer, matching the results on the increased sediment volume after centrifugation. Pectin addition also changes the flow behaviour of ADD from shear-thinning and thixotropic to quasi-newtonian. This means that the system undergoes a transition from a flocculated state with partial surface coverage into a free-flowing state, where surface coverage is completed and particle attraction minimized. This conclusion is corroborated by microscopy and particle size analysis (Rolin *et al.* 1998).

In industry, the pectin dosage required for optimum acidified milk stabilization depends on the formulation and production technology of the product. The stability of acidified milk drinks is evaluated on the basis of the viscosity, the amount of sediment after defined centrifugation and by microscopic examination of the protein particles (Herbstreith and Fox KG Corporate Group 2003).

Important parameters are:

- pH value of the drink (optimum range 3.9-4.1)
- protein content and particle size (1-2 µm~ smooth, 10-20 µm~ chalky, 40-60 µm~ grainy)
- fermentation conditions during yoghurt preparation (temperature, time, bacteria culture used to produce uniform-sized protein particles)
- conditions during direct acidification using juice or acid (direct acidification makes large protein particles)
- heat treatment during the production process (the more intense the heat treatment, the greater the risk of thermal agglomeration of protein particles).
- homogenization (10-20 MPa, to destroy caseinate gel)
- heat treatment of the finished products (it has adverse effect because of the weakest bound molecules dislodge from the complex)
- Addition of Ca^{2+} (more Ca^{2+} reacts with more pectin) (Syrbe *et al.* 1998; Boulenguer and Laurent 2003; Herbstreith and Fox KG Corporate Group 2003; Lucey 2004; Tromp *et al.* 2004; Sedlmeyer *et al.* 2004; Mosteller 2006; Sejersen *et al.* 2007; Ye 2007; Janhøj *et al.* 2008).

Dilution drastically hampers the stabilization of ADD. In fact, except for Ca^{2+} enriched drinks, water-dilution modifies the ionic strength, which induces a decrease in stability (Ye 2007). A high Ca^{2+} concentration inhibits stabilization since it strongly enhances caseinate self-association (Syrbe *et al.* 1998). ADD milk solid non-fat (MSNF) can vary from 1 to 8.5% in commercial application (Laurent and Boulenguer 2003).

Types of pectin used for acid dairy drink (ADD) stabilization

ADDs are commonly stabilized by HM-Pectin (Syrbe *et al.* 1998; Boulenguer and Laurent 2003; Herbstreith and Fox KG Corporate Group 2003; Lucey 2004; Tromp *et al.* 2004; Sejersen *et al.* 2007; Ye 2007; Janhøj *et al.* 2008). LM-pectin with more numerous carboxyl groups should associate more strongly with the positively charged casein particles than HM-pectin. However, LM-pectin exhibits lower stabilization properties than HM-pectin. Tighter binding of pectin molecules could be the reason, leading to a flatter configuration and less polymeric stabilization, but without detailed information on the molar mass and the distribution of carboxyl residues, all this remains speculation (Syrbe *et al.* 1998). HM-apple pectin is especially suited for this stabilization mechanism when a high viscosity ADD is assumed. If a low-viscosity end product is required, HM-citrus pectin is mainly used. Only HM-pectin within a very specific range of esterification (approx. 68-72%) is suited for the stabilization of ADDs. For optimum stabilization, pectin requires a high molar mass and defined Ca^{2+} reactivity (Herbstreith and Fox KG Corporate Group 2003).

CONCLUSION

Pectin is a high value functional food ingredient widely used as a gelling agent and stabilizer. Food scientists and plant scientists therefore share a common goal to better understand the structure and functionalities of pectic polymers at the molecular level. The basic properties of pectin have been known for nearly 200 years, but recently there has been tremendous progress in our understanding of the

very complex fine structure of pectic polymers and pectinolytic enzymes. This has been made possible by synergies between plant and food research and by the application of a range of state-of-the-art techniques including enzymatic fingerprinting, mass spectrometry, NMR, molecular modelling, and monoclonal antibodies. With this increased knowledge, there are increased opportunities for novel applications. Producers are beginning to develop a new generation of sophisticated designed pectins with specific functionalities. However, it will be important that these advances are carefully managed, so that pectin maintains its deserved reputation as a natural product.

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Extracting Intramolecular Sequence Information from Intermolecular Distributions: Highly Nonrandom Methylester Substitution Patterns in Homogalacturonans Generated by Pectinmethylesterase

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Received March 31, 2010; Revised Manuscript Received May 10, 2010

The ratio of the two component sugar residues comprising the anionic polysaccharide homogalacturonan (HG; namely, methylesterified or unmethylesterifed galacturonic acid (GalA)) has been controlled chemically or enzymatically in order to produce samples comprised of varying degrees and distributions of methylesterification (DM). Capillary electrophoresis (CE) has been used to characterize the samples produced and, by mapping the measured electrophoretic mobilities to biopolymer charge density, intermolecular distributions of the DM have been extracted. For chemically modified samples with random intramolecular patterns of methylesterification, the experimentally extracted intermolecular DM distributions agree well with the predictions of calculations based on the binomial theorem, demonstrating that the random nature of the demethylesterification process and, hence, the intramolecular DM patterns themselves, are directly reflected in the intermolecular distribution. Furthermore, this principle has been demonstrated by extending the work to the study of substrates with highly nonrandom DM distributions generated using a processive plant-pectinmethylesterase (pPME). An ensemble of polymer chains, generated in silico by a simulation optimized to match the experimentally measured intermolecular DM distribution, contains all possible information regarding the substrate and can further be interrogated to obtain, for example, the full Gal-A blocklength distribution.

Introduction

Biopolymer Sequencing. The fact that macroscopic samples of nucleotides or proteins can be generated in which all chains have the identical intramolecular sequence of their component residues has been a great benefit in the development of sequencing strategies. This not only ensures that following site-specific chain scissions sufficient identical fragments are liberated to enable their facile detection, but also means that sequence reconstruction can be performed by overlapping different severed fragments liberated by non-sequence-specific digestion that each originate from different chains but contain common residue patterns (Figure 1a). The fact that in samples of heteropolysaccharides each chain does **not**, in general, possess the same sequence of component sugars means that such approaches are not directly applicable to these biopolymers. Nevertheless, progress has been made in the same spirit, particularly where only two residue types are predominant, where either (i) a single "average chain" is reconstructed from fragments derived from some digest of the entire sample[1] or (ii) ensembles of chains are reconstructed from fragments so that they are consistent with, for example, the molecular weight distribution of the starting material.[2] Alternatively, structure−function studies of such biopolymers have concentrated on the measurement of sample-average structural indicators, such as the amount of particular fragments released by particular enzymes,[3−6] NMR spectra or derived triad frequencies,[7−10] or FTIR spectra,[11] which have then been empirically correlated with some quantitative measure of a particular functionality of the sample. Undoubtedly carefully considered work in this area has moved the field forward and, indeed, useful correlations have been obtained in this manner. However, whatever the functionality under investigation, from the strength of gels formed[12−17] to physiological effects implicated in a raft of human health issues,[18−20] it is not clear that the characterization of the fine-structure in terms of the properties of a fictitious "average chain" or a sample average will always reveal sufficient details of the underlying structure−function relationships, even in polysaccharides containing only two residue types. It should be noted that many biologically important structural heteropolysaccharides do, in fact, comprise predominantly only two sugar residue types, including pectins, alginates and carrageenans, chitosans, and dermochondan and chondroitan sulphates, integral components of the biopolymeric scaffold of land plants, seaweeds, crustaceans, and animals, respectively. To reiterate, such samples do not, in general, contain multiple copies of the same chain, and to understand their behavior, the reconstruction of an ensemble of chains of differing intramolecular sequences that faithfully represent the real sample must be sought. While, as described, some work in this direction has been carried out, it has been, to date, focused on reconstructing chains from fragments that have been liberated from the sample by digests of various kinds so that (i) the digest process and its dependence on fine-structure must be well understood[21−23] and (ii) the

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10.1021/bm1003527 © 2010 American Chemical Society
Published on Web 05/27/2010

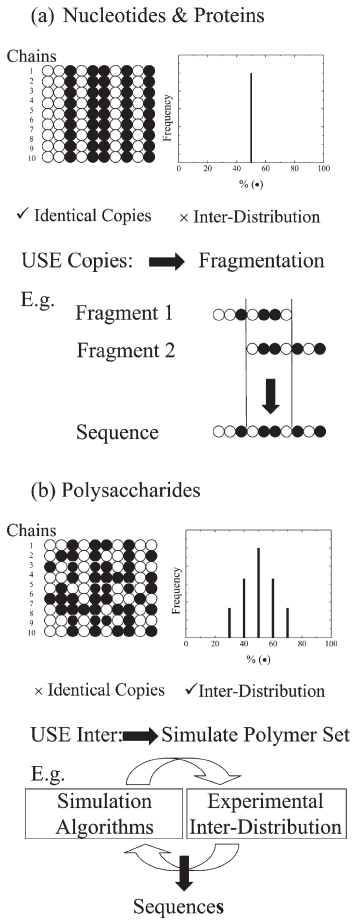

Figure 1. (a) Schematic representation of biopolymer sequencing in systems of multiple identical chains; showing 10 hypothetical chains each of 10 residues in length, with two distinct residue types, and sequence reassembly from fragments. (b) Schematic representation of biopolymer sequencing in systems with an intermolecular distribution of intramolecular sequences; showing 10 hypothetical chains of 10 residues in length, with two distinct residue types, and ensemble reconstruction from intermolecular measurements.

sample itself is destroyed in the "sequencing" process. Here we describe an alternative strategy that circumvents these problems by simulating ensembles of chains that, rather than being constructed to be consistent with fragmentation results, are generated to match a measured intermolecular distribution of the residue contents.

Intermolecular Strategy. Not having multiple copies of the same chain has traditionally been seen as a huge disadvantage for polysaccharides of the type discussed, negating as it does many elegant strategies used for sequencing other biopolymers (e.g., Figure 1a). However, framed in a more positive light, such samples do possess something that DNA and protein samples do not, an intermolecular distribution of the number of specific residue types in each chain (Figure 1b), and it will be argued herein that by exploiting this fact and modeling this intermolecular distribution, significant progress in generating faithful ensembles of chains can be made that contain all relevant fine-structure information.

For the copolymeric example expounded herein, the problem can be illuminated by comparison with simple coin tossing: a sequence of n coin tosses produces a string of heads and tails that can be thought of as corresponding to a biopolymer sequence with n residues, with each having two possible identities. In this analogy, performing a second trial and generating another sequence of n tosses corresponds to generating another polymer in the ensemble. To extract information about the distribution of heads and tails and the process that generated them, two approaches can be taken. First, the sequences of heads and tails seen within each trial can be directly examined: indeed in an analogy to the nucleotides and proteins example (Figure 1a) each trial (chain) would possess the same sequence of heads and tails (residues), and direct examination of the intramolecular sequence is the only option. However, if different sequences of heads and tails are found in the repeated trial sequences (corresponding to different sequences of residues in different chains) then the information describing the ensemble is also contained in how the total number of heads found in each trial is distributed between trials. Thus, far from being a disadvantage the presence of an intermolecular distribution allows information about the intramolecular sequences to be obtained without the need for directly examining them. This means that samples can have their intramolecular residue distribution inferred without having to fragment them: a sample of this type could potentially have its fine-structure measured and, subsequently, functionality studies could be carried out on the very same sample.

Example: Homogalacturonan and pPME. Homogalacturonan samples have been used to investigate the success or otherwise of the intermolecular strategy expounded above. In this case, one of the two residue types is charged, making the extraction of the intermolecular distribution of the fraction of each chain that is comprised from that residue directly measurable by electrophoretic means, as described in detail below. In addition, it is well-known that highly nonrandom demethylesterification processes occur naturally during enzymatic processing with pPME.[24−28] These pPME-mediated demethylesterification processes are of great interest in their own right, and can also be exploited to generate samples in the laboratory with interesting fine-structures.

The HGs used originate from pectin and, indeed, while the detailed structure of the pectin macromolecular assembly in vivo is still a matter of debate to some extent,[29] most commercially available pectin samples can be considered as a collection of polymer chains, each consisting of extended regions of homogalacturonan (HG) interspersed sparsely with regions of rhamonogalacturonan I (RGI).[30] HG consists of a linear backbone of (1,4)-linked α-D-galacturonic acid (GalA) residues, commonly partly methyl-esterified at C-6, while the RG-I backbone consists of [2)-α-L-Rha*p*-(1,4)-α-D-Gal*p*A-(1] repeats.[31] The rhamnosyl (Rha) residues of RG-I backbone are substituted, mainly at *O*-4, with several types of arabinose (Ara)- and galactose (Gal)-containing neutral sugar side chains.[31]

The relative amount of methylesterified GalA residues (degree of methylesterification, DM) and the distribution of the methylesterification of the HG, both among chains, and along individual polymer backbones, is a key determinant of molecular functionality. Indeed, cell wall enzymes routinely tailor methylesterification distributions to exploit structure−function relationships based on the dependence of molecular association on the pattern of methylesterification.[32,33] The measurement of such distributions is then vital in understanding the role that fine-structure modifications play in determining the functionality of pectin both in vivo and in vitro.

Capillary electrophoresis has been shown to be a useful tool for the investigation of just such pectin and HG methylester distributions.[34−41] Most simply it can used in order to measure the average DM of pectin and HG samples, since a linear relationship between the electrophoretic mobility and the average charge per sugar residue has been observed over a large range

of methylesterification degree.[34,35] While many other methods perform this sample averaged DM measurement equally well,[10,42−47] an advantage of the electrophoretic method is its inherent separation quality. For chains with lengths in excess of around 25 residues, a symmetrical scaling of charge and hydrodynamic friction coefficient with the degree of polymerization (DP) is found. This means that larger polymeric chains, regardless of their DP, elute according to their average charge density and, therefore, that each CE migration time marks species with a unique DM. Peak shapes thus reflect the intermolecular methylesterification distribution (the methylesterification distribution among chains) of the sample.[39,48]

In the direct examination of patterns of methylester groups that exist along individual polymer backbones (the intramolecular distribution), two basic approaches have been investigated to date, either using NMR in an attempt to directly measure the frequencies of the possible triad sequences of residues found within the experimental ensemble of chains[7,8,10] or fragmenting the polymer, examining the liberated fragments, and making inferences about the predigested structure. Much work has been carried out employing the latter fragmentation approach using endopolygalacturonase (endo-PG) to digest the polysaccharide which, owing to the enzyme specifity, renders the subsequent digest pattern methyl-ester-sequence dependent. Here, the separation, detection, and quantification of partially methylated oligogalacturonide digest fragments play a key role in the elucidation of the fine-structure of pectin. Initial progress was made in this area primarily using anion exchange chromatography and mass spectrometry,[4,49,50] and more recently electrophoretic methods have also been reported as additional tools in pectic oligosaccharide analysis. These include the use of gel electrophoresis (PACE) coupled with fluorescent labeling,[51−53] and indeed capillary electrophoresis (CE), which has been used in conjunction with the end labeling of digest fragments or even, very recently, hyphenated with mass spectrometry.[54] In addition, CE has also been used simply employing UV detection of the unadulterated oligomers, which can be separated without labeling by exploiting the breaking of the scaling symmetry between charge and size at low DPs alluded to above.[38,40]

While, to date, the intra- and intermolecular distributions of methylesterfication have been treated rather independently, the inherent connection between these distributions is the focus of this work as described above. Herein, numerical simulations have been used to investigate the effects of a nonrandom demethylesterification mechanism (that of pPME) on the intermolecular DM distribution of homogalacturonans, with the goal of generating an ensemble of chains that possess the experimentally measured intermolecular DM distribution. (Figure 1b). Such an ensemble can subsequently be used to reconstruct concomitant intramolecular sequences and, if required, derivative single-number parametrizations of the sample set, such as average blocklength.

Experimental Section

Samples. *Homogalacturonans.* HGs were isolated from commercial citrus pectin (ref 1400 Danisco, Copenhagen, DK). Citrus pectin was deesterified at pH 12, 17 h at 4 °C, precipitated with 3 vol of ethanol, treated by solvent exchange (ethanol 70%, ethanol 95%, acetone) and dried in a ventilated oven at 40 °C. De-esterified pectin was hydrolyzed by 0.1 M HCl at 80 °C for 72 h in sealed tubes.[55] The acid-insoluble fraction, corresponding to HG, was washed twice by 0.1 M HCl then distilled water, and resuspended in distilled water. The pH of the suspension was brought to 7 by tetrabutyl ammonium hydroxyde (TBA), and the resulting solution (HG-TBA) was extensively dialyzed against distilled water before freeze-drying. HG-TBA was dissolved in dimethyl sulfoxide and methyl iodide was added (HG-TBA/CH_3I, 1/1, mol/mol).[56] The solution was kept in the dark under continuous gentle stirring for 24 h. Residual CH_3I was removed through a nitrogen flux (1 h at room temperature). The solution was dialyzed twice against 0.2 M NaCl and then extensively with distilled water before freeze-drying.

Table 1. Chemical and Macromolecular Characteristics of the HG Samples Used in This Study

	HG-B69	HG-B40	HG-B20	HG-P76	HG-P64	HG-P56	HG-P36
GalA (mol %)	>99	>99	>99	>99	>99	>99	>99
Rha (mol %)	<1	<1	<1	<1	<1	<1	<1
DM	69	40	20	76	64	56	36
M_w	15230	15720	16560	19040	18760	21520	19610
DP_w	82	87	93	102	101	117	108
I (M_w/M_n)	1.19	1.18	1.19	1.21	1.19	1.18	1.18
Ca activity	0.65	0.43	0.23	0.23	0.14	0.12	0.08

Random Demethylesterification. Three highly methylesterified HG samples were redissolved in deionized water and their solutions kept under gentle stirring at 4 °C while adequate amounts of 0.2 M NaOH where added to yield samples of known DM values, HGB69, HGB40, and HGB20, respectively,[48] as given in Table 1.

pPME Demethylesterification. Aliquots of the recovered highly methylated HG were de-esterified by pPME (Sigma 5400; 194 U/mg) to yield a series of pPME-de-esterified HG samples of varying DM. Highly methylesterified HG aliquots (4× 1 g) were dissolved in phosphate buffer 100 mM pH 7.6 (91 mL) for 30 min at 30 °C. pPME (1.82 mL at 1 mg/mL) was added to two of the aliquots and solutions were kept under gentle stirring at 30 °C for 25 and 70 min to yield HGP76 and HGP64, respectively. pPME (1.82 mL at 5 mg/mL) was added to the two other aliquots and solutions were kept under gentle stirring at 30 °C for 40 and 120 min to yield HGP56 and HGP36, respectively. At the end of the reaction, solutions were cooled in an ice bath and the pH was brought to 5 with 2 N phosphoric acid. Solutions were then extensively dialyzed, the pH brought to 7 with NaOH, and solutions were finally freeze-dried.

The characteristics of the final HG samples were analyzed as described below and are given in Table 1.

Endo PG II Digests. Endo-PG II (EC 3.2.1.15) from *Aspergillus niger* was prepared as described previously.[57] Digests were carried out by incubating 1.0 mL of the substrate, at a total concentration of 0.5%, and pH 4.2, with 20 μL of the enzyme solution, that was in turn generated by diluting 25 μL of a 7.5 mg mL^{-1} protein stock into 2.0 mL of 50 mM acetate buffer at pH 4.2. All experiments were carried out at (30 ± 1) °C by keeping the digest mixture in a water bath. After 24 h, the enzyme was denatured by rapid heating to 95 °C, and the concentrations of the various oligomeric species liberated recorded using CE as described in due course.

Analytical Methods. GalA contents were determined by the automated *m*-hydroxybiphenyl[58] method. Individual neutral sugars were analyzed as their alditol acetate derivatives[59] by GLC after hydrolysis with 2 M tifluoroacetic acid at 121 °C for 2.5 h. DM was determined by titrimetry.[60]

High Performance Size-Exclusion Chromatography Combined with Multiple-Angle Laser Light-Scattering Detection. HP-SEC was performed at room temperature on a system comprised of one Shodex OH SB-G precolumn followed by two columns in series (Shodex OH-Pack SB-804 HQ and OH-Pack SB-805 HQ, Shodex, Showa Denko KK, Miniato, Japan). Samples were eluted with 0.05 M $NaNO_3$ buffer containing 0.02% NaN_3 as preservative at a constant flow rate of 42 mL/h. A refractometer (RI; ERC 7517A) and a multiple-angle laser light-scattering device (MALLS; Mini Dawn, Wyatt, Santa Barbara, CA) operating at three angles (41, 90, and 138°) were used as detectors. Samples were solubilized in 0.05 M $NaNO_3$ buffer containing 0.02% NaN_3, left overnight with tail-overhead continuous mixing, heated at

40 °C for 15 min, cooled, and filtered (PVDF filter 13 mm diameter, 0.45 μm pore size; Whatman Inc., Sandford, ME) prior to analysis. Samples were injected automatically through a 50 μL loop. Data for molar mass determinations were analyzed using Astra 1.4 software (Wyatt, Santa Barbara, CA), taking d*n*/d*c* 0.146. Unsmoothed M_w and M_n values were obtained from raw data.

Calcium Activity. Calcium activity coefficients at neutralization point were determined in triplicate by means of a dual-wavelength spectrophotometric method using TMMX as an activity probe for calcium ions.[61]

Capillary Electrophoresis. Experiments carried out in this work used an automated CE system (HP 3D), equipped with a diode array detector. Electrophoresis was carried out in a fused silica capillary of internal diameter 50 μm and a total length of 46.5 cm (40 cm from inlet to detector). The capillary incorporated an extended light-path detection window (150 μm) and was thermostatically controlled at 25 °C, although in reality it is possible that the temperature in the capillary during electrophoresis could exceed this value by several degrees.[62] Phosphate buffer at pH 7.0 was used as a CE background electrolyte (BGE) and was prepared by mixing 0.2 M Na_2HPO_4 and 0.2 M NaH_2PO_4 in appropriate ratios and subsequently reducing the ionic strength to 50 or 90 mM. At pH 7.0, the unmethylated GalA residues are fully charged, and while the polysaccharides studied here are susceptible to base-catalyzed β-elimination above pH 4.5, no problems were encountered during the CE runs of some 20 min at room temperature. All new capillaries were conditioned by rinsing for 30 min with 1 M NaOH, 30 min with a 0.1 M NaOH solution, 15 min with water, and 30 min with BGE. Between runs, the capillary was washed for 2 min with 1 M NaOH, 2 min with 0.1 M NaOH, 1 min with water, and 2 min with BGE. Detection was carried out using UV absorbance at 191 nm with a bandwidth of 2 nm. Samples were loaded hydrodynamically (various injection times at 5000 Pa, typically giving injection volumes of the order of 10 nL) and, typically, electrophoresed across a potential difference of 20 kV. All experiments were carried out at normal polarity (inlet anodic) unless otherwise stated. Electrophoretic mobilities, μ, are related to the migration times of the injected samples relative to a neutral marker, t and t_0, respectively, by the equation

$$\mu = \mu_{obs} - \mu_{eo} = (lL/V)(1/t - 1/t_0)$$

where L is the total length of the capillary, l is the distance from the inlet to detector, V is the applied voltage, μ_{obs} is the observed mobility, and μ_{eo} is the mobility of the electroosmotic flow (EOF).[63] In this work, the EOF is indicated by a dip in the signal resulting from the refractive index change associated with the passage of water from the injection plug past the detection window. Electrophoretic mobilities of the HG were mapped to charge density as described in detail elsewhere.[48]

In endo-PG II digests of HG, mono-, di-, and trigalacturonic acid were identified by comparison with previous results. For their absolute quantification, L-methionine ethylester hydrochloride (obtained from Sigma-Aldrich Corp., U.S.A.) was used as an internal standard, thus, circumventing the need to know the injection volume and protecting against its run-to-run variation. This particular compound was selected because it has an absorbance at the digest detection wavelength, 191 nm, and at pH 7 is predominantly positively charged and, thus, migrates on the opposite site of the neutral marker to the anionic digest itself.[40]

Numerical Simulations. *General Strategy.* A set of 10^4 chains with a chosen DP distribution was modeled in silico, each one as a one-dimensional array of a length such that the ensemble distribution approximated the experimentally determined molecular weight distribution. Methylesterification of the residues was simulated simply by selecting array elements at random from the entire set and labeling the element M rather than G. The process was continued until the required sample average DM of the starting substrate was reached. Subsequently, demethylesterification was simulated either (i) through a random process, relevant to base saponification, or (ii) using an algorithm designed to model pPME action, described in detail below, and at each step the sample averaged DM, intermolecular DM distribution, G-blocklength distribution, and average G-blocklength were calculated.

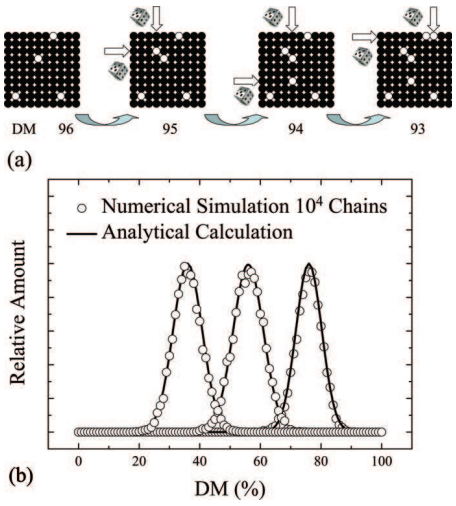

Figure 2. (a) Schematic representation of the operation of the numerical simulation for the random demethylesterification model, for a simplified case of 10 chains, each 10 residues in length. (b) Theoretical intermolecular DM distributions for randomly demethylesterfied homogalacturonans (76, 56, and 36% DM) obtained from both numerical simulation and analytical theory.

First, to gain confidence in the written code and examine the level of noise expected, the numerical simulation was used to generate a set of chains of DP = 100 and number-average DM 96% as a model of the homogalacturonan starting material. From this starting point, three sample sets were randomly demethylesterified until sample average DM values of 76, 56, and 36% were reached, respectively. Figure 2a shows a schematic representation of the operation of the numerical simulation for this model, for a simplified case of 10 chains, each 10 residues in length. These simulations of random demethylesterification processes were undertaken so that an analytical theory was also available for comparison. The particular DM values were selected as they were the values of three of the pPME demethylesterified samples, to be described in due course, and are additionally well spaced throughout the DM range. As expected, the agreement between the numerical simulation (symbols) and the analytical calculation (lines) for all three DM values (Figure 2b) can be seen to be excellent, validating the basic algorithms used and confirming that the number of chains used is sufficient to simulate meaningful distributions.

In light of its importance both in vivo and for functionality control in vitro, pPME action was selected as the mechanism to model in order to generate substrates with highly nonrandom demethylesterification patterns. Specifically, an attempt was made to model pPME action in the numerical simulation so that predicted intermolecular DM distributions could be compared with those measured experimentally.

pPME Model. In the pPME model, the array elements, representing the polymer residues, are attacked at random, with the attack point representing subsite +1 of the PME. It is well established that a charged residue is required at subsite −1 for a successful substrate binding, that is, the enzyme docks at a GM interface.[64] If such an interface is found, then the M-labeled array element is relabeled G with a probability P_{GM} (the optimum value of which will be argued to be ~1) representing demethylesterification of that residue by the enzyme. To capture the processive behavior of the enzyme, if the subsequent array element is also M, again it is relabeled G with probability P_{GM} and so on toward the reducing end of the chain. In the event that after an M → G transition the next residue (array element) encountered is already unmethylesterified, then the enzyme can detach from the chain with probability P_{MG} (the optimum value of which will be argued to be ~0.4) or with probability $(1 - P_{MG})$ the residue is skipped over and the enzyme continues to move along the chain (the elements of the same

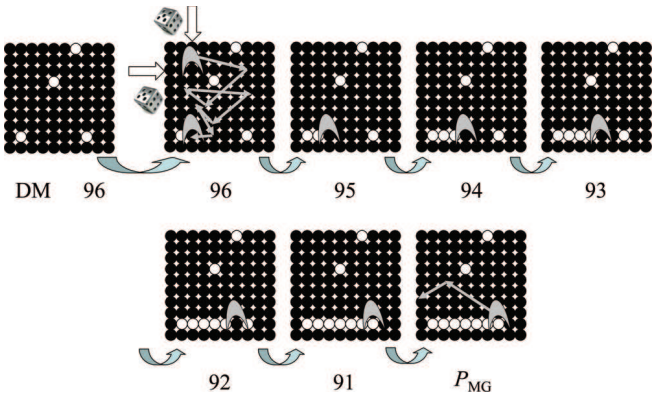

Figure 3. Schematic representation of the operation of the pPME model shown for a simplified case of 10 chains, each 10 residues in length. The "enzyme" can be seen diffusing around until finding a binding site, traveling along a number of methylesterified residues, demethylesterifying them, then detaching from the chain upon encountering an unmethylesterifed residue, and finally continuing to walk around the 2-D (residue label × chain label) array.

1-D array continue to be interrogated) and so on. This is akin to assigning some significance to the identity of the sugar residue at enzyme subsite +2 during the encounter, which seems reasonable. While careful X-ray studies of *Erwina chrysanthemi* PME bound to specific oliogalacturonides suggest that the group of amino acids forming this subsite can comfortably accommodate methylesters,[64] the way in which the binding energy of the site to a galacturonic acid residue depends upon its methylesterification or otherwise is presently unknown. These studies[64] also suggested that subsite +3 of the enzyme may play a more crucial role than +2, with a charged sugar residue at this site favoring detachment of the enzyme. Slightly more elaborate enzyme models that incorporate such information can easily be constructed within the framework described here and are the subject of current work. Such extensions are likely to be particularly useful in investigating cases in which pPME action is initiated on randomly demethylesterifed starting substrates of lower DM, which will hence exhibit a larger variety of tetra- or pentameric sugar residue sequences within the binding cleft length. The substrates described here, however, being of very high initial DM, exhibit a limited number of possible residue patterns extending the length of the binding cleft and, thus, are largely insensitive to such further elaborations.

When a single enzyme−substrate interaction is over, then further searching of the 2-D (residue label × chain label) array for binding sites continues in two possible modes: (i) simple random selection of another array element or (ii) a pseudodiffusive process in which the "enzyme" describes a random walk over other sites. In the simulations described herein, such a walk is implemented for 1000 steps before another "enzyme" (another randomly selected starting point) takes over, corresponding approximately to the experimental conditions of there being one enzyme for 1000 polysaccharide chains. Figure 3 shows a schematic example of this model for a simplified case of 10 chains, each 10 residues in length. The "enzyme" can be seen finding a binding site, traveling along a number of methylesterified residues, demethylesterifying them as it goes, then detaching from the chain upon encountering an unmethylesterifed residue, and finally continuing to walk around the 2-D (residue label × chain label) array.

Results and Discussion

Intermolecular Distributions. *Randomly Demethylesterified Monodisperse Homogalacturonans.* Figure 4a shows the measured (symbols) and the calculated (lines) intermolecular DM distributions for two randomly demethylesterified HG substrates of sample average DMs given by 40 and 69%. A good match of the experimental data with the results of the

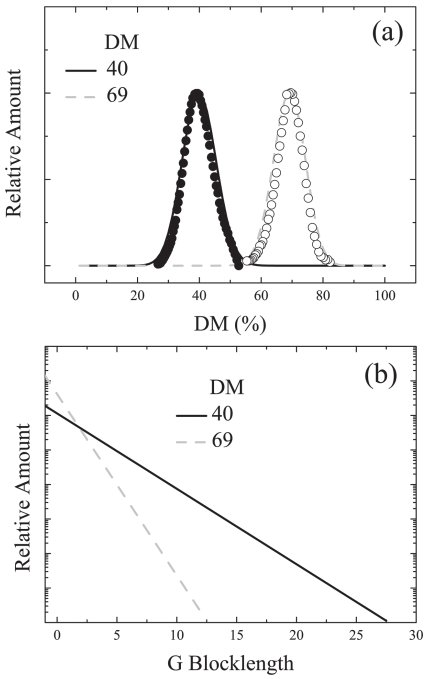

Figure 4. (a) Measured (symbols) and calculated (lines) intermolecular DM distributions for randomly demethylesterified substrates and (b) the relative number of occurrences of particular lengths of contiguous unmethylesterified GalA that are consistent with (a).

calculation can be seen, which is based on the binomial theorem. This implies that the demethylesterification was indeed a random process and, thus, that the statistical properties of the intramolecular distributions are also given by the same random model, enabling the full G-blocklength distribution (the relative number of occurrences of particular lengths of contiguous unmethylesterified GalA) to be calculated (Figure 4b). That is to say that the theoretical ensemble of chains that have the intermolecular DM distribution shown in Figure 4a also has the G blocklength distribution given in Figure 4b. In some sense then, while the distribution shown in Figure 4b is the result of a calculation, it has been indirectly "measured" by insisting that its accompanying intermolecular distribution be consistent with its direct measurement by experiment.

The properties of chemically demethylesterifed samples are particularly amenable to analytical calculation, being the result of a random process, and thereby permit a simple demonstration of the central principle of this work: matching intermolecular DM distributions can yield a model from which blocklength distributions can be calculated.[48] However, the matter of nonrandom distributions generated by intrinsically nonrandom mechanisms has not previously been examined. To address this problem, numerical simulations of demethylesterification have been developed.

pPME Demethylesterified Monodisperse Homogalacturonans. Figure 5a shows the experimentally determined DM distributions obtained from four pPME-modified homogalacturonans that were demethylesterified from a 96% DM sample to sample average DMs of 76, 64, 56, and 36, as described in the Experimental Section. A number of things are immediately apparent. First, as expected, the distributions are clearly not Gaussian functions centered on the sample-averaged DM values, as would be expected for a random demethylesterification[48] (compare with the results of such a model displayed in Figure 2b). Second, all the samples still contain a significant proportion of chains that have a DM close to that of the starting material.

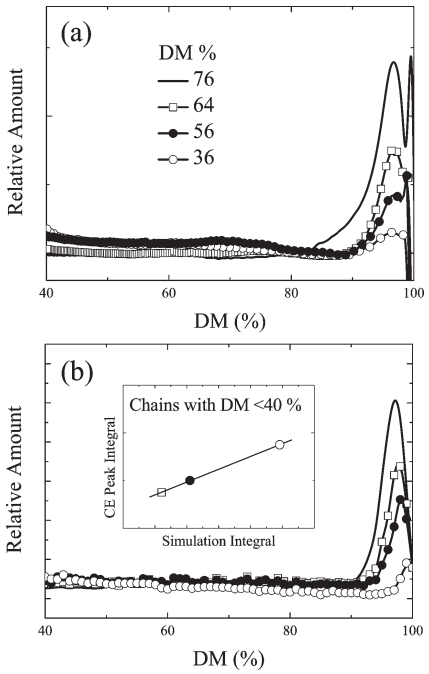

Figure 5. (a) Experimentally determined DM distributions of four pPME-modified homogalacturonans produced as described in the Experimental Section, and (b) the results of numerical simulations carried out as described in the text with $P_{GM} = 1$ and $P_{MG} = 0.4$. (Inset) Integrated area of the experimental distribution below 40% compared with the number of chains found below this limit in the simulation results.

Third, there also appears to be a very broad distribution of chains with other DMs. It should be noted that the "spikes" seen at DM 100% in the experimental data are artifacts originating from the passage of the neutral marker past the detection window. Clearly, ignoring the DM distribution among chains and imagining that each polysaccharide in these modified samples has the same DM as the sample average would be a serious misrepresentation of the samples.

Figure 5b shows the results of numerical simulations carried out as described above with $P_{GM} = 1$ and $P_{MG} = 0.4$. It can be seen that there is a reasonable agreement between the predicted and the measured DM distributions above DM 40%. As discussed previously[48] for DM values below ~35%, it becomes progressively more difficult for CE to resolve the DM distribution with confidence, owing to a counterion-condensation-like phenomenon. Nevertheless, it is possible to integrate the area of the distribution below this limit and compare it with the number of chains found below this limit in the simulation results (Figure 5b, inset). The linear relationship so obtained suggests that the model also captures the intermolecular distributions for DMs below 40% reasonably well.

Figure 6a displays the concomitant intramolecular distributions of the ensemble of chains possessing the measured intermolecular distribution in the form of a G blocklength distribution. It should be noted again that the simulation yields full unmethylesterfied GalA-blocklength distributions for the sample under study, which is of pivotal importance if detailed predictive structure−function models for such things as calcium binding are to be created and tested. The distribution of small blocklengths (<4) simply reflects the presence of blocks that were in the original high DM substrate. Indeed, fitting the slope of the plot in this region yields a probability for the addition of an extra G to the contiguous block, which corresponds to that found for a random substrate with a DM of (96.8 ± 1.0)%. Subsequently, for all samples examined here, all other possible

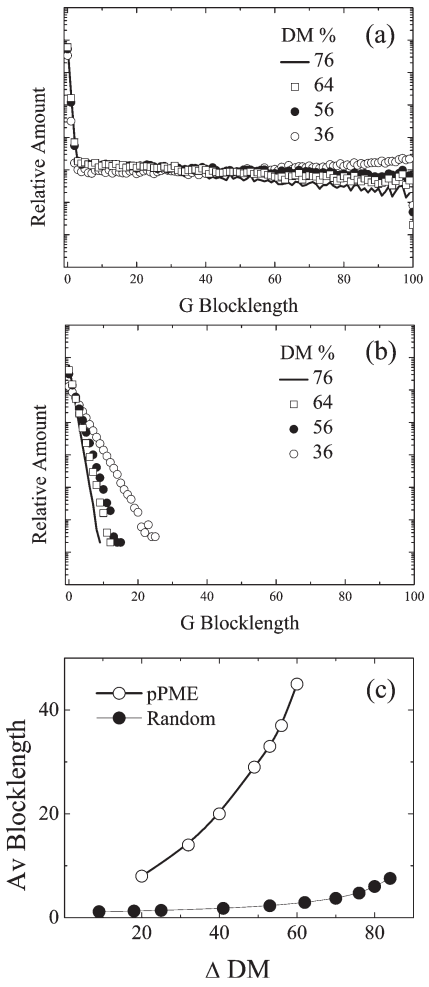

Figure 6. (a) Information on the intramolecular sequences of the pPME-generated fine-structures, represented as G-blocklength distributions, from the chain ensembles, consistent with the experimentally determined intermolecular DM distributions. (b) Full G-blocklength distributions for substrates randomly demethylesterified to sample-averaged DM values equivalent to those of the pPME-generated samples. (c) Number-average blocklengths generated via the application of the pPME or random demethylesterification algorithms described in the text. Symbols show the results of the simulations for both chain ensembles corresponding to the experimental samples and some further points calculated for other DM values. The solid lines are simply a guide to the eye.

blocklengths (100 > BL > 5) are represented to some degree. The full G-blocklength distributions for substrates randomly demethylesterified to equivalent sample-averaged DM values are also shown for comparison in Figure 6b, and it can at once be seen that, in contrast, this mechanism fails to generate any chains with a G-blocklength >25, even at the smallest DMs. With the full G-blocklength distribution in hand, a simple number-average blocklength generated by the two demethylesterification models exemplified in Figure 6a,b is shown in Figure 6c, plotted against the reduction in the sample-average DM achieved. It should be noted that these average blocklengths are not equivalent to those that would be estimated assuming processivity but neglecting the intermolecular distribution (i.e., where the blocklength would be the change in DM).

It can be seen from Figure 6c that to obtain the same average G-blocklength obtained by reducing the DM of a high DM substrate with pPME by only 20%, instead using random demethylesterification, around an 80% reduction in DM would be required. This fact, revealed by the modeling employed, is in good agreement with the results of calcium

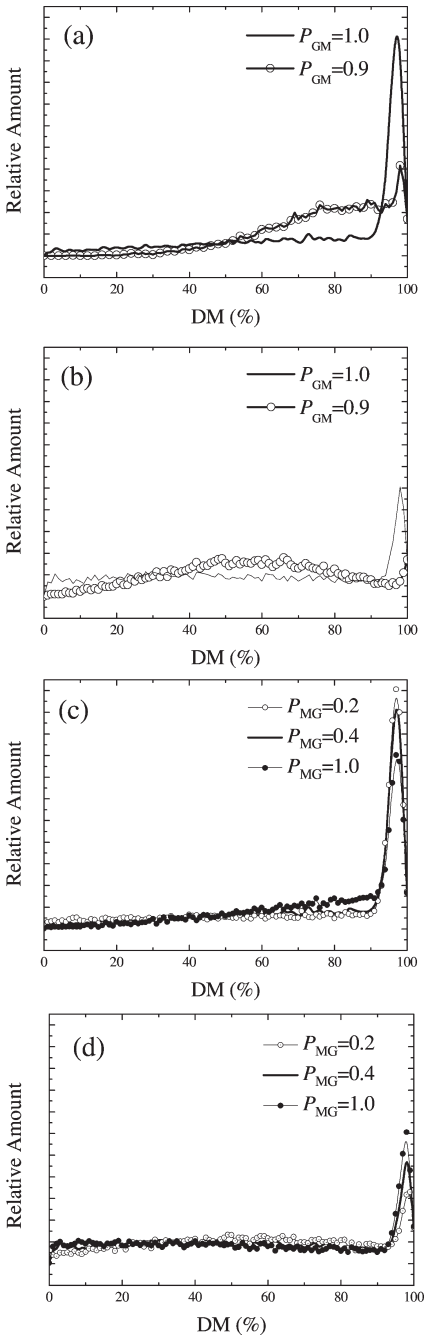

Figure 7. Results of numerical simulations of intermolecular DM distributions for pPME-modified homogalacturonans of (a and c) 76% and (b and d) 56% DM, carried out as described in the text illustrating the sensitivity of the results to variations in P_{GM} (a and b) and P_{MG} (c and d).

activity measurements that were undertaken, which showed that, with base saponification, only by reducing the high DM starting substrate right down to 20% sample-average DM could a calcium-binding response akin to that found with any of the pPME-processed samples examined herein be obtained (see Table 1). It should be noted, however, that for the extremely broad intermolecular DM distributions seen here for the pPME processed samples a single average value is of questionable significance.

Figure 7 addresses the sensitivity of the model to the parameters used to generate the data shown in Figure 5. It is clear from Figure 7a and b, in particular, in the simulation of the 76% DM substrate, that even reducing P_{GM} from 1.0 to 0.9 results in a considerably less satisfactory agreement with the

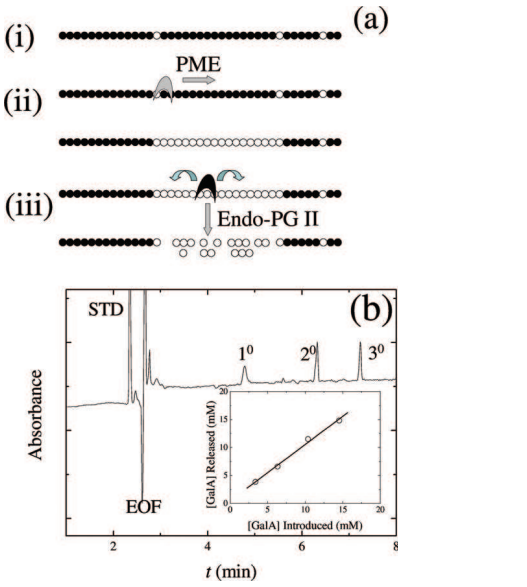

Figure 8. (a) Schematic model illustrating how the pectin structures are modified by the attendant enzymes used in the current work, showing (i) the starting substrate, (ii) the pPME modified substrate, and (iii) the polygalacturonse digest of the substrate shown in (ii). (b) Electropherogram obtained from an endo-PG II digest of a pPME generated homogalacturonan sample (corresponding to a (iii)), carried out as described in the Experimental Section. Mono-, di-, and trigalacturonic acid, the electro-osmotic flow, and the standard L-methionine ethylester hydrochloride are labeled (1°, 2°, 3°, EOF, and STD, respectively). (Inset) Amount of GalA introduced by the pPME, known from the decrease in average DM, against the amount released by endo-PG II.

measured intermolecular distribution, While Figure 7c and d show that it is more difficult to distinguish the value of P_{MG}, 0.4 is reasonable (and indeed is supported by preliminary work on pectin substrates to be reported elsewhere).

Polygalacturonase Digests of the pPME-Generated Monodisperse Homogalacturonans. The intramolecular sequences of methylesterification predicted by the model described here, which successfully reproduces the measured intermolecular distributions, have implications for the endo-PG digests of these substrates. According to the model predictions, all of the pPME introduced G-blocks should be able to be released by endo-PG II, as illustrated in Figure 8a. Figure 8b shows the electropherogram obtained from the endo-PG II digest of the pPME generated 76% HG sample, carried out as described in the Experimental Section. As expected, the digest results in the release of mono-(1°), di-(2°), and tri-(3°) galacturonic acid.[40]

In this experiment a quantitative assessment of the GalA released by the endo-PG II was carried out using an internal standard, as described in the Experimental Section and previously.[40] The inset in Figure 8b shows the results of plotting the amount of GalA introduced by the pPME, known from the decrease in average DM, against the amount of GalA endo-PG II released. There can be seen to be close quantitative agreement between the results, as predicted (a simple linear regression analysis yields a slope of 1.00 ± 0.06 (R = 0.997)).

Conclusions

Regarding the General Strategy. For samples of heteropolysaccharides that do not possess multiple chains of identical sequence, the presence of an intermolecular distribution can nevertheless be used as an advantage, allowing information

about the intramolecular sequences to be obtained without the need for directly examining them.

Regarding HG and pPME. For pectic substrates that possess random intramolecular patterns of methylesterification experimentally extracted intermolecular distributions agree well with the predictions of calculations based on the binomial theorem, indeed demonstrating the close connection between the inter- and intramolecular distributions of the methylesterification of pectin samples. Herein, the case of highly nonrandom, but nevertheless extremely biologically relevant, sequences of methylesterification are considered, as generated by the enzyme pPME. A numerical simulation of enzyme's action is able to reasonably reproduce measured intermolecular distributions of pPME-generated homogalacturonan fine-structures. The fact that the pPME model works reasonably well and essentially only directly considers a small number of subsites suggests that the simple docking of the GM interface is the dominant feature of the interaction. It is hoped that future work might shed light on the two probabilities used in the model in terms of binding energies of the enzyme to different oligosaccharide motifs. Furthermore, the ensemble of chains emerging from the simulation is reasonably unique and allows the prediction of the full Gal-A blocklength distributions for these substrates. It has also been shown that endo-PG digests of these pPME-generated substrates are consistent with the implications of the proposed intramolecular sequences.

Acknowledgment. We thank Marie-Jeanne Crépeau for skillful technical assistance in the preparation and characterization of HG samples.

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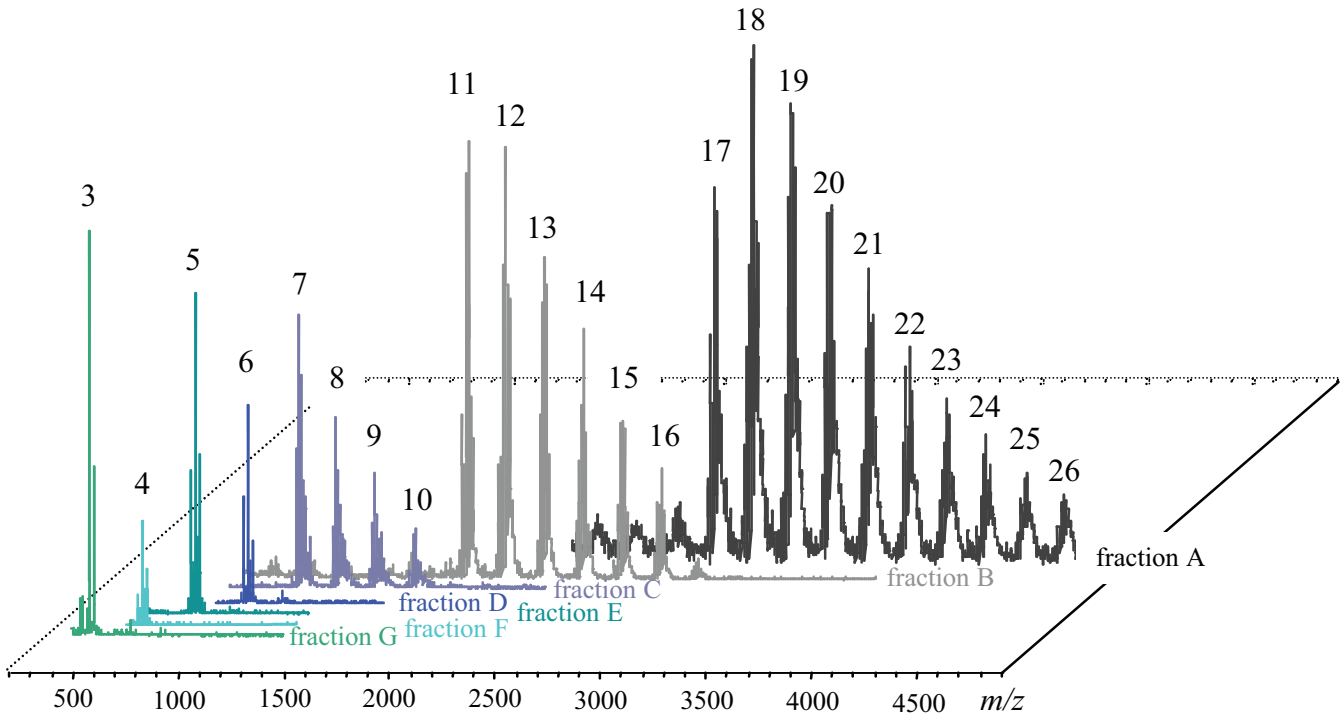
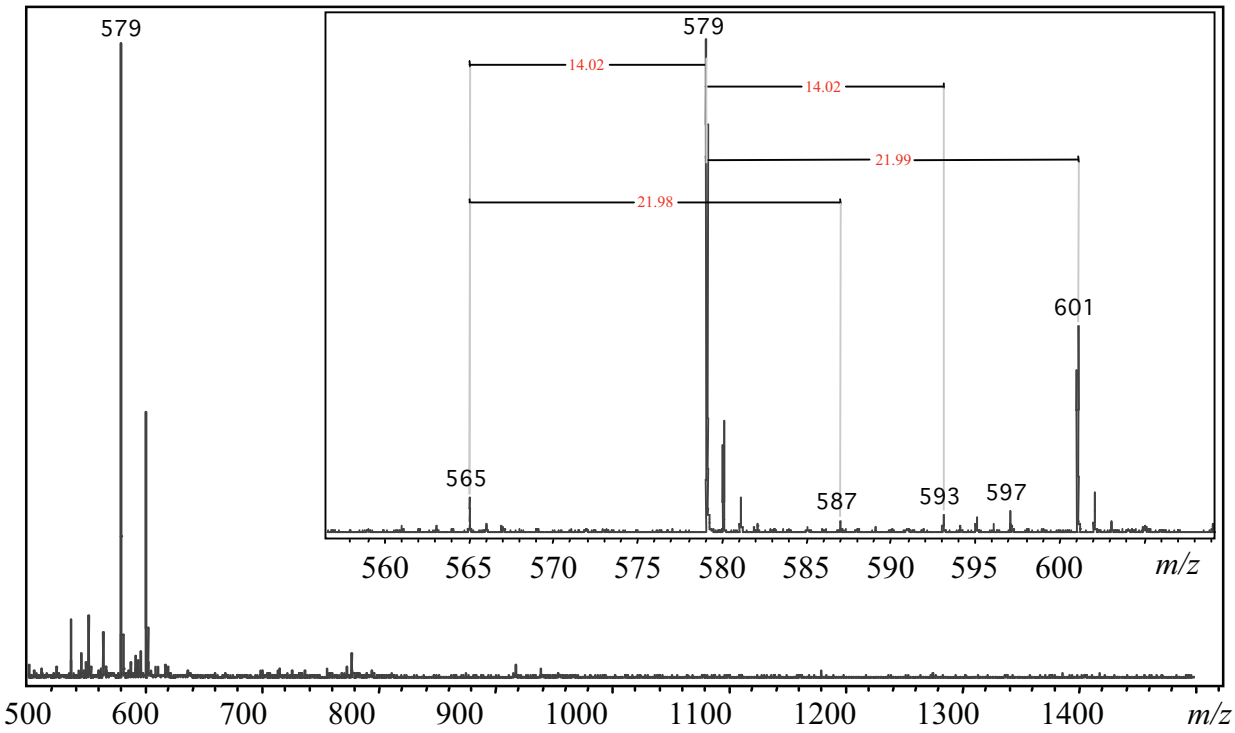
ANNEXE 3: Mass spectra of HG-B69 pectin lyase degradation products after SEC fractionation

Overview of the mass spectra



HG-B69 + PL
Fraction G

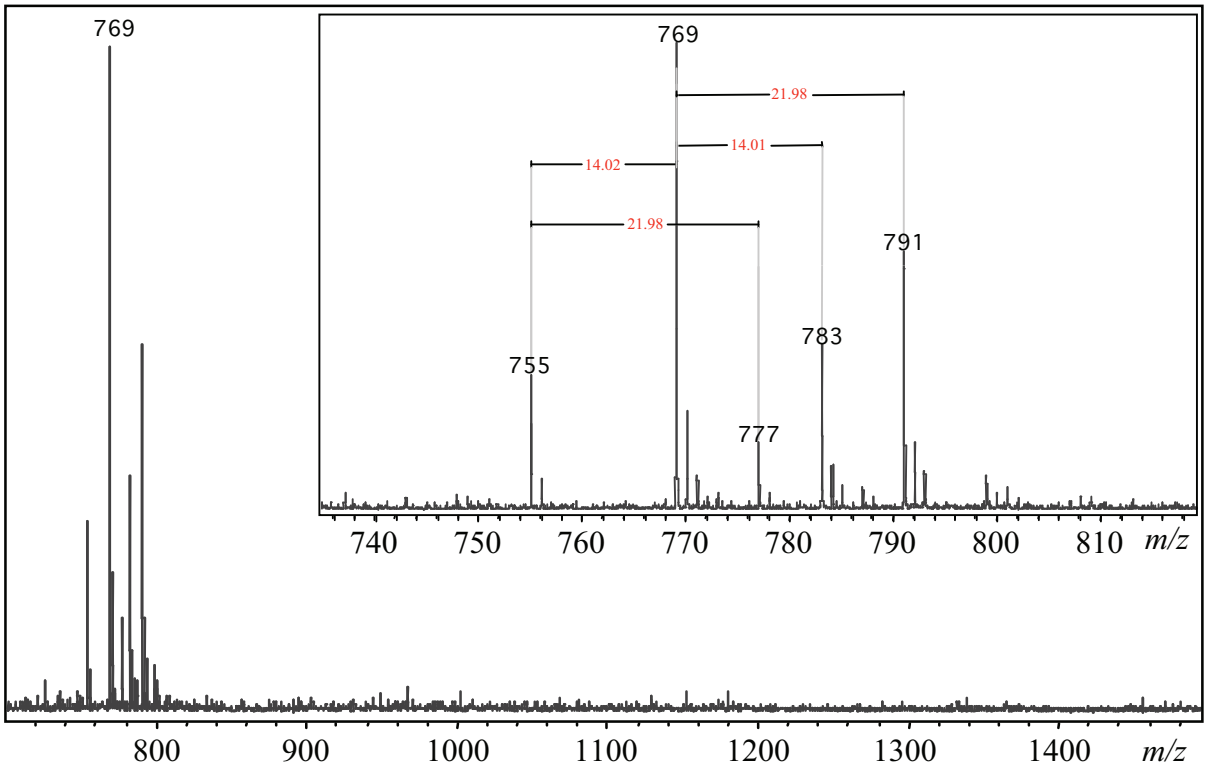
Intensity



m/z	Nature
536,966	contaminants
544,952	contaminants
550,968	matrix
565,056	**[3(1)+Na]+**
579,076	**[3(2)+Na]+**
587,035	[3(1)+(Na-H)+Na]+
593,1	**[3(3)+Na]+**
597,084	[3(2)+Na]+ saturated
601,061	[3(2)+(Na-H)+Na]+

HG-B69 + PL
Fraction F

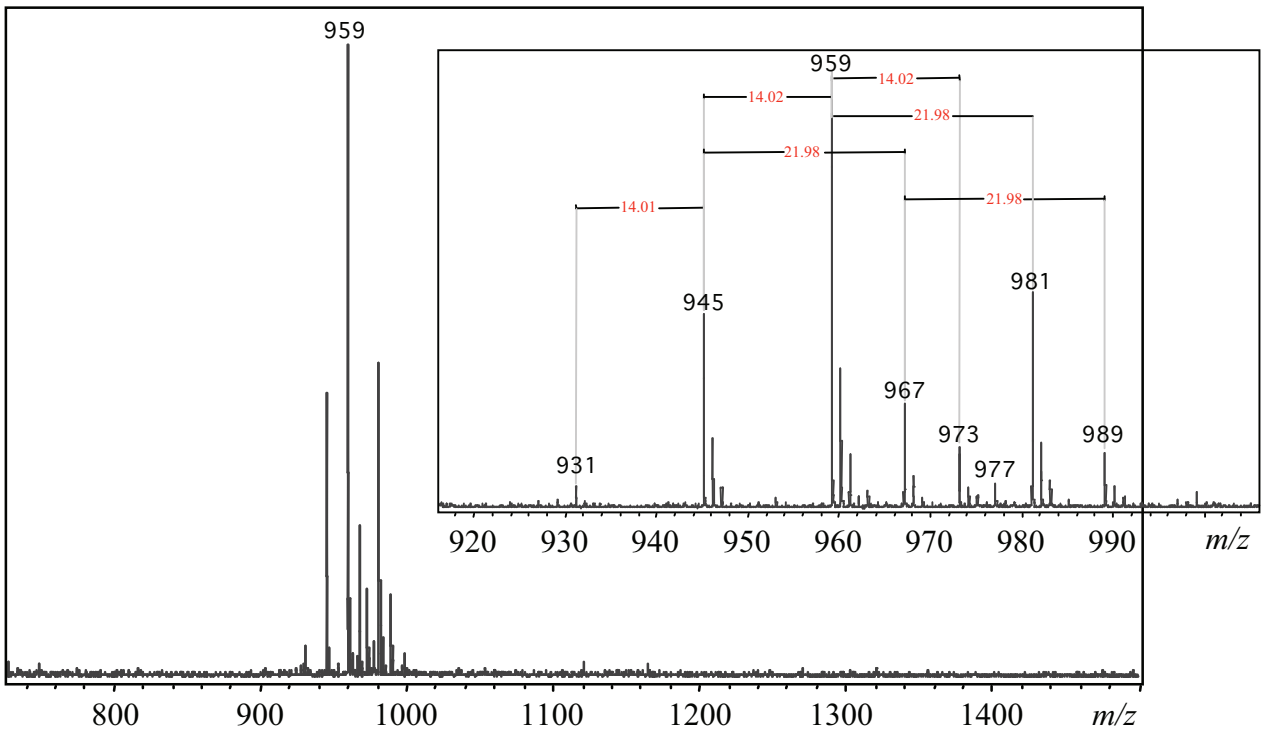
Intensity



m/z	Nature
755,123	**[4(2)+Na]+**
769,144	**[4(3)+Na]+**
777,102	[4(2)+(Na-H)+Na]+
783,157	**[4(4)+Na]+**
791,121	[4(3)+(Na-H)+Na]+

HG-B69 + PL
Fraction E

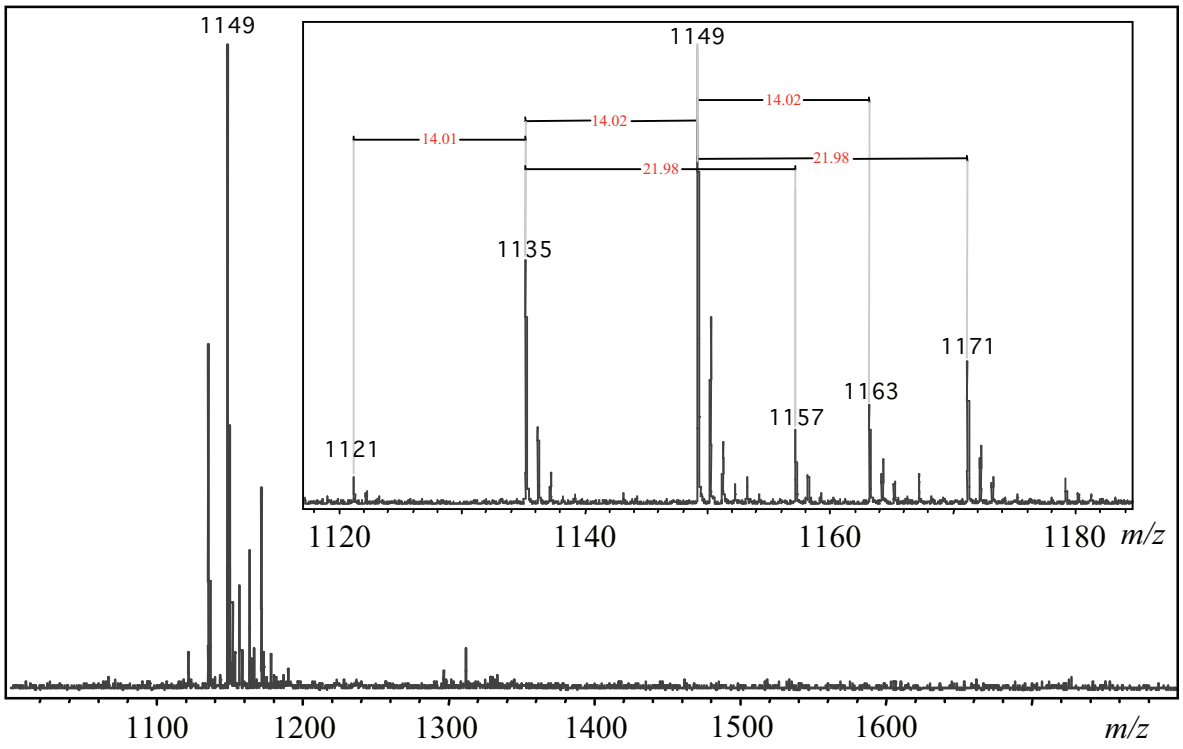
Intensity



m/z	Nature
931,154	**[5(2)+Na]+**
945,167	**[5(3)+Na]+**
959,184	**[5(4)+Na]+**
967,15	[5(3)+(Na-H)+Na]+
973,2	**[5(5)+Na]+**
977,188	[5(4)+Na]+ saturated
981,165	[5(4)+(Na-H)+Na]+
989,134	[5(3)+2(Na-H)+Na]+

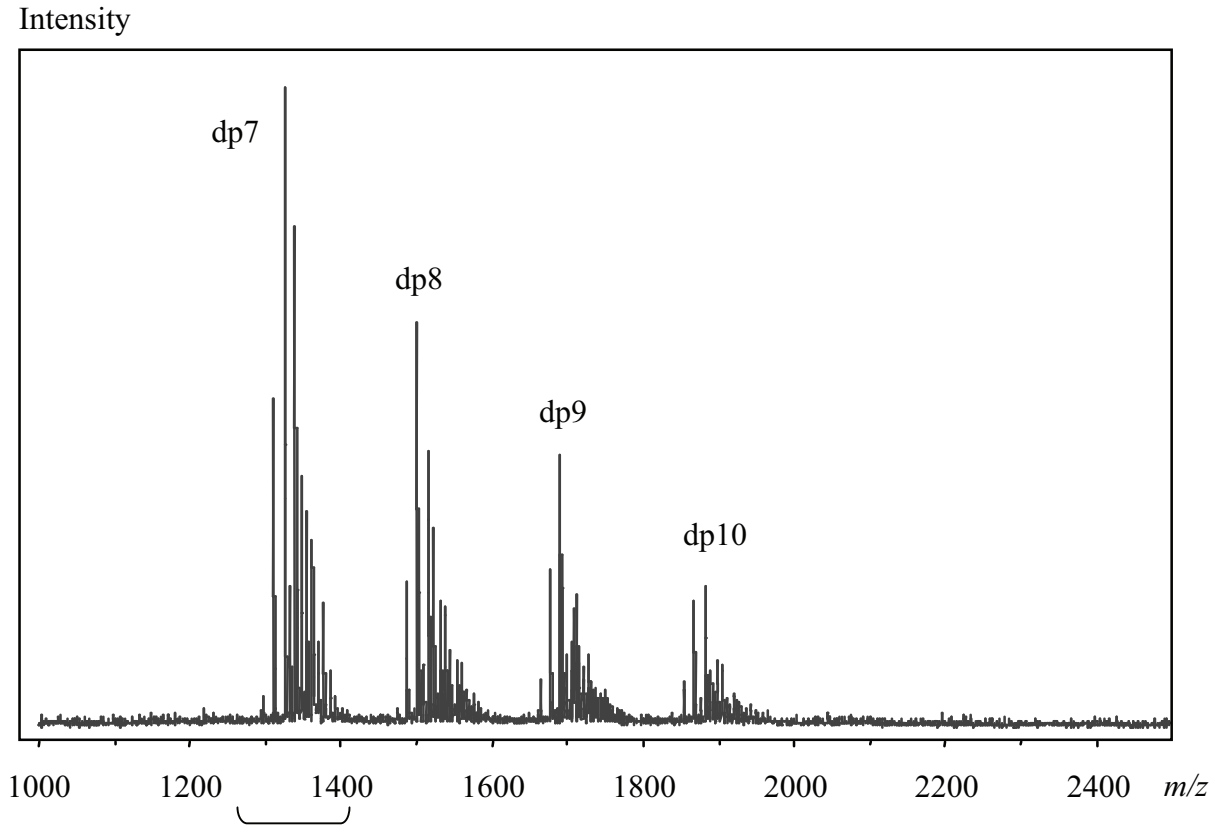
HG-B69 + PL
Fraction D

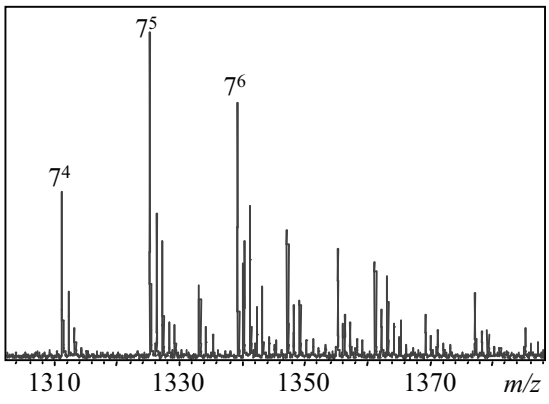
Intensity



m/z	Nature
1121,203	**[6(3)+Na]+**
1135,213	**[6(4)+Na]+**
1149,232	**[6(5)+Na]+**
1157,198	[6(4)+(Na-H)+Na]+
1163,249	**[6(6)+Na]+**
1171,212	[6(5)+(Na-H)+Na]+

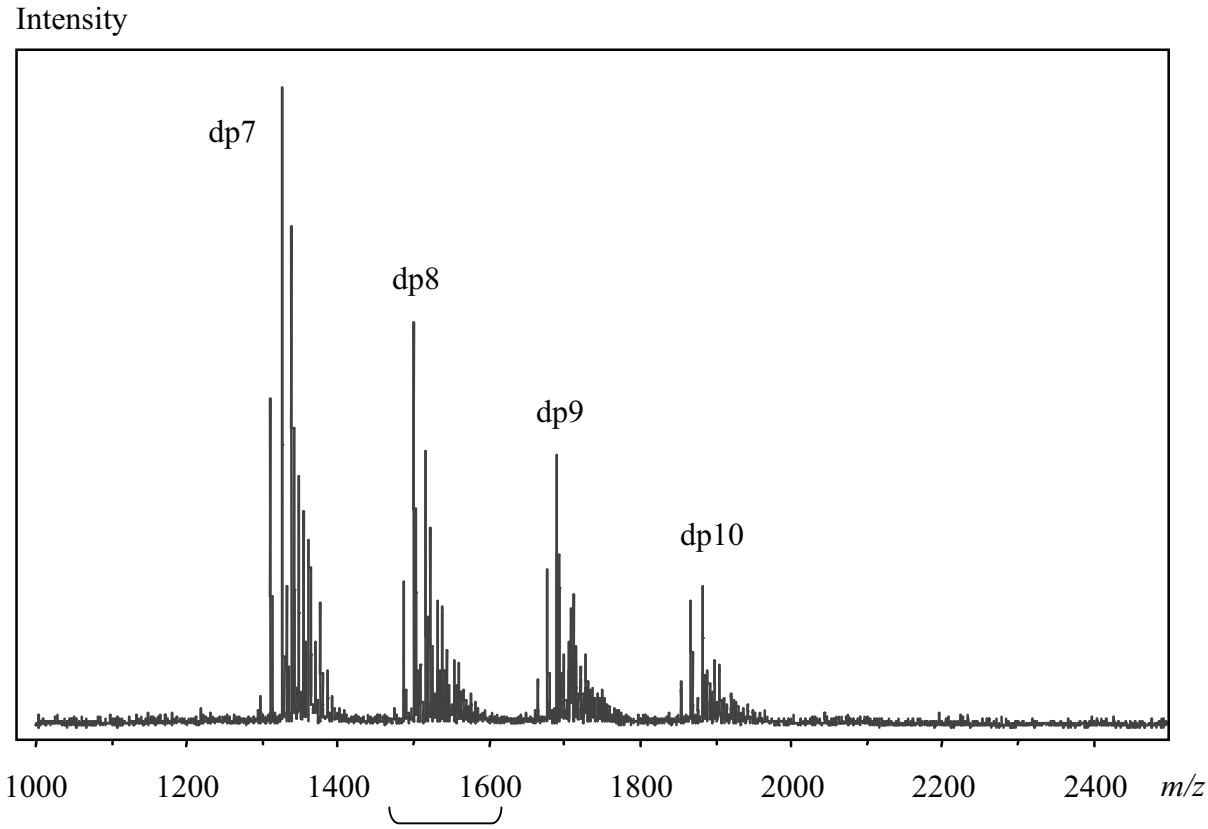
HG-B69 + PL
Fraction C

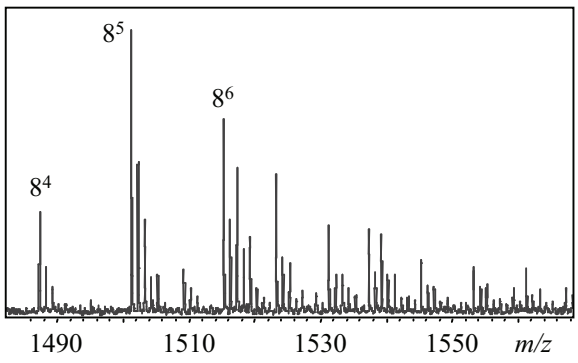




m/z	Nature
1311,246	**[7(4)+Na]+**
1325,261	**[7(5)+Na]+**
1333,231	[7(4)+(Na-H)+Na]+
1339,276	**[7(6)+Na]+**
1341,238	[7(5)+K]+
1347,241	[7(5)+(Na-H)+Na]+
1355,246	[7(4)+2(Na-H)+Na]+
1361,255	[7(6)+(Na-H+Na]+
1363,22	[7(5)+(Na-H)+K]+
1369,224	[7(5)+2(Na-H)+Na]+
1377,236	[7(4)+3(Na-H)+Na]+

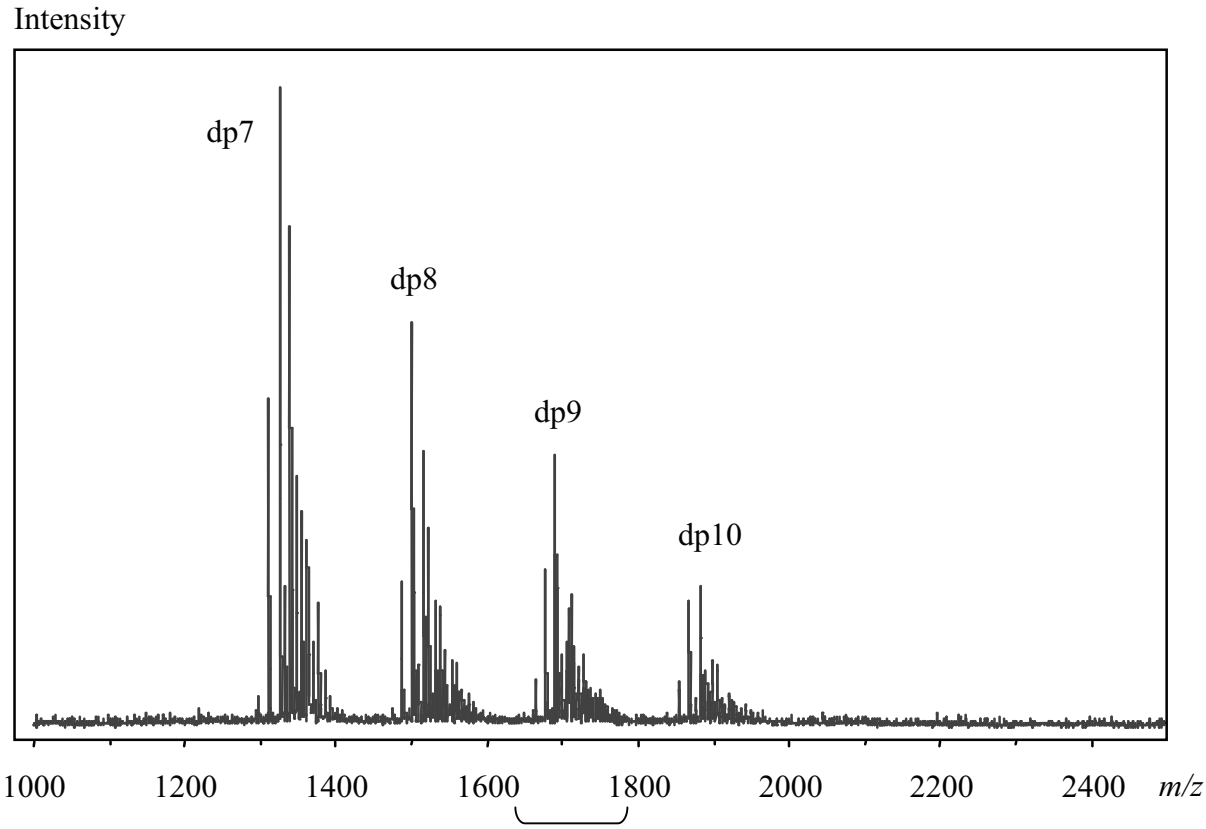
HG-B69 + PL
Fraction C



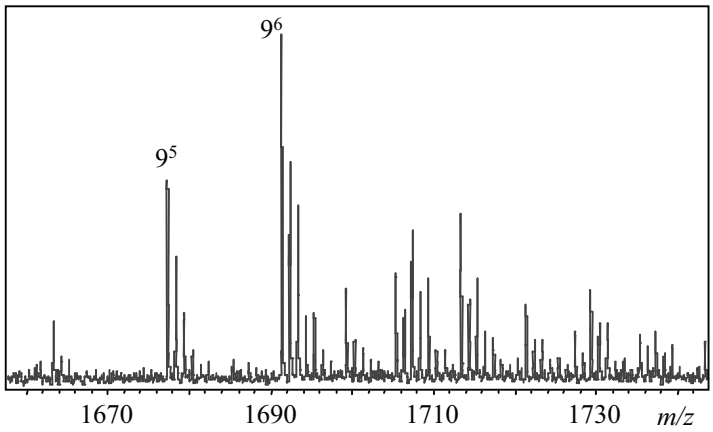


m/z	Nature
1487,277	**[8(4)+Na]+**
1501,302	**[8(5)+Na]+**
1515,313	**[8(6)+Na]+**
1517,273	[8(5)+K]+
1523,284	[8(5)+(Na-H)+Na]+
1531,286	[8(6)+K]+
1537,297	[8(6)+(Na-H)+Na]+

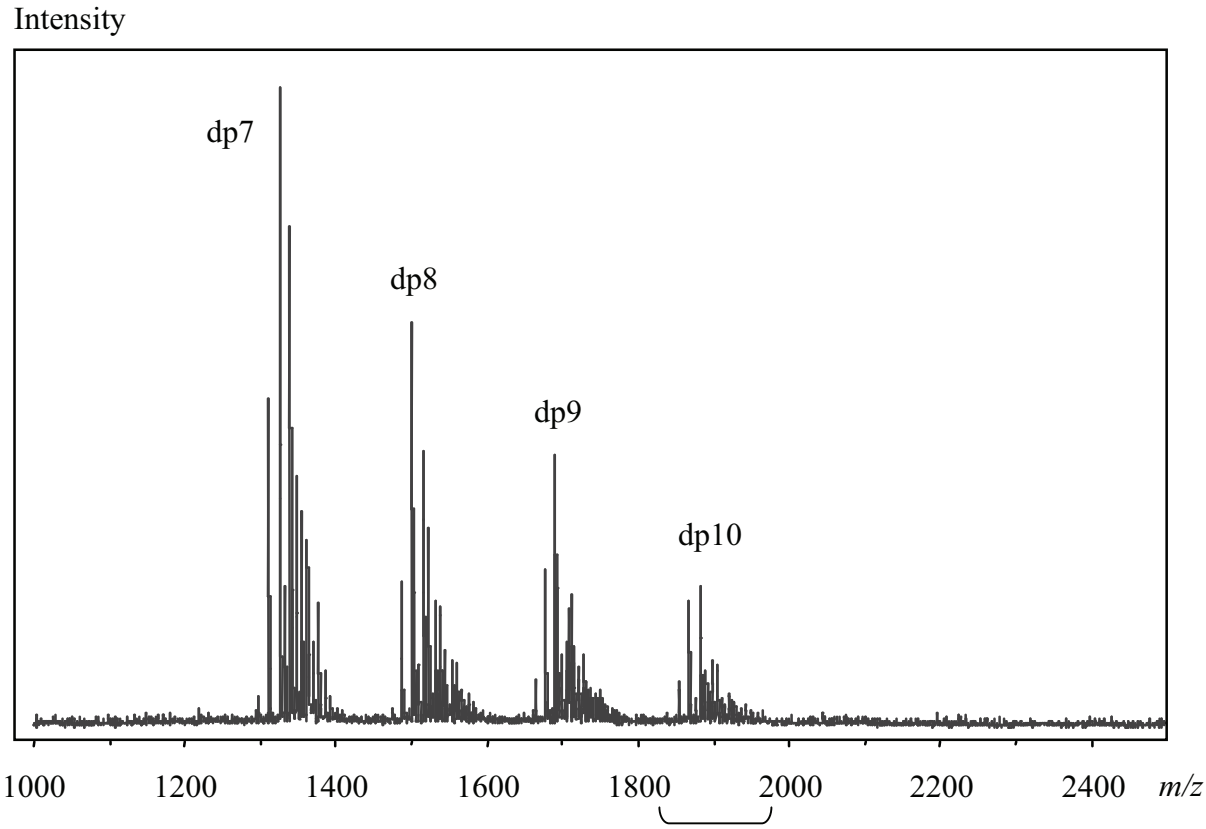
HG-B69 + PL
Fraction C

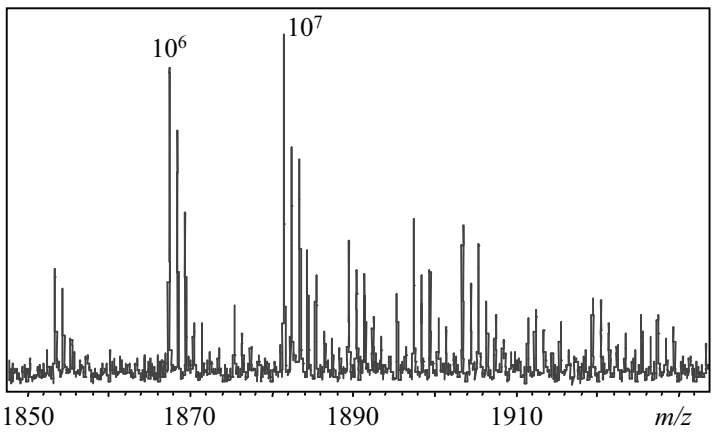


m/z	Nature
1677,353	**[9(5)+Na]+**
1691,373	**[9(6)+Na]+**
1707,353	[9(6)+K]+
1713,353	[9(6)+(Na-H)+Na]+



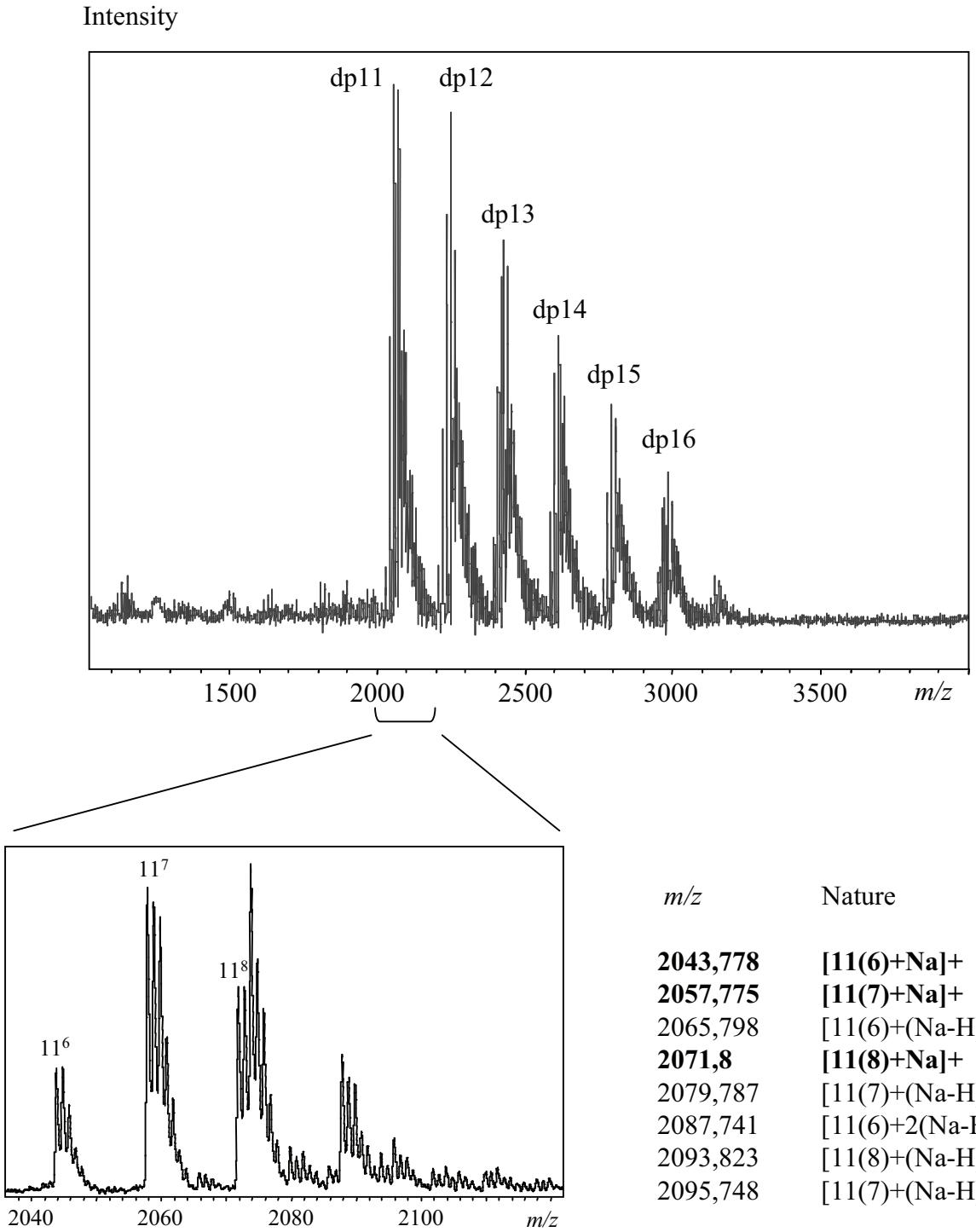
HG-B69 + PL
Fraction C





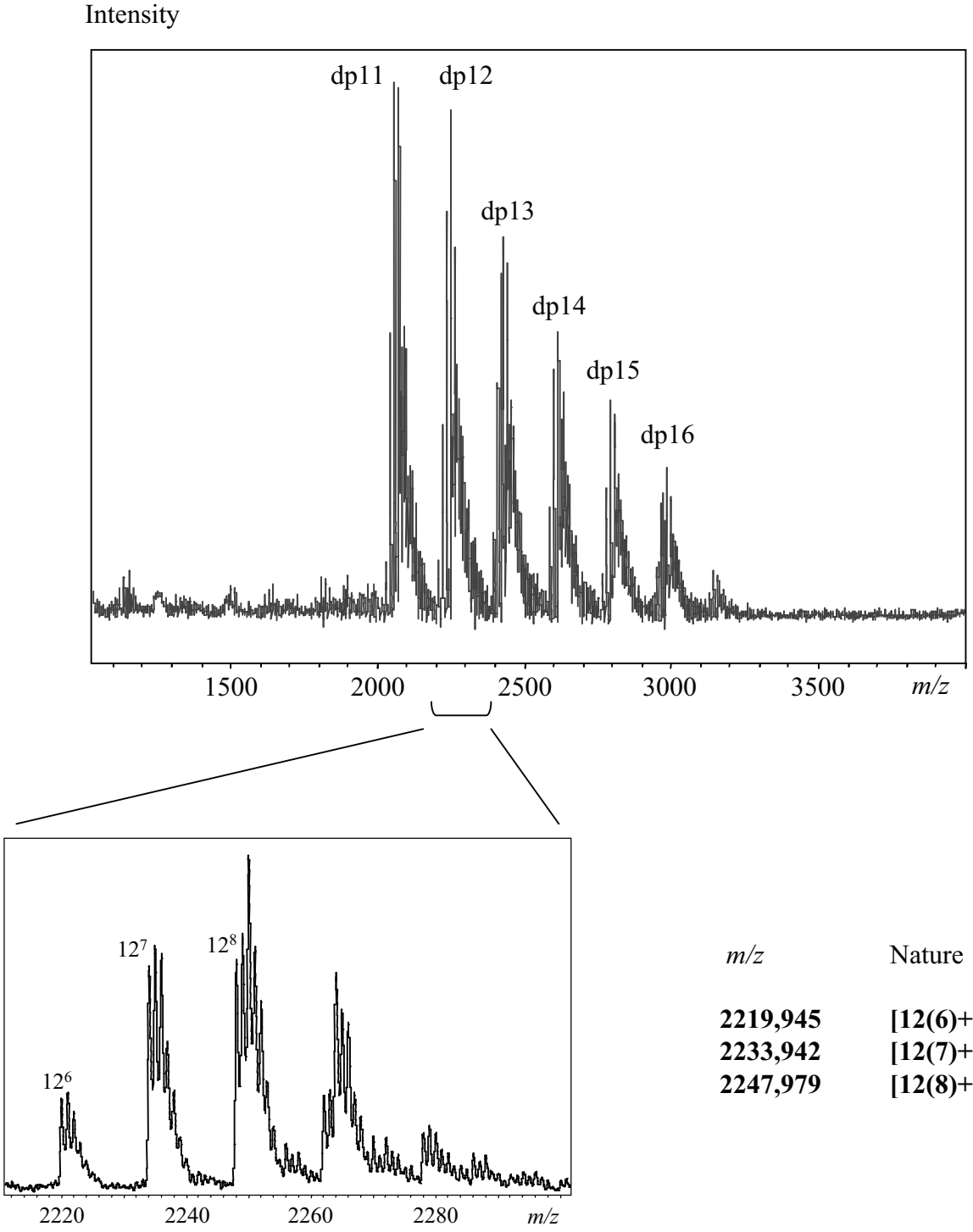
m/z	Nature
1867,442	**[10(6)+Na]+**
1881,46	**[10(7)+Na]+**

HG-B69 + PL
Fraction B



m/z	Nature
2043,778	**[11(6)+Na]+**
2057,775	**[11(7)+Na]+**
2065,798	[11(6)+(Na-H)+Na]+
2071,8	**[11(8)+Na]+**
2079,787	[11(7)+(Na-H)+Na]+
2087,741	[11(6)+2(Na-H)+Na]+
2093,823	[11(8)+(Na-H)+Na]+
2095,748	[11(7)+(Na-H)+K]+

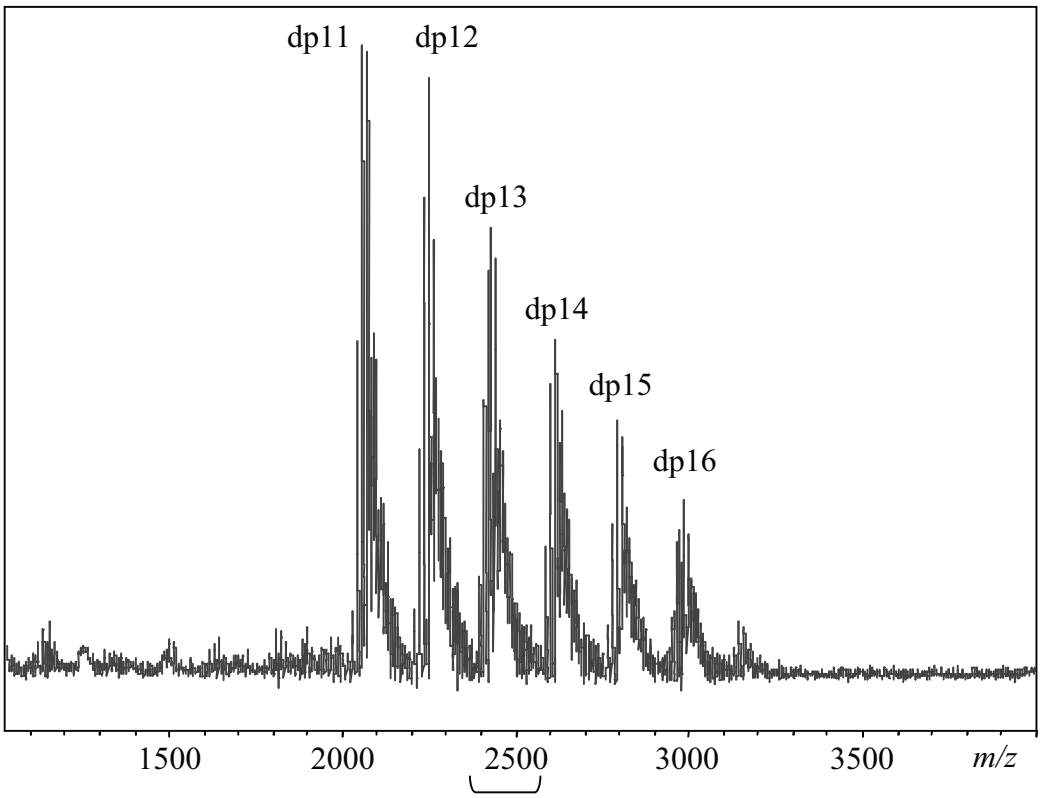
HG-B69 + PL
Fraction B

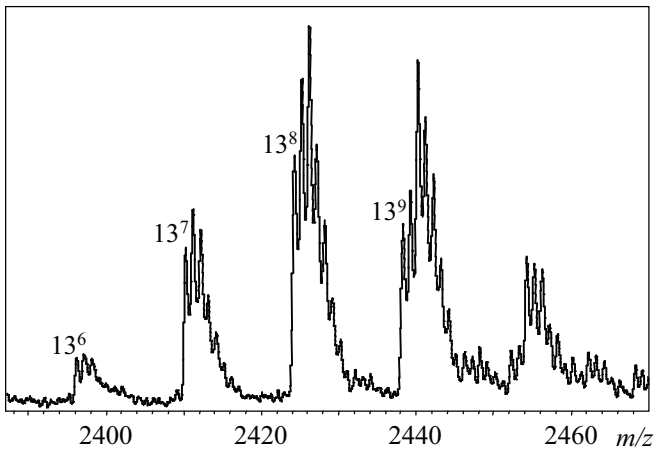


m/z	Nature
2219,945	**[12(6)+Na]+**
2233,942	**[12(7)+Na]+**
2247,979	**[12(8)+Na]+**

HG-B69 + PL
Fraction B

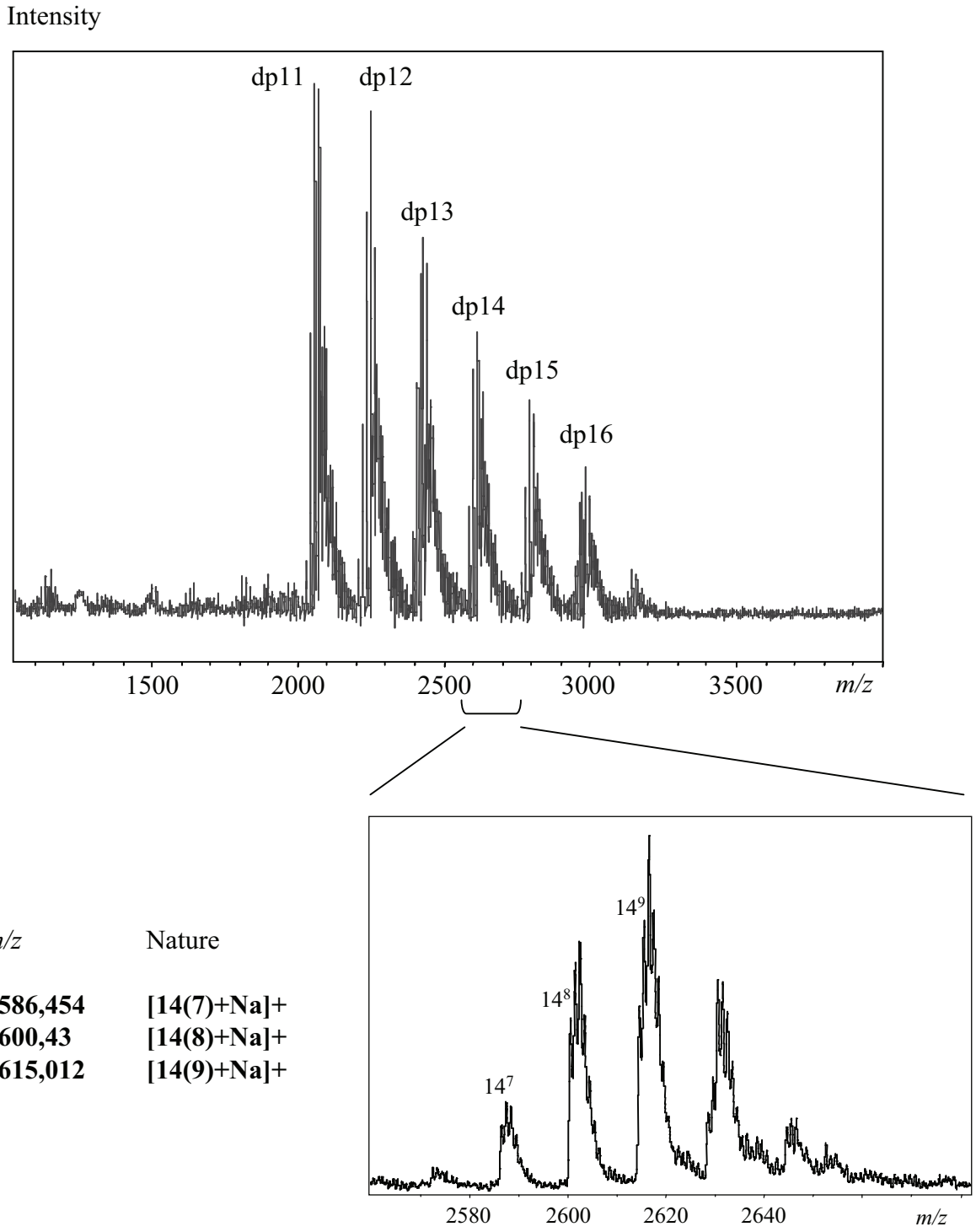
Intensity





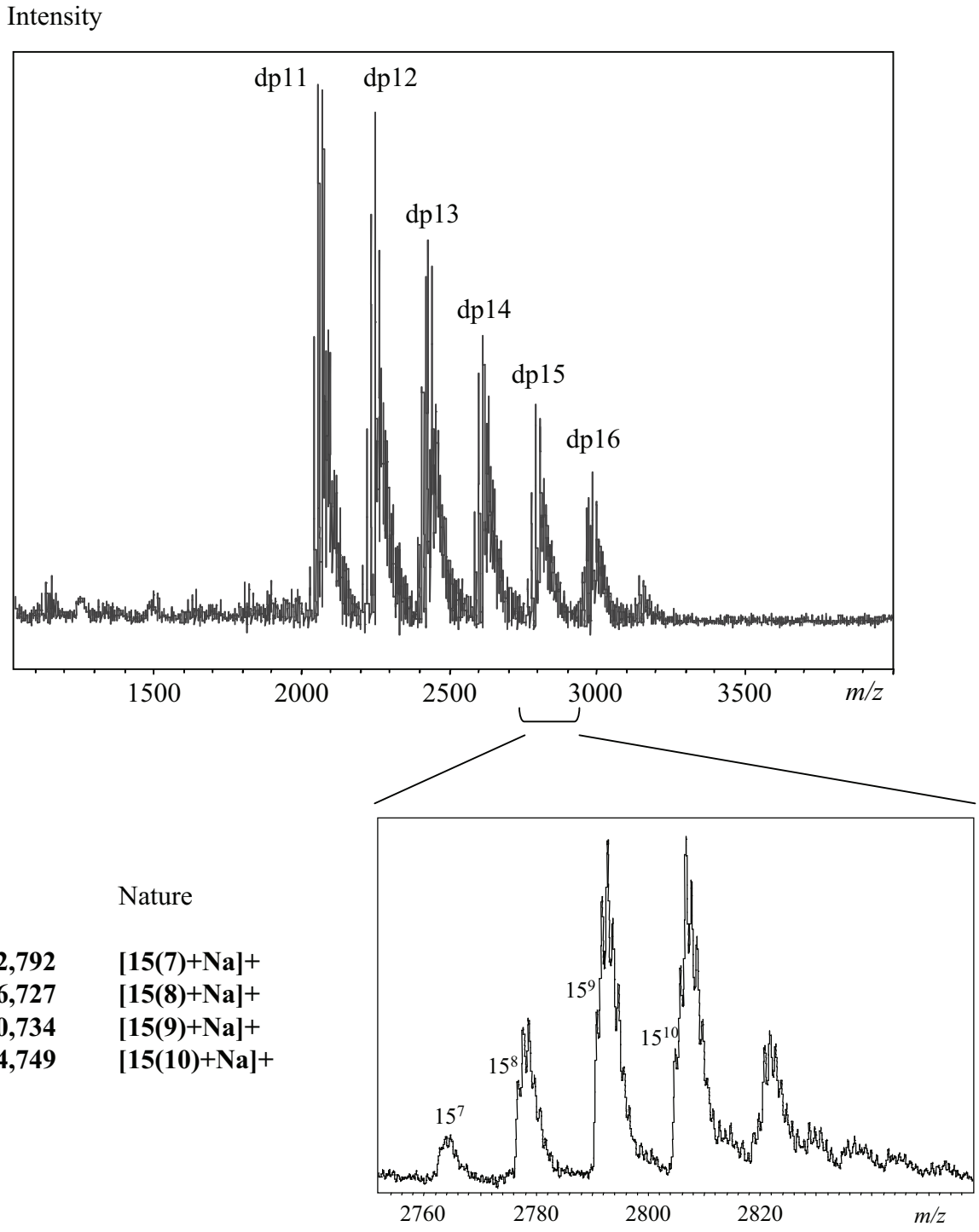
m/z	Nature
2396,167	**[13(6)+Na]+**
2410,177	**[13(7)+Na]+**
2424,138	**[13(8)+Na]+**
2438,679	**[13(9)+Na]+**

HG-B69 + PL
Fraction B



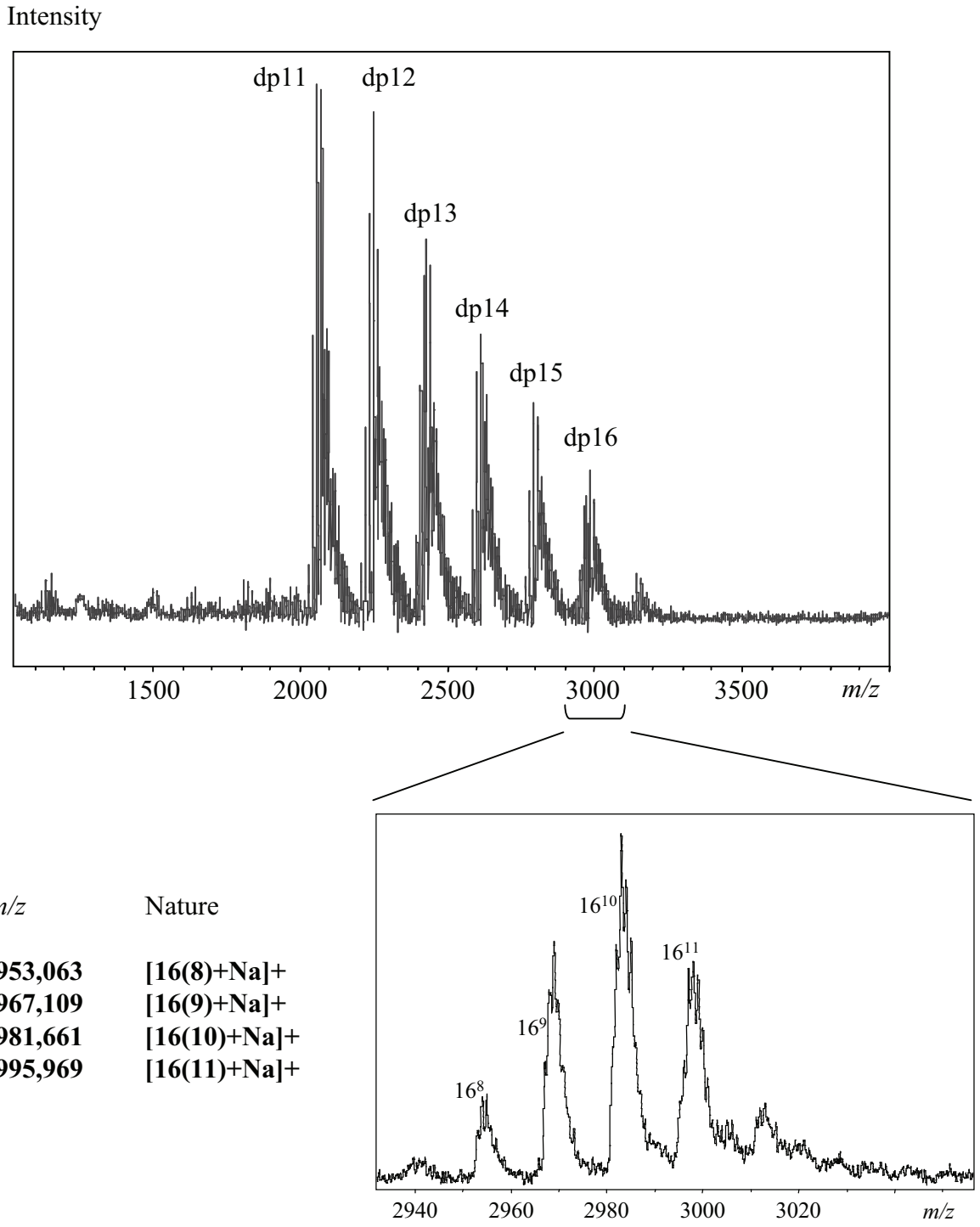
m/z	Nature
2586,454	**[14(7)+Na]+**
2600,43	**[14(8)+Na]+**
2615,012	**[14(9)+Na]+**

HG-B69 + PL
Fraction B



m/z	Nature
2762,792	**[15(7)+Na]+**
2776,727	**[15(8)+Na]+**
2790,734	**[15(9)+Na]+**
2804,749	**[15(10)+Na]+**

HG-B69 + PL
Fraction B



m/z	Nature
2953,063	**[16(8)+Na]+**
2967,109	**[16(9)+Na]+**
2981,661	**[16(10)+Na]+**
2995,969	**[16(11)+Na]+**

HG-B69 + PL
Fraction A

Intensity