Carbohydrate Research 344 (2009) 1798–1807

Contents lists available at ScienceDirect

Carbohydrate Research

journal homepage: www.elsevier.com/locate/carres

Mass spectrometry for pectin structure analysis

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ARTICLE INFO

Article history:
Received 16 July 2008
Received in revised form 27 August 2008
Accepted 29 August 2008
Available online 11 September 2008

Keywords:
Homogalacturonan
Rhamnogalacturonan
Pectic oligosaccharides
Cell wall polysaccharides

ABSTRACT

Pectin are extremely complex biopolymers made up of different structural domains. Enzymatic degradation followed by purification and structural analysis of the degradation products proved to be efficient tools for the understanding of pectin fine structure, including covalent interactions between pectic structural domains or with other cell wall polysaccharides. Due to its high sensitivity, high throughput and capacity to analyze mixtures, mass spectrometry has gained more and more importance as a tool for oligosaccharides structural characterization in the past 10 years. This review will focus on the combined use of mass spectrometry and enzymatic digestion for pectins structural characterization.

1. Introduction

All plant cells are encapsulated in a cell wall, whose most prominent components are polysaccharides: cellulose, hemicelluloses and pectins. These polymers collectively determine cell wall shape and mechanical properties. Pectins, because of their multiple interaction properties, are likely to be key components for the architecture of plant cell walls. Pectin is probably the most complex polysaccharide in nature as it can be composed of 17 different monosaccharides, some of them being esterified by methyl, acetyl or feruloyl groups.[1,2] By degrading cell wall materials or extracted pectins by purified pectolytic enzymes, it clearly appeared that the different monosaccharides were not randomly distributed along the pectin macromolecule but were concentrated within different pectic structural domains.[3–11] Over the years, many pectic structural domains have been described and nowadays pectins are viewed as multiblock co-biopolymers containing the following structural domains: (i) homogalacturonan (HG), (ii) xylogalacturonan (XGA), (iii) rhamnogalacturonan I backbone, encompassing arabinan and arabinogalactan I and II side-chains (RG-I), and (iv) rhamnogalacturonan II (RG-II).[12,13] De Vries (1982)[5] recognized a pattern of 'smooth' HG regions and ramified 'hairy' regions, in which the neutral sugars are located. The amount, fine structure, and maybe length of each domain vary widely with respect to plant origin,[12] between different cell types, at different stages of cellular development, and even within the thickness of a given wall.[14,15] This variability most likely impinges on the physicochemical and hydrodynamic properties of pectins and hence their functionality in the specific cellular context as well as in the food application field.

Pectins being extremely complex multiblock biopolymers, analyses on extracted pectin whole macromolecules are not sufficient to give insight into the pectin fine structure. To reveal its structural characteristics, pectin is commonly degraded into oligosaccharides by chemical and/or enzymatic means. After fractionation of the degradation products, isolated structural elements, which are in the analytical range of a broad set of analytical techniques, can be obtained.[13,16] Although mass spectrometry was the only tool in the very early days of structural analysis, this has been largely replaced by the use of NMR. The main drawbacks of NMR techniques are, however, sample purity and concentration requirements. The high sensitivity, high throughput and capacity to analyze mixtures of the new generation of mass analyzers led to a re-emergence of the use of mass spectrometry for oligosaccharides structural characterization in the past 10 years.[17] In particular, the development of soft ionization techniques like matrix-assisted laser desorption (MALDI) and electrospray (ESI), together with the development of powerful analyzers with MS/MS or MS^n capability, allowed the application of mass spectrometry techniques to the structural characterization of carbohydrates. Among these techniques, derivatization, such as permethylation[18–20] and metal adduction,[21–27] in combination with collision-induced dissociation (CID) allowed determination of the linkage, sequence and branching of oligosaccharides. Structural analysis of underivatized oligosaccharides using negative ion electrospray mass spectrometry was also investigated.[28,29]

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doi:10.1016/j.carres.2008.08.036

This review will focus on the combined use of mass spectrometry and enzymatic digestion for the structural characterization of pectins.

2. Analysis and sequencing of HG-derived oligomers

2.1. Sequencing of partly methylated oligogalacturonates

HG is the simplest and most abundant pectic structural domain. It consists of a linear backbone of (1,4)-linked α-D-galacturonic acid (GalA) residues. The minimum estimated length of this domain is ~100 GalA residues.[30] GalA residues are commonly partly methyl-esterified at C-6 and both the degree of methyl-esterification (DM) and the location of methyl-esterified residues onto HG domains have a profound impact on pectin functional properties.[31] Many studies were thereby devoted to the analysis of partly esterified oligogalacturonates resulting from the digestion of pectins differing in their methyl-esterification pattern by *endo*-polygalacturonases, *exo*-polygalacturonases or pectin lyases. MALDI-time-of-flight (TOF) MS, in conjunction with anion-exchange chromatography was first used.[32–34] This method allowed determination of the number of methyl esters on the different oligogalacturonates of various degrees of polymerization but it was however impossible to distinguish partially methyl-esterified oligogalacturonates isomers, that is, solely differing in methyl ester distribution.

ESI-MS^n for partially methylated oligogalacturonates was revealed to be a powerful technique as it allowed location of methyl-esterified GalA residues in oligomers up to a degree of polymerization of 10.[35] Quadrupole ion traps (IT) possess the attractive feature that multiple steps of ion isolation and fragmentation (MS^n) can be achieved. According to Domon and Costello (1988) nomenclature,[36] four different ion series arise from cleavage of the glycosidic bond, two of which contain the reducing end (Y- and Z-series), and two of which contain the non-reducing end (B- and C-series) (Fig. 1A). Cross-ring fragmentation has been denoted by A_i and X_j, the exact position of the sugar ring cleavage being described by a superscript at the upper left side (Fig. 1B). For saturated oligogalacturonates, B- and Z-ions and C- and Y-ions are isomeric, that is, exhibit exactly the same molar mass. By introduction of an ^{18}O label at the reducing end, the masses of the Y^*- and Z^*-ions increase by 2 Da, compared to the B- and C-ions, respectively, and unambiguous analyses can be performed (Fig. 1A).[22,37,38] ^{18}O labelling is of particular interest as it is very efficient on pectin-derived oligosaccharides and extremely simple to implement.[35,39] Oligogalacturonates fragmentation pattern according to Domon and Costello (1988) nomenclature[36] was established from MS^2,[35] and MS^2 and MS^3 analyses[40] performed in both positive and negative ion modes. The negative ion mode provided the simplest CID spectra. Fragmentation of the singly charged $[M-H]^-$ ions was dominated by C- and Z-ions and a sole specific cross-ring cleavage ion ($^{0,2}A$) (−60 Da), indicative of 1,4 type glycosidic linkage,[41] was observed.[35,40] Quéméner et al. (2003) additionally noticed a loss of methanol (−32 Da) that was always correlated to the presence of a methyl-ester group on the

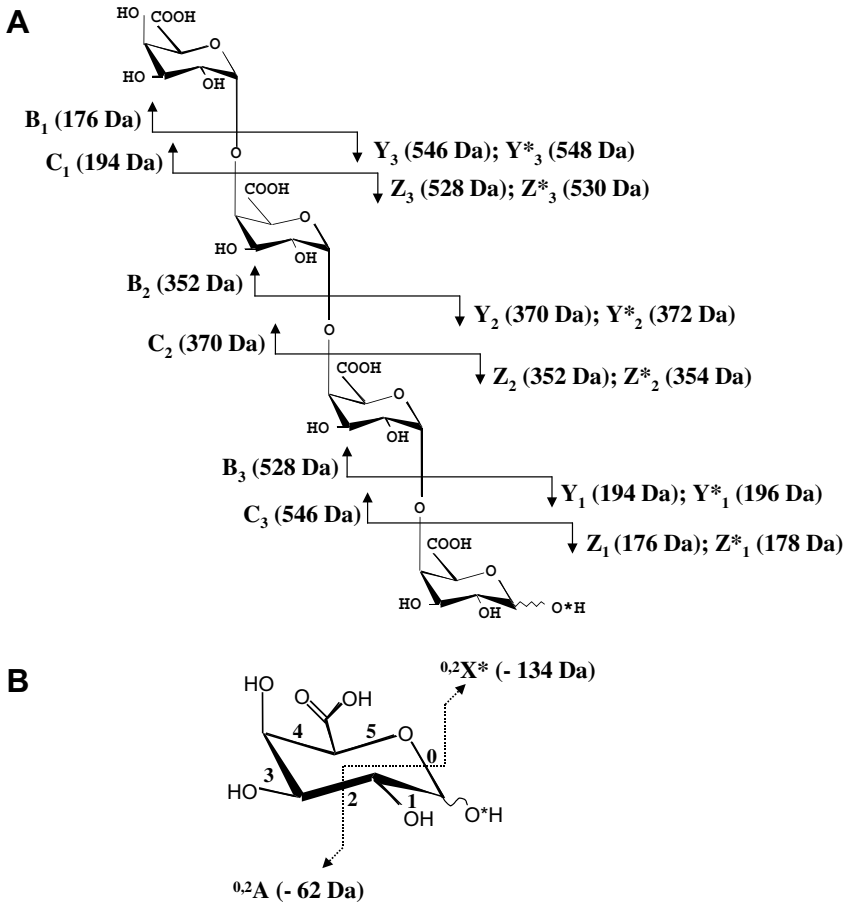

Figure 1. Illustration of the carbohydrate fragmentation nomenclature introduced by Domon and Costello (1988) [36] for a saturated tetramer of galacturonic acid. * is for ^{18}O-labelling of the reducing end. (A) Glycosidic bonds cleavages. The B_i and the C_i fragment ions contain the non-reducing end. The index '*i*' determines the number of glycosidic bonds calculated from the non-reducing end. The Y_j and Z_j fragment ions contain the reducing end. The index '*j*' determines the number of glycosidic bonds calculated from the reducing end. (B) Cross-ring cleavages. Cross-ring fragmentation has been denoted A_i (i.e., contains the non-reducing end) and X_j (i.e., contains the reducing end). The exact position of the sugar ring cleavage is described by superscript at the upper left side.

GalA residue at the reducing end.[40] CID spectra of sodium- and lithium-cationized species acquired in positive mode were more complex with B-, Y- and C-type ions together with the ^{0,2}A-type cross-ring cleavage ion. Potassium- and ammonium-cationized species showed poor or complex fragmentation.[35] Post-source decay (PSD)/MALDI-TOF-MS was also used for the structural analysis of partly methyl-esterified oligogalacturonates.[42] PSD/MALDI-TOF-MS and nano ESI-MS/MS in the negative mode resulted in similar fragmentation patterns.[35,42] Unsaturated partly methyl-esterified oligogalacturonates were also studied and their fragmentation patterns were established.[35,42]

Several groups then applied tandem MS to get precise sequence information on partially methylated oligogalacturonates that was correlated to the specificity of several pectolytic enzymes. The mode of action of purified *endo*-polygalacturonases, *exo*-polygalacturonases, pectin lyases and pectin-methyl esterases was established, and pathogen-related enzyme action was determined.[43–50]

2.2. Sequencing of partly methylated and acetylated oligogalacturonates

In some plant species, HG domains are not only partly methyl-esterified at C-6, but also partly acetyl-esterified at *O*-2 and/or *O*-3. Acetylation of HG domains is well known to strongly alter pectin associative properties.[51–55] As for methyl-esterification, both the degree of acetyl-esterification (DAc) and the location of acetyl-esterified residues onto the HG domains affect pectin functional properties. To get a better understanding of the relationship between the acetylation of pectins and their associative properties, more information about the distribution of acetyl groups onto

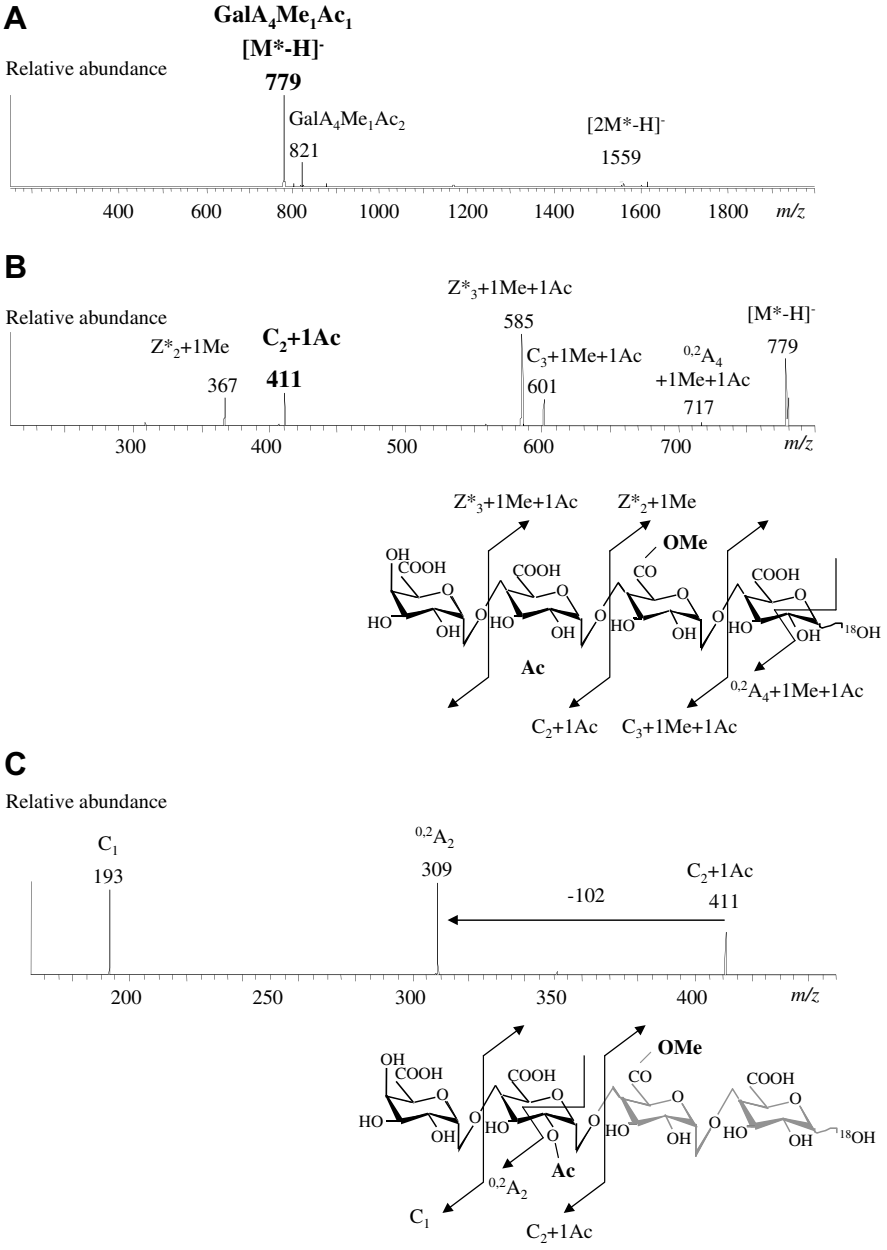

Figure 2. Negative ESI-IT-CID mass spectra of the ^{18}O-labelled monomethylated and monoacetylated tetragalacturonate ($GalA_4Me_1Ac_1$) and observed cleavages at each MS step. (A) Full-scan MS of the fraction of interest recovered by anion-exchange chromatography of sugar beet pectin enzymatic digest. (B) MS^2 experiment (*m/z* 779 > products). C. MS^3 experiment (*m/z* 779 > 411 > products.

HG domains is thereby necessary. In that context, ESI-IT-MS^n was assessed for determining the exact location of acetyl groups on *O*-2 and/or *O*-3 of GalA residues in HG.[56] Sugar beet pectin, which is acetylated onto HG and RG-I domains,[7] was first hydrolyzed by a combination of *endo*-polygalacturonase, pectin-methyl-esterase, *endo*-arabinanase and *endo*-galactanase. The end-products were fractionated by anion-exchange chromatography and several oligogalacturonates fractions were recovered.[56] The two major fractions were identified as monomethylated and monoacetylated trigalacturonate ($GalA_3Me_1Ac_1$) and monomethylated and monoacetylated tetragalacturonate ($GalA_4Me_1Ac_1$) on the basis of biochemical and high-performance anion-exchange chromatography analyses. The MS^n spectra of the ^{18}O-labelled fractions were then recorded. The full MS spectra confirmed the previous identification with major singly charged $[M^*-H]^-$ ions at *m/z* 779 and 603 for $GalA_4Me_1Ac_1$ and $GalA_3Me_1Ac_1$, respectively, (Figs. 2A and 3A). Other singly charged ions at *m/z* 821 ($GalA_4Me_1Ac_2$), 645 ($GalA_3Me_1Ac_2$) and 561 ($GalA_3Me_1$) were also identified together with some cluster $[2M^*-H]^-$ ions. After isolation and CID of the $[M^*-H]^-$ precursor ions, complementary ions of the C- and Z-series were observed (Figs. 2B and 3B), which allowed the following structures to be proposed: (GalA–AcGalA–MeGalA–GalA) and (AcGalA–MeGalA–GalA) for $GalA_4Me_1Ac_1$ and $GalA_3Me_1Ac_1$, respectively. The CID spectra also revealed the presence of specific cross-ring cleavage ions of the ^{0,2}A-series corresponding to the loss of labelled $C_2H_4O^{18}O$ fragments (−62 Da). The ions of the C-series, after sequential trapping and CID, also lose only the $C_2H_4O_2$ species (−60 Da), indicative of 1,4 type glycosidic linkage.[41] The assessment of the precise location of the acetyl groups onto GalA residues was based on these specific cross-ring cleavages. The

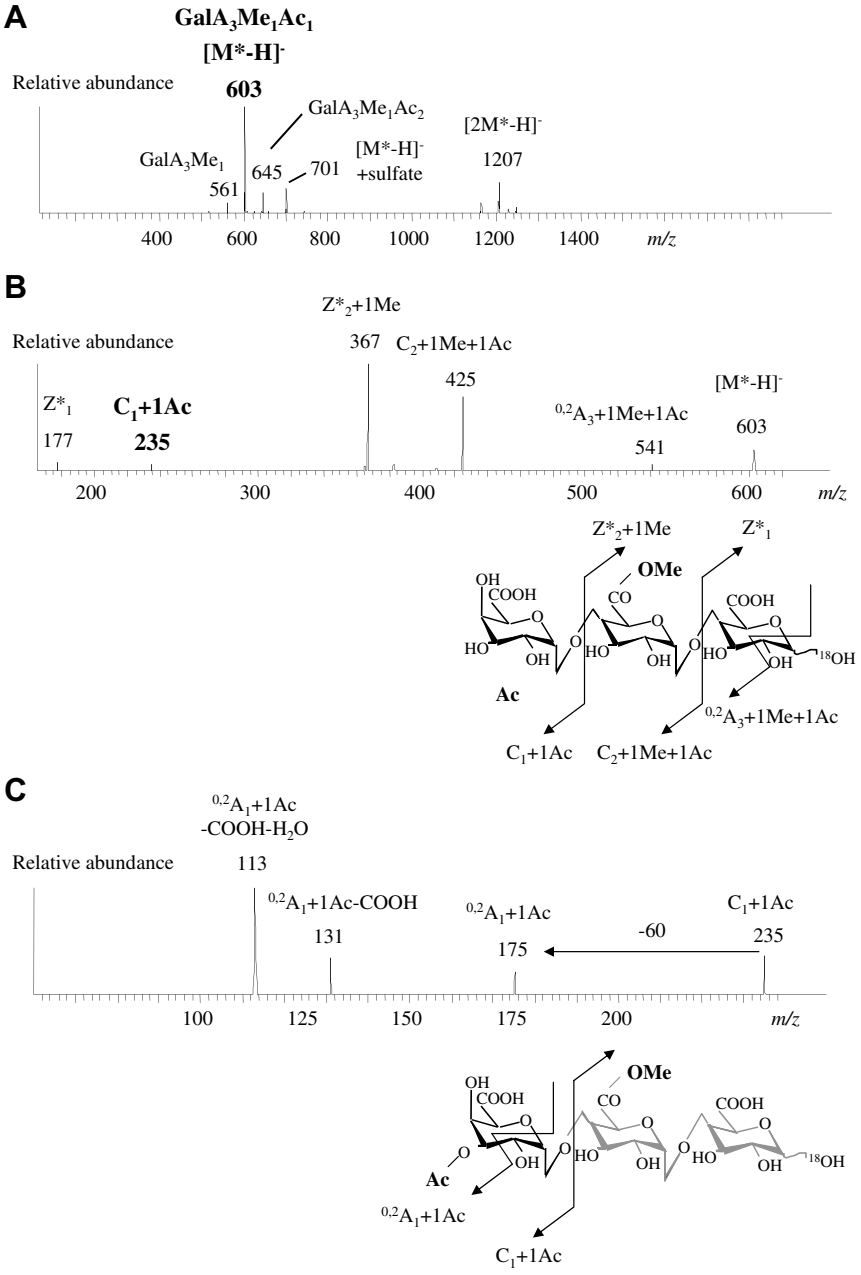

Figure 3. Negative ESI-IT-CID mass spectra of the ^{18}O-labelled monomethylated and monoacetylated trigalacturonate ($GalA_3Me_1Ac_1$) and observed cleavages at each MS step. (A) Full-scan MS of the fraction of interest recovered by anion-exchange chromatography of sugar beet pectin enzymatic digest. (B) MS^2 experiment (*m/z* 603 > products). C. MS^3 experiment (*m/z* 603 > 235 > products.

[C_2+1Ac] ion at *m/z* 411 and the [C_1+1Ac] ion at *m/z* 235 for $GalA_4Me_1Ac_1$ and $GalA_3Me_1Ac_1$, respectively, were isolated and fragmented in an MS^3 analysis (Figs. 2C and 3C). Ions corresponding to a loss of 102 Da (60+42) were produced when the acetyl group was linked on *O*-2, which indicates that the acetyl-ester bond is not broken during the CID process and the acetyl group remains linked to (and is removed together with) the $C_2H_4O_2$ fragment. Accordingly, ions corresponding to a loss of 60 Da were produced when the acetyl group was linked on *O*-3 (Figs. 2C and 3C). This work about assignment of acetyl groups to *O*-2 or *O*-3 of pectic oligogalacturonates,[56] together with works about tri- and tetra-antennary *N*-glycans,[57] mucin *O*-glucans,[58] and urinary *O*-glycans,[59,60] provided evidence that $^{0,2}A_n$ cross-ring cleavage ions are highly diagnostic ions for oligosaccharides linkage and branching patterns analysis in negative ion mode. Thanks to this first study about assignment of acetyl groups to *O*-2 or *O*-3 of pectic oligogalacturonates, most of the sugar beet pectin hydrolysates fractions recovered after anion-exchange chromatography could be analysed for their fine structure by ESI-IT-MS^n.[61] The quantitative recovery with respect to both GalA and acetyl contents of HG-derived fractions, the sequencing of most of the compounds present in these fractions by ESI-IT-MS^n, and the known chain length of HG domains,[30,62] permitted depiction as a quantitative representation of the different HG-derived oligogalacturonates recovered. It was shown that both *O*-2 and *O*-3 acetylation were present, that 2,3-di-*O*-acetylation was absent and that GalA residues carrying both acetyl and methyl esters were rare.[61] Recently, an extensive degradation of sugar beet pectin with *Aspergillus aculeatus* pectin methyl esterase, in combination with one or the other of three different *endo*-polygalacturonases known to display variable tolerance towards methyl and/or acetyl groups, was carried out.[63] The oligogalacturonates generated were quantified and their sequence determined by ESI-IT-MS^n. An 'overlap' method was then used to assess acetyl groups distribution onto HG domains. This approach allowed the proposal of a blockwise distribution of acetyl groups onto sugar beet HG domains with, on average, four zones of 7–15 contiguous non-acetylated GalA units per HG chain. Although further work is needed to overcome expected DAc inter-chain heterogeneity, this is a clear step forward in the understanding of HG domains substituent distribution.

3. Analysis and sequencing of substituted galacturonans-derived oligomers

3.1. Sequencing of XGA oligomers

XGA exists in various plant cell walls. It has been isolated, by chemical or enzymatic means, from apple,[9] soy,[64] pea,[65,66] and watermelon.[67] XGA can be considered as a 'substituted galacturonan' since it consists of an HG backbone substituted at *O*-3 with monomeric and short oligomeric β-D-xylose (Xyl) side-chains. Gum tragacanth has a similar structure but with galactose (Gal) or fucose (Fuc) linked to *O*-2 of some of the Xyl residues.[68] Sakamoto et al. (2002) used ESI-IT-MS to study the specificity of two *exo*-polygalacturonases from *A. niger* towards XGA from pea hulls.[69] Both *exo*-PGs produced mainly GalA and GalAXyl dimer. The discovery,[70] over-expression in *A. niger* and further purification[71,72] of a xylogalacturonan hydrolase (XGH) that has no detectable activity on HG, allowed the generation of XGA oligosaccharides for structural characterization. Apple- and potato-derived oligosaccharides using XGH were studied in comparison with gum tragacanth XGA.[73,74] Hydrolysates were analyzed by MALDI-TOF-MS and also separated by HPAEC. GalAXyl was the main product and significant amounts of $GalA_2Xyl_2$, $GalA_2$, $GalA_2Xyl$, $GalAXyl_2$ and $GalA_2Xyl_3$ were also produced. Off-line MALDI-TOF MS analysis of HPAEC peaks was achieved and some peaks were structurally investigated using ESI-IT-MS^n. MS^2 and MS^3 steps were decisive in showing that dimeric Xyl side-chains were present in $GalAXyl_2$ and $GalA_2Xyl_3$.[74] Semi-quantification by HPAEC and structural characterization by MS of the different XGA oligosaccharides was crucial for gaining insight into XGH mode of action.[73,74] An exolytic action and the processive character of XGH were demonstrated. It was further shown that xylosylated GalA units and GalA units carrying a Xyl dimer can accommodate subsites −1 and +1 of XGH. The position −1 in the active site of *endo*-polygalacturonases from *A. niger* or *A. nidulans* (most probably PGII) can also accommodate a Xyl residue on *O*-3 of the GalA residue.[75] The experimental approach involving HPAEC and MALDI-TOF MS provided evidence that XGA exists in cell walls from various tissues of *Arabidopsis thaliana*.[76] The predominant production of $GalA_3Xyl$ and the release of linear oligogalacturonates (degree of polymerization 1–6) pointed to a lower degree of Xyl substitution in XGA from *A. thaliana* than in XGA from apple and potato. The HPAEC/MALDI-TOF MS approach was also used for the identification of a XGA xylosyltransferase and contributed to evidence that insertion mutants of the At5g33290 locus were XGA deficient.[77] Separation of complex mixtures of APTS-labeled XGA oligosaccharides by capillary electrophoresis (CE) and their subsequent identification by on-line ESI-IT-MS^n was recently achieved.[78] Typical XGA oligosaccharides were identified. By analyzing a more complex mixture encompassing RG-I, XGA and HG-derived oligosaccharides, a large set of structural data was obtained in a single analysis and in a relatively short time.[78]

3.2. Sequencing of RG-II side chains

RG-II is a pectic polysaccharide of about 5 kDa that is found in the primary cell walls of vascular plants. It is composed of 12 different monomers, including rare glycosyl residues such as D-apiose (Api), L-aceric acid (AceA), 2-keto-3-deoxy-D-*lyxo*-heptulosaric acid (Dha), 2-keto-3-deoxy-D-*manno*-octulosonic acid (Kdo), 2-*O*-methyl L-fucose (2-OMe-Fuc) and 2-*O*-methyl D-xylose (2-OMe–Xyl), interconnected by about 20 linkages.[1] Four side-chains are attached to the oligogalacturonide backbone constituted of at least six α-(1,4)-D-Gal*p*A residues. The A and B chains are two structurally complex oligosaccharides both linked to the backbone via an Api residue. Kdo- and Dha-containing disaccharides are named C and D side-chains, respectively. RG-II exists in the wall as a dimer that is cross-linked via a borate diester between the Api residues of two A side-chains.[79,80] Despite its complexity, both the structure of RG-II and its ability to dimerise via a borate diester are conserved in vascular plants.[81] This suggests that proteins involved in RGII synthesis appeared early in land plant evolution and that it has fundamental functions in the primary wall organization. Due to its high complexity, structural information about a whole RG-II molecule is hardly feasible. As a consequence, to get insight about the RG-II structure, this pectic polymer is usually fragmented into the galacturonan backbone and the constitutive side-chains by mild acid hydrolysis, taking advantage of the location of the acid-labile Kdo, Dha and Api at the junction between the side-chains and the oligogalacturonide backbone.[81–88] Oligosaccharide fragments resulting from this mild hydrolysis were then analyzed by MS. First structural identifications were carried out on permethylated oligosaccharides.[82,84,89] Emergence of soft ionization techniques such as MALDI and ESI then allowed the investigation in both negative and positive modes of native RG-II side-chains either after isolation or by MS^2 analysis of molecular ions detected in the MS spectrum of the crude oligosaccharide fragment mixture.[85–87] Thus MS^2 sequencing allows both the sequence identification of A and B chains, the two structurally complex side-chains, and the location of acetates although this analysis is complicated by the

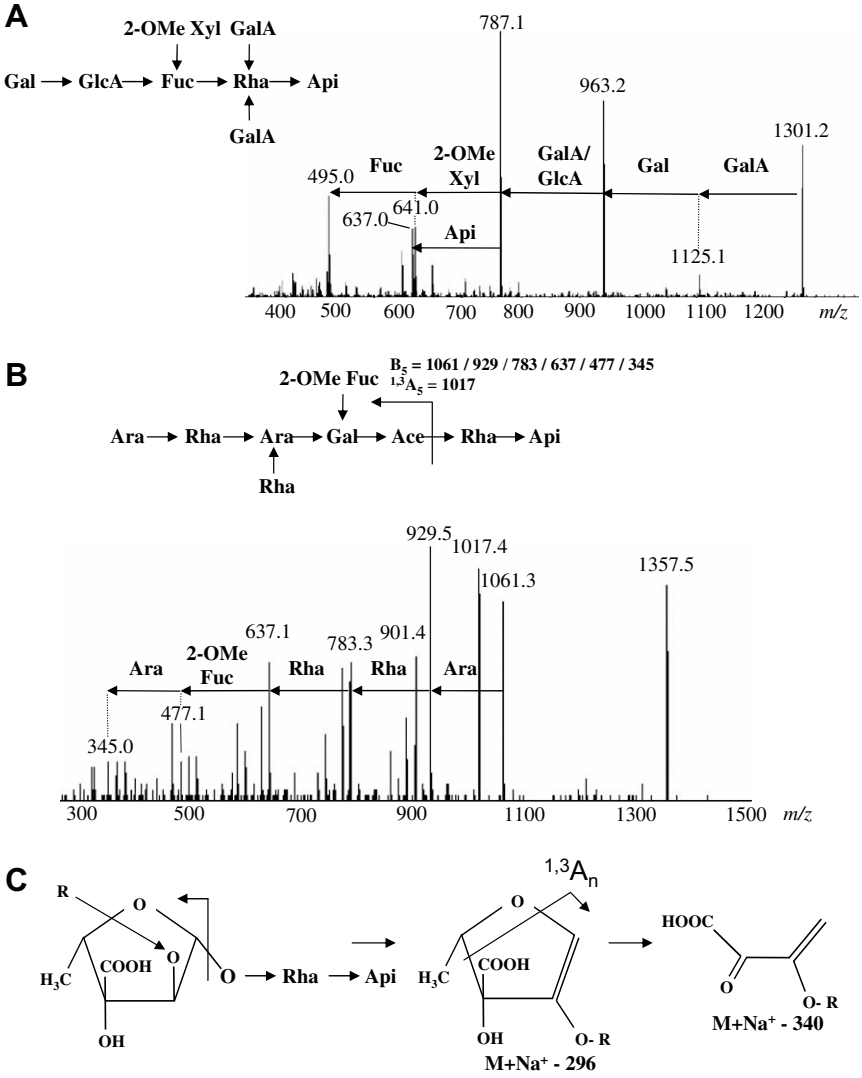

Figure 4. Positive ESI-MS-MS of ions at *m/z* 1301 and 1357 assigned, respectively, to RG-II side-chains A and B. (A) MS^2 experiment (*m/z* 1301 > products) and side-chain A sequence determination. (B) MS^2 experiment (*m/z* 1357 > products) and side-chain B sequence determination. (C) Glycosidic bond cleavage between Ace and Rha together with $^{1,3}A$ cross-ring cleavage of Ace in side-chain B.

presence of isobaric monomers in these oligosaccharides (GalA/GlcA, AceA/2–OMe-Fuc, Api/Ara). As illustrated in Figure 4A, ESI MS^2 fragmentation of A side-chain exhibited mainly Y-type fragment ions allowing its sequence determination (Lerouge et al., unpublished results). The MS^2 fragmentation pattern of B side-chain in positive mode is more sophisticated (Fig. 4B). Two major fragments corresponding to M-296 (B fragment) and M-340 ($^{1,3}A$ fragment) are observed which result from the cleavage of the glycosidic bond between Ace and Rha together with the ring cleavage of Ace (Fig. 4C). These diagnostic ions gave rise to a Y ion series allowing the oligosaccharide sequence investigation.

4. Analysis and sequencing of RG-I-derived oligomers

The RG-I backbone consists of [2)-α-L-Rha*p*-(1,4)-α-D-Gal*p*A-(1] repeats.[4,90,91] RG-I domains isolated from several plant species, appeared highly acetylated at *O*-2 and/or *O*-3 of GalA units.[11,61,92] No strong evidence has been published that GalA units in RG-I domains are methyl-esterified. The rhamnosyl (Rha) residues of RG-I backbone are substituted, mainly at *O*-4, with several types of arabinose (Ara) and Gal-containing neutral sugars side-chains.[31] The proportion of branched Rha residues vary widely depending on plant species.[91] Furthermore, an intermolecular variation of side-chains arrangement within the thickness of a given cell wall has also been evidenced.[15] The intramolecular arrangement of those side-chains is not as yet fully understood. Neutral sugars side-chains are very versatile in their length and fine structure.[93]

MS for identification of RG-I-derived oligosaccharides was first applied for the characterization of charged oligosaccharides liberated by rhamnogalacturonan hydrolase during the incubation of pectic hairy regions of apple.[94] On-line HPAEC-thermospray-MS was implemented. The poor peak shape observed when operated in full-scan mode was improved by a sequential run in multiple-ion detection. A search was carried out for all *m/z* values calculated for combinations of hexoses, deoxyhexoses and uronic acids. Several RG-I-derived oligosaccharides (Rha_2GalA_2, $Rha_2GalA_2Gal_1$, $Rha_2GalA_2Gal_2$, Rha_3GalA_3, $Rha_3GalA_3Gal_2$ and $Rha_3GalA_3Gal_3$) were identified. The exact location of the Gal residues could, however, not be established. The separation of APTS-labelled RG-I oligosaccharides by capillary electrophoresis (CE) and their subsequent identification by on-line ESI-IT-MS^n was also achieved.[78] Some new oligomers were detected (Rha_1GalA_2, Rha_2-

$GalA_3$, $Rha_2GalA_2Gal_2Ara?_1$ and $Rha_2GalA_2Gal_3$, $Rha_2GalA_2Gal_4$). MS^2 analyses led to a tentative structure for $Rha_2GalA_2Gal_3$, with a Rha–GalA–Rha–$GalA^*$ backbone, a dimeric Gal chain on the first Rha residue next to the GalA reducing end ($GalA^*$) and a monomeric Gal on the second Rha residue.[78] However, some isomeric forms were also present.

On *A. thaliana* seed imbibition, a 'pectinaceous' mucilage is released.[95–99] Two distinct layers of mucilage can be distinguished,[95,96] both layers being extremely rich in unbranched unesterified RG-I.[100] ESI-IT-MS analysis of hydrolysates obtained from digestion of the inner mucilage layer with rhamnogalacturonan hydrolase revealed the presence of Rha_1GalA_1, Rha_1GalA_2, Rha_2GalA_2 and Rha_2GalA_3.[101] After isolation and CID of the *m/z* 515 $[M-H]^-$ precursor ions, complementary ions of the C- and Z-series, especially a C_2 ion corresponding to the loss of a GalA residue, could be observed (Fig. 5A). The specific cross-ring cleavage ions $^{0,2}A_3$ (−60 Da), indicative of 1,4 type glycosidic linkage,[41] was also observed. This agrees with the presence of a 4-linked GalA residue at the reducing end. The $[C_2]$ ion at *m/z* 339 was isolated and fragmented in an MS^3 analysis (Fig. 5B). C_1 ions corresponding to the loss of a Rha residue were observed. Interestingly, an intense water loss and fragment ions corresponding to a loss of 104 Da were produced. This agrees with fragmentations observed for 1,2-linked disaccharides and corresponds to $^{0,2}X$ ion types.[41,102] This agrees with the presence of a 1,2-linked Rha residue. This peculiar fragmentation pattern allowed sequencing of Gal-containing RG-I oligosaccharides that were specifically present in the mucilage of At5g63800 mutants.[101]

Single chain-derived oligomers (i.e., devoid of RG-I backbone residues) were also successfully sequenced using ESI-MS/MS in the positive mode.[103] Soybean pectin was hydrolyzed by arabino-galactan-degrading enzymes yielding arabino-, galacto- and arabino-galacto-oligomers. After purification by size-exclusion chromatography and HPAEC, monosaccharide linkage analyses and ESI-MS/MS were used to assess monosaccharide sequence and branching pattern of the different oligosaccharides. These analyses indicated the presence of common linear (1,4)-linked galacto-oligosaccharides and both linear and branched arabino-oligosaccharides. Two types of arabino-galacto-oligosaccharides (Gal_5Ara_1) were isolated and, in both cases, CID of the per-O-methylated parent ions was determinant for sequence analysis. In particular, series of Y ions provided evidence for the presence of (i)

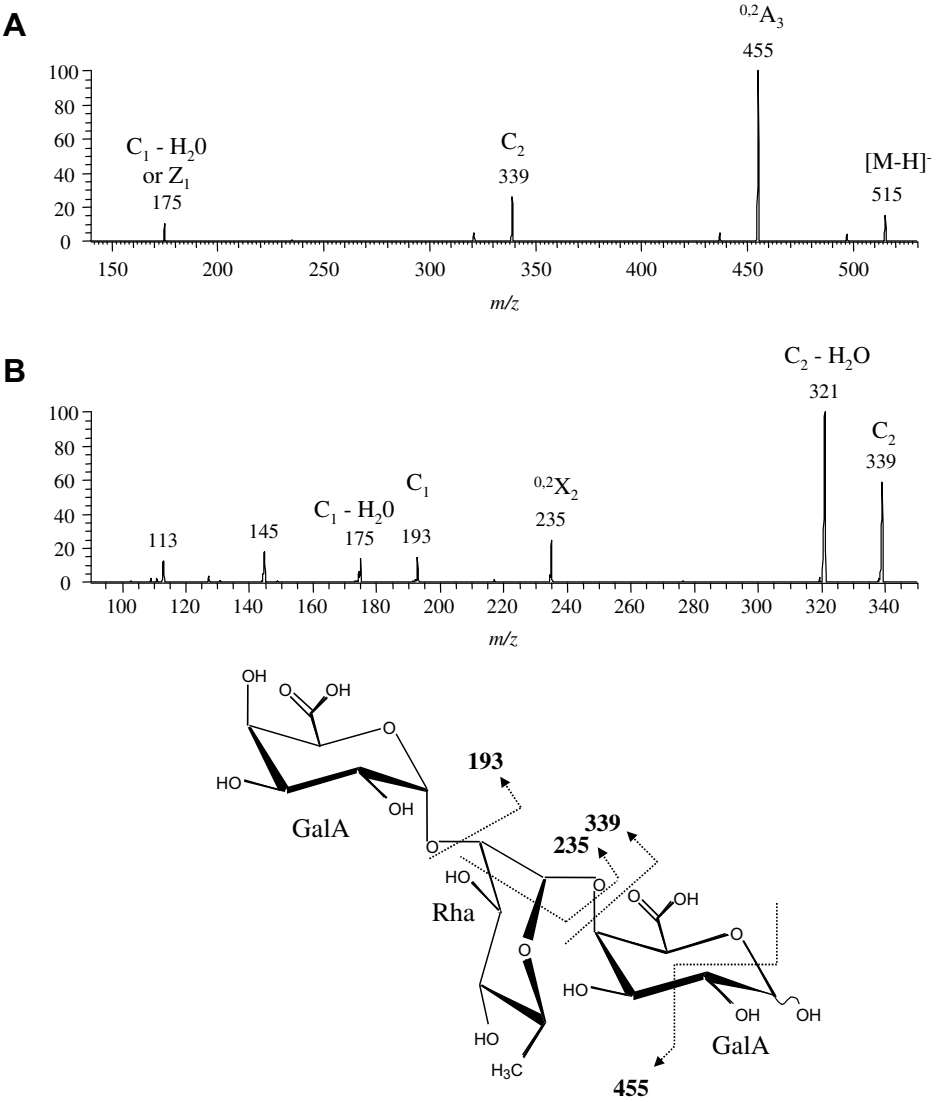

Figure 5. Negative CID mass spectra of the major oligomer (Rha_1GalA_2 *m/z* 515) recovered after digestion of the *A. thaliana* seeds inner mucilage layer with rhamnogalacturonan hydrolase. (A) MS^2 experiment (*m/z* 515 > products). (B) MS^3 experiment (*m/z* 515 > 339 > products). (C) Chemical structure of Rha_1GalA_2 and observed cleavages at each MS step.

linear oligosaccharides containing (1,4)-linked Gal residues bearing an Ara*p* residue at the non-reducing end, and (ii) linear oligosaccharides constructed of (1,4)-linked Gal residues interspersed with an internal (1,5)-linked Ara*f* residue.[103]

ESI-MS^n in both positive and negative modes was also used for sequencing of arabino- and galacto-oligomers bearing a phenolic acid. Indeed, in species of the *Amaranthaceae* family, for example, sugar beet and spinach, pectic arabinan and galactan chains are esterified by ferulic acid moieties.[7,104–106] Various feruloylated Ara- and Gal-containing mono- and disaccharides with known linkage configurations were analyzed by ESI-IT or ESI-Q-TOF.[39] CID experiments using the two mass analyzers generated similar fragmentation patterns. As already seen for HG-derived oligosaccharides, the negative ion mode produced simpler CID patterns than the positive ion one. Cross-ring fragment ions were highly diagnostic for feruloyl group location on Ara and Gal rings. In particular, the CID spectrum of an Ara residue substituted at *O*-2 by a ferulic acid residue was dominated by a diagnostic cross-ring cleavage ion at (−108 Da; i.e., −110 Da for ^{18}O-labelled-oligosaccharides) and an intense water loss, while a high relative abundance of the diagnostic cross-ring cleavage ion at (−60 Da; i.e., −62 Da for ^{18}O-labelled-oligosaccharides) together with moderate water loss were characteristic of linkage of ferulic acid through the *O*-5 of the Ara unit.[39]

5. Connecting elements

Although a lot of progress has been made in the structural characterization of the different pectic domains, the knowledge of the covalent interconnections between pectic structural domains or with other cell wall polysaccharides is very limited. Until those cross-links have been isolated and structurally characterized, they will remain controversial.[107] However, the scarcity of the connecting elements, their potential structural heterogeneity, and the difficulties in specifically isolating and purifying them, make progress slow and arduous.

Direct evidence that pectic arabinan and galactan side-chains are covalently cross-linked (intra- or inter-molecularly) through dehydrodifeulates (diFA) in sugar beet cell wall has been provided.[108] A sequential isolation procedure including acid and enzymatic (Driselase® + Ronozym®) hydrolyses was applied to sugar beet cell wall material. DiFA-rich fractions, although present in very limited amounts, could be purified thanks to their hydrophobic character. These fractions were analyzed by HPAEC and ESI-IT-MS^n. Considering that the three diFA-rich fractions were recovered in very low amounts and that two of them were mixtures, MS was undoubtedly decisive for their structural characterization. Furthermore, the use of ^{18}O-labelling, and the established fragmentation patterns of feruloylated oligomers with known linkage configuration,[39] allowed full sequencing of the different diFA-oligosaccharides. The diFA moieties were shown (i) to connect arabinan chains via the *O*-2 of internal Ara residues from (1,5)-linked chains, (ii) to connect arabinan chains via the *O*-5 of Ara residues, presumably at non-reducing ends of (1,5)-linked chains, and (iii) to connect arabinan and galactan chains via the *O*-2 of internal Ara residues from (1,5)-linked chains and the *O*-6 of internal Gal residues from (1,4)-linked chains.

Recently, Coenen et al. (2007) succeeded in identifying some connection elements between HG or XGA and RG-I.[109] Modified hairy regions (MHR) were isolated from fresh apple using a pectinase-rich enzymatic mixture (Rapidase Liq$^{+®}$).[110] The apple MHR was saponified and submitted to controlled acid hydrolysis to yield a mixture of HG-, XGA- and RG-I-derived oligosaccharides amongst which were oligomeric fragments involved in the connection between HG or XGA and RG-I. Oligosaccharides were fractionated by HPAEC and 42 peaks were annotated by off-line MALDI-TOF-MS. Among them, 4 were of 'hybrid' nature, namely $GalA_3Rha_1$, $GalA_4Rha_2$, $GalA_5Rha_3$ and $GalA_6Rha_3Xyl_1$. MS^n spectra were interpreted on a sequencing point of view. When submitted to a MS^2 analysis in positive mode, the parent ion at *m/z* 717 ($GalA_3Rha_1^*$) yielded complementary ions from the B- and Y*-series together with some ions from the C-series. This fragmentation pattern agreed with the presence of the Rha residue at the reducing end.[109] The Y^*_2 ion ($GalA_1Rha_1^*$) (*m/z* 365) was further fragmented in a MS^3 analysis. Although the presence of cross-ring cleavage ions was not discussed in the article, both ^{0,2}A ions (-62 Da) at *m/z* 303, indicative of 1,4 linkages, and ^{0,2}X ions (-104 Da) at *m/z* 261, indicative of 1,2 linkages,[102] were observed. NMR analysis of another 'hybrid' oligosaccharide ($GalA_5Rha_3$) was, however, in favour of 1,2-linked Rha only.[109] Further work is needed to separate the different possible isomers with respect to GalA–Rha linkage.

6. Conclusion

Structural analysis of pectins is a big challenge due to the complexity of this branched macromolecule, which contains various structural domains. The fine structure of various heterogeneous oligosaccharides may be determined by NMR analysis but this technique generally requires purified and homogeneous samples at micromole/nanomole level. Therefore, mass spectrometry, in combination with the use of glycolytic enzymes to release the constituent oligosaccharides, has been extensively used in the last few years to provide extensive structural information including monomer composition, branching, substituent type, and linkage information in the picomole–femtomole range. This enzymatic fingerprinting approach, based on the use of pectolytic enzymes of various specificities in combination with tandem mass spectrometry, was decisive as described in this review. The capability to analyze mixtures without need of purification steps, the high sensitivity, mass accuracy and mass resolution of the new generation of analyzers, will provide definite benefits in the structural analysis of oligosaccharides by mass spectrometry. In this way, MALDI TOF/TOF instruments able to perform high-energy CID required for producing not only abundant cross-ring—but also 'internal'-cleavage ions resulting from elimination of substituents from the *pyranose* ring, are very promising as new fragmentation routes may be obtained.[111–113] This tandem MS technique will be useful for the characterization of isomeric structures, provided that these isobaric structures have been separated by means of HPLC techniques such as HPAEC with Carbohydrate Membrane Desalter (CMD) used as an in-line desalter prior to mass analysis.[114] As long as isomeric structures are concerned, ion mobility mass spectrometry (IMS), which has the unique ability to separate gas-phase ions based upon their size, shape and charge, also provides an exciting alternative to more traditional separating techniques.[60,115,116] By coupling IMS to the TOF MS analyzers, ions may be separated in two dimensions, by their ion mobility and *m/z* ratio. New instrumental developments have improved ion mobility as a separation technique more efficient than liquid chromatography.[116] In addition to filtering of ions by their mobility, IMS CID MS/MS fragmentation of species of interest may be also performed for increasing sequence assignment coverage.[60] This new strategy may be particularly useful for the analysis of branched isoforms released by enzymic digestion of RG-I domains. Finally, the recent hybrid linear quadrupole ion trap in conjunction with Fourier-transform ion-cyclotron resonance-mass spectrometer (ESI-LTQ-FT-ICR-MS) is also full of promise for its ability to provide more sensitivity than conventional ion trap spectrometer, mass accuracy and high-resolution on a fast chromatographic timescale. In particular, some isobaric oligosaccharides released from enzymic digestion or mild

acid hydrolysis and containing the isobaric monomers AceA/2-OMeFuc or uronic acid/ferulic acid should be distinguished taking into account their respective monoisotopic masses. All these new mass spectrometry approaches to address isomeric structures should give a better insight into pectin fine structure and interconnections, either between pectic structural domains or between a given domain and other cell wall polysaccharides.

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