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Evidence for a blockwise distribution of acetyl groups onto homogalacturonans from a commercial sugar beet (*Beta vulgaris*) pectin

Marie-Christine Ralet *, Marie-Jeanne Crépeau, Estelle Bonnin

INRA, UR1268 Biopolymères Interactions Assemblage, Rue de la Géraudière, B.P. 71627, F-44300 Nantes, France

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ABSTRACT

Commercial acid-extracted sugar beet pectin was extensively hydrolysed using an *endo*-polygalacturonase (*An*PGI from *Aspergillus niger* or *An*PGII from *A. niger* or *Fm*PG from *Fusarium moniliforme*) in combination with *Aspergillus aculeatus* pectin methyl-esterase (*Aa*PME). The homogalacturonan-derived oligogalacturonates released were quantified by high-performance anion-exchange chromatography and their structure determined by mass spectrometry. The different *endo*-polygalacturonases exhibited variable tolerance towards acetyl groups. *An*PGI was the most active and *Fm*PG the less. A hypothetical homogalacturonan was constructed using the *An*PGI-recovered oligogalacturonates as building blocks and the validity of the model was checked taking into account *Fm*PG observed requirements and hydrolysis products. A blockwise repartition of the acetyl groups onto sugar beet pectin homogalacturonan is proposed.

1. Introduction

Pectins are amongst the most abundant polysaccharides in many plant primary cell walls. Pectin molecules are mainly composed of four different structural elements: homogalacturonans (HGs), xylogalacturonans (XGAs), type I rhamnogalacturonans (RGs-I) and type II rhamnogalacturonans (RGs-II), which have been extensively described in previous reviews (Voragen et al., 1995; Ridley et al., 2001; Ralet et al., 2002). HGs consist in a repetition of α–(1→4)-linked D-galacturonic acid (GalA) units that can be partly methyl-esterified at C-6 and, in some species, partly acetyl-esterified at O-2 and/or O-3. Many of the properties and biological functions of pectins are believed to be mediated by ionic interactions between HG domains (Ridley et al., 2001; Willats et al., 2001). Not only the degree of esterification, but also the distribution of methyl and acetyl groups onto HGs has a deep impact on those interactions (Kohn et al., 1983; Thibault and Rinaudo, 1985; Ralet et al., 2003). In particular, acetylation of HG domains is well known to strongly alter pectins associative properties (Pippen et al., 1950; Kohn and Furda, 1968; Kohn and Malovikova, 1978; Renard and Jarvis, 1999; Ralet et al., 2003). 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 pectin molecules, and more particularly onto HGs, is necessary. In that purpose, enzymatic degradation of polysaccharides followed by structural analysis of the degradation products proved to be efficient tools (Massiot and Thibault, 1989; Sakamoto and Sakai, 1995; Needs et al., 1998; Perrone et al., 2002; Ralet et al., 2005). Basically, HG is the best substrate for *endo*-polygalacturonases (poly [1→4-α-D-galacturonide] glycanohydrolase, EC 3.2.1.15) (PGs), as they hydrolyse 1→4 linkages between two GalA residues (as reviewed by Jayani et al., 2005). They are widely distributed in plants, fungi, yeasts, and bacteria (Fogarty and Kelly, 1983) and display variable tolerance toward methyl- and acetyl-esterification (Chen and Mort, 1996; Benen et al., 1999; Bonnin et al., 2002a, 2003; André-Leroux et al., 2005). In a previous work (Ralet et al., 2005), acetylated oligogalacturonates were recovered – after enzymatic hydrolysis of sugar beet pectin using an *An*PGI from *Aspergillus niger* in combination with a pectin-methyl esterase from *Aspergillus aculeatus* – purified, and structurally characterized by mass spectrometry. A list of oligogalacturonates sequences was obtained but the puzzle of in which order the oligogalacturonates were linked up in the original HG had still to be solved. The distribution of acetyl groups onto HG domains remained thereby speculative.

In the present work, an extensive degradation of commercial acid-extracted sugar beet pectin was carried out with three different PGs, *An*PGI and *An*PGII from *A. niger* and *Fm*PG from *Fusarium moniliforme*, in combination with *A. aculeatus* pectin-methyl esterase (*Aa*PME). Those PGs are known to display variable tolerance towards methyl and/or acetyl groups (Bonnin et al., 2002a, 2003; André-Leroux et al., 2005) so that an "overlap method" could be tentatively used to assess acetyl groups distribution onto HG domains. The oligogalacturonates generated by the three PGs were quantified and their sequences determined by mass spectrometry.

* Corresponding author. Tel.: +33 240675196; fax: +33 240675084.
E-mail address: ralet@nantes.inra.fr (M.-C. Ralet).

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A hypothetical HG was constructed using the *An*PGI-recovered oligogalacturonates as building blocks and the validity of the model was checked taking into account the hydrolysis products released by the other PGs.

2. Results and discussion

2.1. Analysis of hydrolysis products by high-performance anion-exchange chromatography

An acid-extracted commercial sugar beet pectin was extensively hydrolysed by a given PG (*An*PGI, *An*PGII or *Fm*PG) in combination *Aa*PME. PGs activity is most often reduced by increasing DM (Chen and Mort, 1996; Benen et al., 1999) and combined activity of PG and PME is necessary to extensively degrade pectin HG regions. Around 75% of the methyl groups initially present in sugar beet pectin (DM 62) were removed by *Aa*PME, leading to an overall mean final DM of ~14. The enzymatic digests were analysed by HPAEC pH 13 (Fig. 1). Due to the high pH of the eluent, all ester groups were removed and separation of the oligogalacturonates took place according to their degree of polymerisation (dp). *An*PGI generated high amounts of oligogalacturonates of dp ⩽ 6. Oligogalacturonates of dp 7–13 were also detected. Altogether, 93% of the GalA initially present in sugar beet pectin was recovered as HG-derived oligogalacturonates. A very similar value of GalA repartition between HG and RG-I was obtained by a different experimental approach consisting of degrading RG-I regions to leave intact HG domains (Thibault et al., 1993; Bonnin et al., 2002b). It was thereby considered that the totality of the GalA present in HG domains was recovered as oligogalacturonates of dp ⩽ 13 after digestion of sugar beet pectin with *An*PGI in combination with *Aa*PME. The oligogalacturonates dp distribution was significantly altered when *An*PGII was used since lower amounts of oligogalacturonates of dp < 7, particularly dp 3 and 4, and higher amounts of larger oligogalacturonates were produced. Oligogalacturonates up to dp 16 were detected. Using *An*PGII, 82% of the GalA initially present in sugar beet pectin (i.e. 89% of the GalA present in HG domains) was recovered as oligogalacturonates of dp ⩽ 16. At last, hydrolysis by *Fm*PG generated much lower amounts of oligogalacturonates of dp ⩽ 5, again particularly dp 3 and 4. Oligogalacturonates up to dp 18 were detected. Using *Fm*PG, 76% of the GalA initially present in sugar beet pectin (i.e. 83% of the GalA present in HG domains) was recovered as oligogalacturonates of dp ⩽ 18.

2.2. Quantification and identification of partly methylated and/or acetylated oligogalacturonates

Preparative anion-exchange chromatography was used to purify the different oligogalacturonates present in the sugar beet enzymatic digests (Fig. 2). As previously shown (Ralet et al., 2005), oligogalacturonates of dp ⩽ 6 arising from HGs were eluted between 0 and 0.24 M NaCl. RGI together with oligogalacturonates of dp > 6 were eluted for higher NaCl concentrations. Appropriate fractions were pooled and desalted. Fractions were analysed for their GalA content, for their DAc and for their structure by ESI-IT-MS^n. Major oligogalacturonates in each fraction are given in Fig. 2. The electrospray ionization followed by formation of fragment ions by collision-induced dissociation (CID) allows sensitive mapping and sequencing of oligosaccharides. Carbohydrates undergo two types of fragmentation: those of glycosidic cleavages and those of cross-ring fragmentation. For sequencing, glycosidic cleavage ions along the chain are major tools while linkage and branching patterns can be established according to cross-ring fragmentations (Vakhrushev et al., 2004). In particular, we previously demonstrated that cross-ring cleavage ions are highly diagnostic ions allowing the precise location of acetyl groups on O-2 or O-3 of GalA residues (Quéméner et al., 2003). In the present study, a similar experimental MS^n approach was used to perform structural assignment of the different oligogalacturonates generated from sugar beet pectin by *An*PGI, *An*PGII and *Fm*PG. However, in some cases (dp ⩾ 6), the presence of multiple isomeric forms impeded full structural assignment. For clear illustration of the oligosaccharides sequencing analyses performed, MS^n experiments achieved on one major partly methylated and acetylated oligogalacturonate ($4^{1,1}$; $dp^{DM,DAc}$) are presented on Fig. 3. After isolation and CID of the $[M-H]^-$ parent ion at *m/z* 777, glycosidic cleavage ions at *m/z* 601 [C_3 + 1Me + 1Ac], 583 [Z_3 + 1Me + 1Ac], 411 [C_2 + 1Ac] and 365 [Z_2 + 1Me] were detected together with a cross-ring cleavage ion at *m/z* 717 [$^{0,2}A_4$] (Fig. 3A). This fragmentation pattern agrees with the presence of the following structure:

GalA-AcGalA-GalA(*O*-Me)-GalA. The MS_3 analysis of the [C_2 + 1Ac] glycosidic cleavage ion at *m/z* 411 (Fig. 3B) produced a cross-ring cleavage ion at *m/z* 309 [$^{0,2}A_2$] characteristic of the pres-

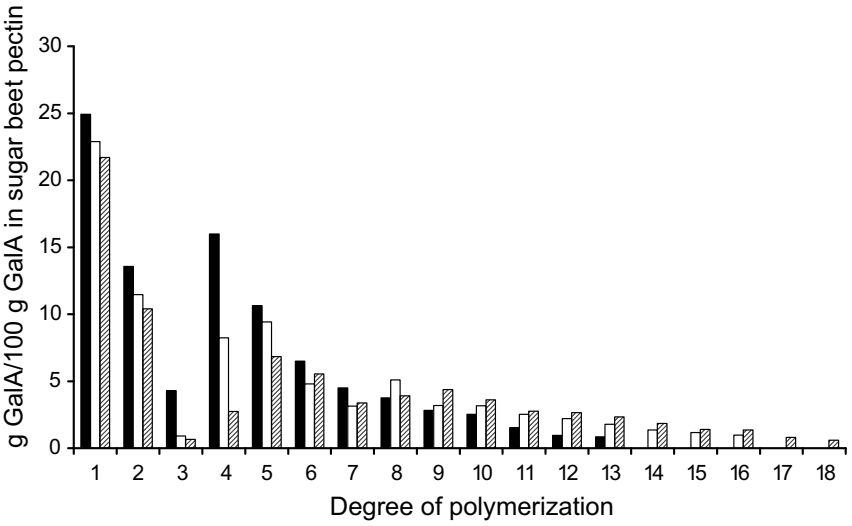

Fig. 1. Oligogalacturonates dp distribution of sugar beet pectin enzymatic digests with *An*PGI (black), *An*PGII (white) or *Fm*PG (grey) in combination with *Aa*PME. Analyses were performed by high-performance anion-exchange chromatography at pH 13.

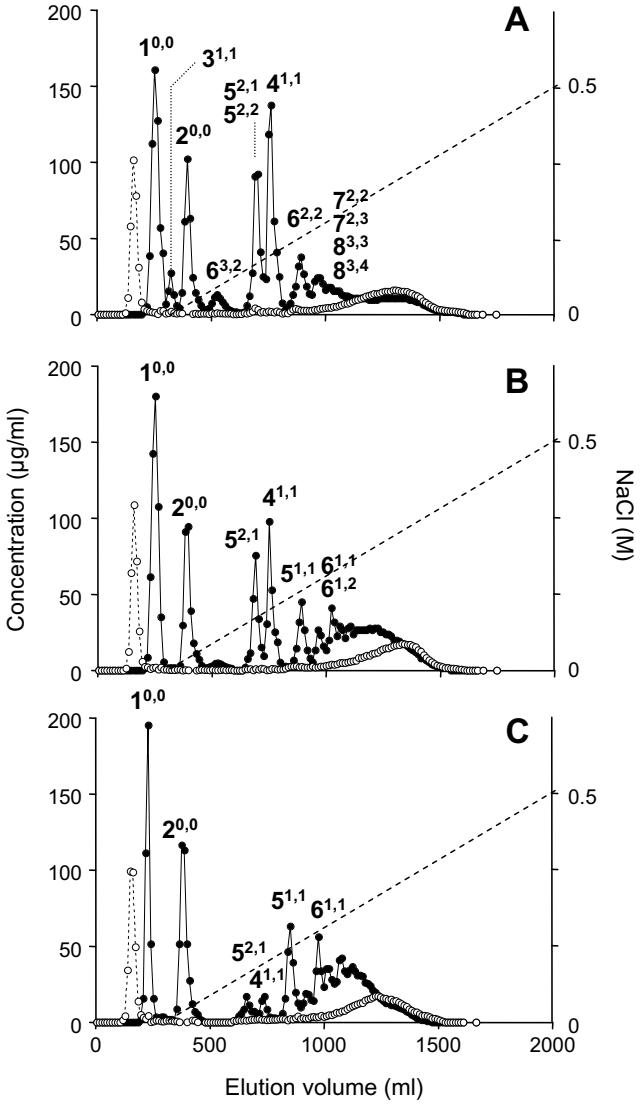

Fig. 2. Anion-exchange (DEAE Sepharose CL-6B) elution patterns of sugar beet pectin hydrolysates (●) GalA; (○) neutral sugars; (----) NaCl gradient. (A) *An*PGI + *Aa*PME. (B) *An*PGII + *Aa*PME. (C) *Fm*PG + *Aa*PME Major oligogalacturonates detected in each peak are quoted as follows: $dp^{Me,Ac}$, degree of $polymerisation^{number\ of\ methyl\ groups,number\ of\ acetyl\ groups}$.

ence of the acetyl group on O-2 of the GalA residue (Quéméner et al., 2003). The structure of $4^{1,1}$ was thereby assigned to GalA-2-*O*-AcGalA-GalA(*O*-Me)-GalA (Fig. 3C).

Knowing (i) the oligogalacturonates dp repartition in the enzymatic extracts (Fig. 1), (ii) the quantitative recovery of the different DEAE-fractions (Fig. 2), (iii) the sequence of most of the compounds present in these fractions, and (iv) the chain length of sugar beet pectin HG domains (dp ~ 80–100 residues; Thibault et al., 1993; Bonnin et al., 2002b), a quantitative representation of the different HG-derived oligogalacturonates after enzymatic hydrolysis of sugar beet pectin with *An*PGI, *An*PGII or *Fm*PG in combination with *Aa*PME, was drawn using an average dp of ~85 for HG (Fig. 4A). For *An*PGI, besides unsubstituted dp1 and dp2 (corresponding to 33 monomers/87), the major product (16 monomers/87) was the dp4 oligogalacturonate GalA-2-*O*-AcGalA-GalA(*O*-Me)-GalA described in the previous chapter. This oligosaccharide has been repeatedly recovered as a major hydrolysis product from various plant cell walls (Needs et al., 1998; Perrone et al., 2002; Ralet et al., 2005). Some rare dimers, trimers or tetramers were 3-*O*-acetylated on reducing or non-reducing GalA residues (Fig. 4A). All oligogalacturonates of dp ⩾ 4 exhibited a non substituted reducing end followed by a methyl-esterified GalA residue. Finally, oligogalacturonates of dp 4 and 5, and most probably those of dp 6, 7 and 8, considering their high DM and DAc, did not have two consecutive unsubstituted GalA residues. For *Fm*PG, unsubstituted dp 1 and dp2 were also major products (27 monomers/86). Oligomers of dp 3 and dp 4 were virtually absent (< one oligomer/HG domain). Oligomers of dp 5 and dp 6 exhibited similar overall structures with, from the reducing end, two unsubstituted GalA residues followed by one methyl-esterified GalA residue, one 2-*O*-acetylated GalA residue and one or two unsubstituted GalA residues. Some rare dimers were 3-*O*-acetylated on reducing GalA residues but, contrary to *An*PGI findings, no 3-*O*-acetylation onto non-reducing GalA residues was detected (Fig. 4A). Results obtained after *An*PGII hydrolysis were intermediate between those obtained after *An*PGI and those obtained after *Fm*PG hydrolysis with the recovery of dp 4 and dp 5 oligogalacturonates with, from the reducing end, one (as for *An*PGI) or two (as for *Fm*PG) unsubstituted GalA residues followed by one methyl-esterified GalA residue, one 2-*O*-acetylated GalA residue and one unsubstituted GalA residues (Fig. 4A). As for *Fm*PG, some rare dimers were 3-*O*-acetylated on reducing GalA residues but no 3-*O*-acetylation onto non-reducing GalA residues was detected.

2.3. Acetyl distribution onto homogalacturonan domains

A hypothetical HG was constructed using the *An*PGI-recovered oligogalacturonates as building blocks since this enzyme allowed the recovery of the highest amount of fully sequenced oligogalacturonates. Only oligogalacturonates of dp 6, 7 and 8 were only partially sequenced by MS^n and putative structures were drawn taking into account fractions DM and DAc and structures established for oligogalacturonates of dp 4 and 5. Several models were manually generated with *An*PGI-recovered oligogalacturonates, from homogeneously distributed acetyl groups to maximal blockiness of acetyl groups. Then, the cleavage sites of *Fm*PG were drawn taking into account *Fm*PG requirements (two consecutive unsubstituted GalA residues at reducing ends). We next compared the amounts of the different oligogalacturonates that should be theoretically recovered for *Fm*PG hydrolysis and the experimental results obtained for this enzyme in order to check the validity of the different representations. The best adequacy between theoretical and experimental values was obtained using a blockwise repartition of acetyl groups displaying four zones of 7–15 contiguous non-acetylated GalA units (Fig. 4B) on a chain of 87 monomers.

Our results agree with a blockwise distribution of acetyl groups onto sugar beet HG domains. It has however to be kept in mind that pectin extraction can lead to some deacetylation (Levigne et al., 2002a). Though, acid extraction is well known to possibly lead to a random deesterification and should therefore not be responsible of the blockwise distribution of acetyl groups observed. Interestingly, high yields of sugar beet pectins (~50% of the pectins initially present in sugar beet cell wall material) with very high DAc values (~40–50%) can be obtained by water extraction after thermal or thermomechanical treatments such as autoclaving or extrusion-cooking (Ralet et al., 1994; Oosterveld et al., 1996; Sakamoto et al., 2002). The commercial acid-extracted sugar beet pectin used in the present study (DAc 30) is thereby probably not fully representative of pectins present in cell wall material. Furthermore, pectins are characterized by a high degree of heterogeneity with respect to molar mass, composition and degree of substitution (Voragen et al., 1995). For sugar beet pectins, some limited heterogeneity with respect to the DAc was evidenced when different extraction conditions are used (Rombouts and Thibault, 1986; Renard and Thibault, 1993; Oosterveld et al., 1996; Levigne

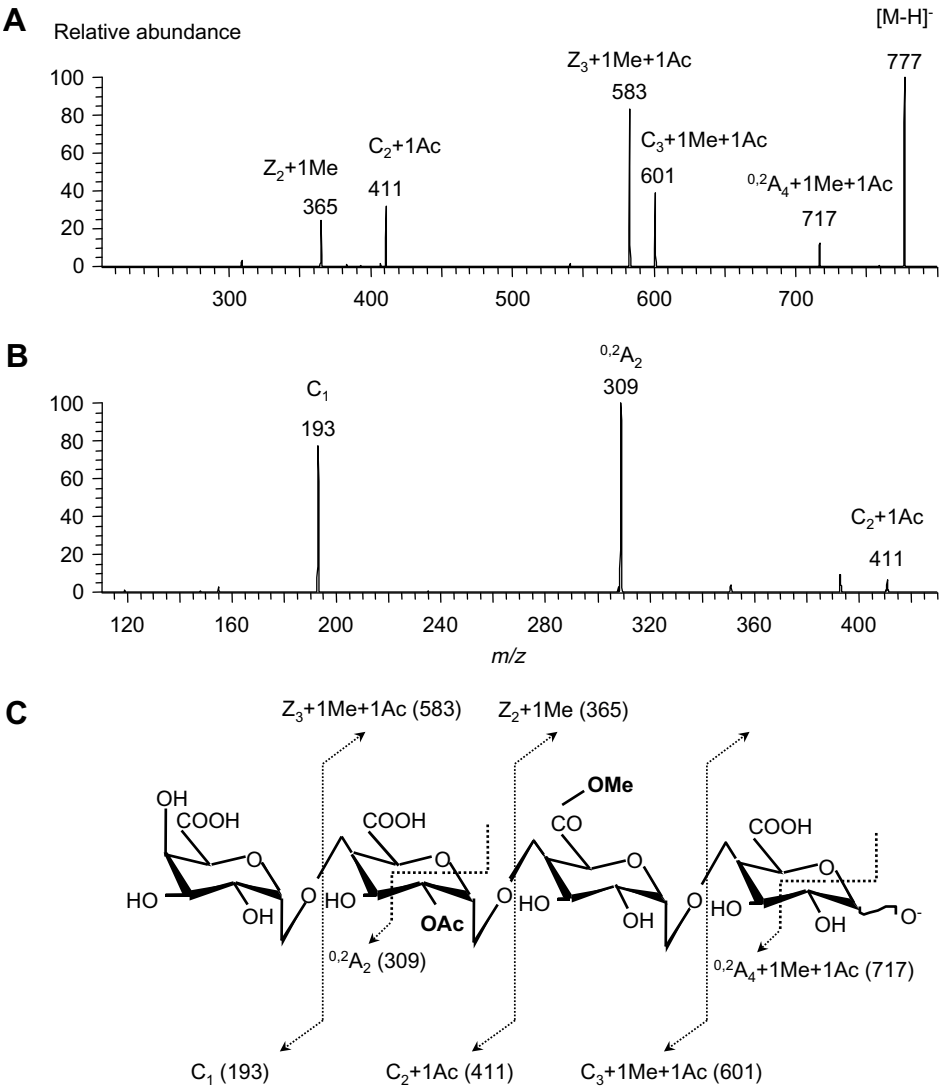

Fig. 3. Chemical structure and negative CID mass spectra of $4^{1,1}$ ($dp^{Me,Ac}$, degree of polymerisation$^{number\ of\ methyl\ groups,number\ of\ acetyl\ groups}$). (A) MS^2 experiment (*m/z* 777 > products). (B) MS^3 experiment (*m/z* 777 > 411 > products). (C) Chemical structure of $4^{1,1}$ and observed cleavages at each MS step. Fragments are labelled according to Domon and Costello's nomenclature.

et al., 2002a). However, interchain heterogeneity with respect to DAc in a given tissue is extremely difficult to appraise due to the deficiency in extraction methods allowing the recovery of the bulk of pectins while preserving acetyl substitution and to the lack of adequate separation techniques based on DAc.

3. Conclusion

In the present work, the various tolerance of *An*PGI and *An*PGII from *A. niger* and *Fm*PG from *F. moniliforme* to methyl and acetyl-substituants was used to built a homogalacturonan answering all the structural as well as the enzymatic requirements, i.e. chain length, DM, DAc and cleavage sites. The obtained polymer shows a rather blockwise distribution of acetyl groups with zones of 7–15 contiguous non-acetylated GalA units. As the substrate used in this study was extracted by chemical means, which are known not to reinforce the blockwise distribution onto pectin, and keeping in mind that some interchain heterogeneity can occur, one can think that this structure is close to that which can be found *in planta*. Even if further work is needed to appraise this interchain heterogeneity within a given tissue or a given cell wall, it can already be concluded that the blockwise distribution shown here has heavy consequences on the interacting capacities of pectin within the cell wall.

4. Experimental

4.1. Enzymes

The pectin methylesterase (PME, UniProt Q12535) from *A. aculeatus*, *An*PGI (UniProt P26213) and *An*PGII from *A. niger* were kindly provided by Novozymes (Bagsvaerd, Denmark). The two PGs were provided as monocomponent enzymes and were further purified before use. The purification of *An*PGII was previously described (Bonnin et al., 2002a). *An*PGI was purified by anion-exchange chromatography on a Mono Q HR 5/5 column (0.5 × 0.5 cm, GE Healthcare) equilibrated with 20 mM piperazine buffer pH 6 and eluted at 1 ml/min with 0.6 M NaCl in the same buffer. The following gradient was applied: 5 column volumes of starting buffer, 20 column volumes up to 0.2 M NaCl in piperazine buffer, 5 column volumes up to 0.6 M NaCl in piperazine buffer. The purified enzyme had a specific activity of 1850 nkat/mg and

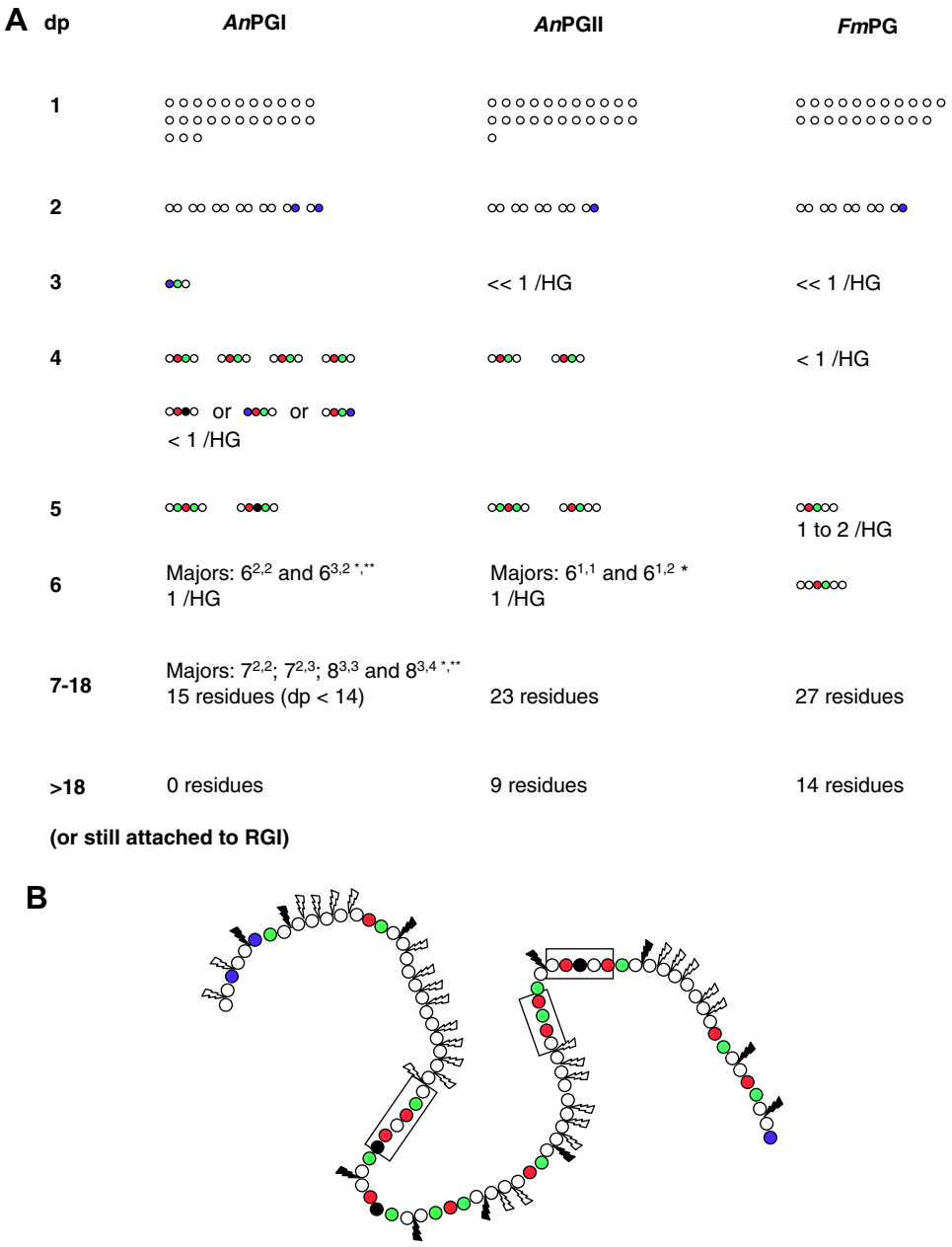

Fig. 4. (A) Quantitative representation of the different HG-derived oligogalacturonates after enzymatic hydrolysis of sugar beet pectin with *An*PGI, *An*PGII or *Fm*PG in combination with *Aa*PME. Structures are written with the reducing terminus to the right. $^{*}dp^{Me,Ac}$, degree of polymerisation$^{number\ of\ methyl\ groups,number\ of\ acetyl\ groups}$ **Partly characterized: unsubstituted reducing end followed by one methyl esterified GalA. (○) unsubstituted GalA residue; (●) methylated GalA residue; (●) 2-*O*-acetylated GalA residue; (●) 3-*O*-acetylated GalA residue; (●) methylated and acetylated GalA residue. (B) HG domain constructed using the *An*PGI-recovered oligogalacturonates and taking into account *Fm*PG requirements and observed hydrolysis products. White arrows represent hydrolysis sites common to both *An*PGI and *Fm*PG. Black arrows represent hydrolysis sites specific to *An*PGI. Repartition of acetylated and methylated GalA residues in boxes are putative and were constructed taking into account the dp, DM and DAc of the major products of dp $\geqslant$ 6 in *An*PGI digest.

was electrophoretically homogenous in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *F. moniliforme* PG (*Fm*PG, UniProt Q07181) was purified to homogeneity from a transformed strain of *Saccharomyces cerevisiae* (Caprari et al., 1996) as previously described (Bonnin et al., 2001).

4.2. Degradation of pectin

Sugar beet pectin (DM 62, DAc 30), was kindly supplied by Danisco (Brabrand, DK). Sugar beet pectin (55 mg) was solubilized at 2 mg/ml in 50 mM acetate buffer pH 4. Three series were prepared (one for each PG) and extensively degraded at 30 °C by adding successive amounts of a given PG and *Aa*PME at $t = 0$, 24 and 48 h (50 pkat PG/mg substrate and 2 nkat *Aa*PME/mg substrate). After a new period of 24 h, the reaction was stopped and an aliquot of the reaction mixture was analysed by HPAEC. The bulk of the hydrolysates was chromatographed on DEAE-Sepharose CL-6B (see section 4.5).

4.3. Analytical methods

GalA and neutral sugars (expressed arbitrarily as Ara) contents were determined by the automated *m*-hydroxybiphenyl (Thibault, 1979) and orcinol (Tollier and Robin, 1979) methods, respectively,

the latter being corrected for interfering GalA. Acetic acid was quantified by the enzymatic method of Enzytec Scil Diagnostics, Darmstadt (Germany). Methanol was released by alkaline deesterification in the presence of $CuSO_4$ and quantified by HPLC on a C18 column (Levigne et al., 2002b). Isopropanol was used as internal standard. DM and DAc were calculated as the molar ratio of methanol and acetic acid to GalA, respectively.

4.4. High-performance anion-exchange chromatography

High-performance anion-exchange chromatography (HPAEC) was performed on a Waters system with pulsed amperometric detection. The Carbopac PA1 column was eluted with 250 mM Na-acetate containing 100 mM NaOH (0–20 min), followed by one linear gradient phase of 500–700 mM Na-acetate containing 100 mM NaOH (20–60 min). The column was reconditioned by washing with 800 mM Na-acetate containing 100 mM NaOH (60–65 min) and then re-equilibrated with the starting buffer.

Response factors were previously calculated for oligogalacturonate standards from laboratory collection (dp 1–12) (Ralet et al., 2005) and the following equation was established

$$\text{Response factor} = 3.0041 \times 10^5 \times \text{dp}^{-0.69275} (R^2 = 0.995)$$

Monomer, dimer and trimer of GalA (Sigma, L'Isle d'Abbeau, France) as well as a pentamer from laboratory collection were used as standards in the present work to validate the previously established equation (Fig. 5).

4.5. Low-pressure chromatography

Anion-exchange chromatography was performed at room temperature on DEAE-Sepharose CL-6B column (30 × 2.6 cm) equilibrated with degassed 50 mM Na-succinate buffer pH 4.5, at a flow rate of 90 ml/h. Hydrolysates were loaded onto the column and the gel was washed with 400 ml of 50 mM Na-succinate buffer. The bound material was eluted with a linear NaCl gradient (0–500 mM) in 50 mM Na-succinate buffer (1600 ml). Twelve-ml fractions were collected and analysed for their content in GalA and neutral sugars. Appropriate fractions were combined, concentrated by in vacuum rotary evaporation at 40 °C and desalted using a column (100 × 1.6 cm) of Sephadex G-10 at 1 ml/min eluted by deionised water. GalA contents were determined by the automated *m*-hydroxybiphenyl method. Pooled desalted fractions were further analysed by mass spectrometry.

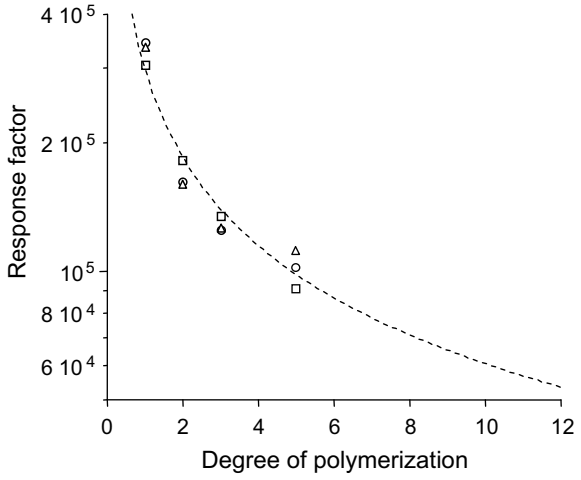

Fig. 5. HPAEC pH13 response factors for oligogalacturonates. (----) curve fit previously established (Ralet et al., 2005) for oligogalacturonates from dp 1 to dp 12 (R^2 = 0.995). (○), (□), (Δ) experimental values for dp 1, 2, 3 and 5.

4.6. Mass spectrometry

ESI-IT-MS experiments were achieved on a LCQ Advantage ion trap mass spectrometer (ThermoFinnigan, USA) using negative electrospray as ionization process. Sample solutions were appropriately diluted in water. Pure methanol was then added (1/1; v/v) to favour the spray formation into the electrospray source. Infusion was performed at a flow rate of 2.5 μl/min. Nitrogen was used as a sheath gas (20 arbitrary units). The MS analyses were carried out under automatic gain control conditions, using a typical needle voltage of 4.2 kV and a heated capillary temperature of 200 °C. For MS^n analyses, the various parameters were adjusted for each sample in order to optimize signal and get maximal structural information from the ion of interest. More than 50 scans were summed for MS^n spectra acquisition.

4.7. Sample handling

As acetyl groups migrate easily (Kamerling et al., 1987), freeze-drying was not used. "Never-frozen samples" were stabilised by adding methanol (final concentration 25% v/v) and kept at 4 °C.

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Mass spectrometry analyses were performed within the "Biopolymers-Interaction-Structural Biology" platform located at the INRA Centre of Nantes. http://www.nantes.inra.fr/plateformes_et_plateaux_techniques/plateforme_bibs.

References

André-Leroux, G., Tessier, D., Bonnin, E., 2005. Action pattern of *Fusarium moniliforme* endopolygalacturonase towards pectin fragments: comprehension and prediction. Biochim. Biophys. Acta 1749, 53–64.

Benen, J.A.E., Kester, H.C.M., Visser, J., 1999. Kinetic characterization of *Aspergillus niger* N400 endopolygalacturonases I, II and C. Eur. J. Biochem. 259, 577–585.

Bonnin, E., Le Goff, A., Körner, R., van Alebeek, G.J.W.M., Christensen, T.M.I.E., Voragen, A.G.J., Roepstorff, P., Caprari, A., Thibault, J.-F., 2001. Study of the mode of action of endopolygalacturonase from *Fusarium moniliforme*. Biochim. Biophys. Acta 1526, 301–309.

Bonnin, E., Le Goff, A., Körner, R., Vigouroux, J., Roepstorff, P., Thibault, J.-F., 2002a. Hydrolysis of pectins with different degrees and patterns of methylation by the endopolygalacturonase of *Fusarium moniliforme*. Biochim. Biophys. Acta 1596, 83–94.

Bonnin, E., Dolo, E., Le Goff, A., Thibault, J.-F., 2002b. Characterisation of pectin subunits released by an optimised combination of enzymes. Carbohydr. Res. 337, 1687–1696.

Bonnin, E., Le Goff, A., van Alebeek, G.-J.W.M., Voragen, A.G.J., Thibault, J.-F., 2003. Mode of action of *Fusarium moniliforme* endopolygalacturonase towards acetylated pectins. Carbohydr. Polym. 52, 381–388.

Caprari, C., Mattei, B., Basile, M.L., Salvi, G., Crescenzi, V., De Lorenzo, G., Cervone, F., 1996. Mutagenesis of endopolygalacturonase from *Fusarium moniliforme*: Histidine residue 234 is critical for enzymatic and macerating activities and not for binding to polygalacturonase-inhibiting protein (PGIP). Mol. Plant-Microbe Interact. 9, 617–624.

Chen, E.M.W., Mort, A.J., 1996. Nature of sites hydrolyzable by endopolygalacturonase in partially-esterified homogalacturonans. Carbohydr. Polym. 29, 129–136.

Fogarty, W.M., Kelly, C.T., 1983. Pectic enzymes. In: Fogarty, W.M. (Ed.), Microbial Enzymes and Biotechnology. Applied Science Publishers, London, pp. 131–182.

Jayani, R.S., Saxena, S., Gupta, R., 2005. Microbial pectinolytic enzymes: a review. Process Biochem. 40, 2931–2944.

Kamerling, J.P., Schauer, R., Shukla, A.K., Stoll, S., Van Halbeek, H., Vliegenthart, J.F.G., 1987. Migration of *O*-acetyl groups in *N*,*O*-acetylneuramic acid. Eur. J. Biochem. 162, 601–607.

Kohn, R., Furda, I., 1968. Binding of calcium ions to acetyl derivatives of pectin. Collect. Czech. Chem. Commun. 33, 2217–2225.

Kohn, R., Malovikova, A., 1978. Dissociation of acetyl derivatives of pectic acid and intramolecular binding of calcium ions to those substances. Collect. Czech. Chem. Commun. 43, 1709–1719.

Kohn, R., Markovic, O., Machova, E., 1983. Deesterification mode of pectin by pectin esterases from *Aspergillus foetidus*. Collect. Czech. Chem. Commun. 48, 790–797.

Levigne, S., Ralet, M.-C., Thibault, J.-F., 2002a. Characterisation of pectins extracted from fresh sugar beet under different conditions using an experimental design. Carbohydr. Polym. 49, 145–153.

Levigne, S., Thomas, M., Ralet, M.-C., Quemener, B., Thibault, J.-F., 2002b. Determination of the degrees of methylation and acetylation of pectins using a C18 column and internal standards. Food Hydrocoll. 16, 547–550.

Massiot, P., Thibault, J.-F., 1989. Enzymic analysis of carrot cell wall polysaccharides. Carbohydr. Res. 190, 121–136.

Needs, P.W., Rigby, N.M., Colqhoun, I.J., Ring, R.G., 1998. Conflicting evidence for non-methyl galacturonoyl esters in *Daucus carota*. Phytochemistry 48, 71–77.

Oosterveld, A., Beldman, G., Schols, H.A., Voragen, A.G.J., 1996. Arabinose and ferulic acid rich pectic polysaccharides extracted from sugar beet pulp. Carbohydr. Res. 288, 143–153.

Perrone, P., Hewage, C.M., Thomson, A.R., Bailey, K., Sadler, I.H., Fry, S.C., 2002. Patterns of methyl and *O*-acetyl esterification in spinach pectins: new complexity. Phytochemistry 60, 67–77.

Pippen, E.L., McCready, R.M., Owens, H.S., 1950. Gelation properties of partly acetylated pectins. J. Am. Chem. Soc. 72, 813–816.

Quéméner, B., Cabrera Pino, J.C., Ralet, M.-C., Bonnin, E., Thibault, J.-F., 2003. Assignment of acetyl groups to O-2 and/or O-3 of pectic oligogalacturonides using negative electrospray ion trap mass spectrometry. J. Mass Spectrom. 38, 641–648.

Ralet, M.-C., Della Valle, G., Thibault, J.-F., 1994. Solubilization of sugar-beet pulp cell wall polysaccharides by extrusion-cooking. Lebensm. Wiss. Technol. 24, 107–112.

Ralet, M.-C., Bonnin, E., Thibault, J.-F., 2002. Pectins. In: De Baets, S., Vandamme, E.J., Steinbüchel, A. (Eds.), Polysaccharides II – Polysaccharides from Eukariotes. Wiley-VCH Verlag, Weinheim, pp. 345–380.

Ralet, M.-C., Crépeau, M.-J., Buchholt, H.C., Thibault, J.-F., 2003. Polyelectrolyte behaviour and calcium binding properties of sugar beet pectins differing in their degrees of methylation and acetylation. Biochem. Eng. J. 16, 191–202.

Ralet, M.-C., Cabrera, J.C., Bonnin, E., Quéméner, B., Hellin, P., Thibault, J.-F., 2005. Mapping sugar beet pectin acetylation pattern. Phytochemistry 66, 1832–1843.

Renard, C.M.G.C., Jarvis, M., 1999. Acetylation and methylation of homogalacturonans 2. Effect on ion-binding properties and conformations.. Carbohydr. Polym. 39, 209–216.

Renard, C.M.G.C., Thibault, J.-F., 1993. Structure and properties of apple and sugar-beet pectins extracted by chelating agents. Carbohydr. Res. 244, 99–114.

Ridley, B.L., O'Neill, M.A., Mohnen, D., 2001. Pectins: structure, biosynthesis, and oligogalacturonide-related signalling. Phytochemistry 57, 929–967.

Rombouts, F.M., Thibault, J.-F., 1986. Feruloylated pectic substances from sugar-beet pulp. Carbohydr. Res. 154, 177–187.

Sakamoto, T., Sakai, T., 1995. Analysis of structure of sugar-beet pectin by enzymatic methods. Phytochemistry 39, 821–823.

Sakamoto, T., Bonnin, E., Thibault, J.-F., 2002. Purification and characterisation of two *exo*-polygalacturonases from *Aspergillus niger* able to degrade xylogalacturonan and acetylated homogalacturonan. Biochim. Biophys. Acta 1572, 10–18.

Thibault, J.-F., 1979. Automatisation du dosage des substances pectiques par la méthode au méta-hydroxydiphenyl. Lebensm. Wiss. Technol. 12, 247–251.

Thibault, J.-F., Rinaudo, M., 1985. Interactions of mono- and divalent counterions with alkali- and enzyme-deesterified pectins in salt-free solutions. Biopolymers 24, 2131–2143.

Thibault, J.-F., Renard, C.M.G.C., Axelos, M.A.V., Roger, P., Crépeau, M.-J., 1993. Studies of the length of homogalacturonic regions in pectins by acid hydrolysis. Carbohydr. Res. 238, 271–286.

Tollier, M.-T., Robin, J.-P., 1979. Adaptation de la méthode à l'orcinol sulfurique au dosage automatique des oses neutres et totaux. Ann. Technol. Agric. 28, 1–15.

Vakhrushev, S.Y., Zamfir, A., Peter-Katalinic, J., 2004. ^{0,2}An cross-ring cleavage as a general diagnostic tool for glycan assignment in glycoconjugate mixtures. J. Am. Soc. Mass Spectrom. 15, 1863–1868.

Voragen, A.G.J., Pilnik, W., Thibault, J.-F., Axelos, M.A.V., Renard, C.M.G.C., 1995. Pectins. In: Stephen, A.M. (Ed.), Food Polysaccharides and their Applications. Marcel Dekker, New York, pp. 287–339.

Willats, W.G.T., McCartney, L., Mackie, W., Knox, J.P., 2001. Pectins: cell biology and prospects for functional analysis. Plant Mol. Biol. 47, 9–27.