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Enzymatic degradation studies of xylogalacturonans from apple and potato, using xylogalacturonan hydrolase

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Abstract

Action of xylogalacturonan hydrolase (XGH) towards xylogalacturonan (XGA) present in the alkali saponified "*modified* hairy regions" from potato and apple pectin was studied.

Analysis of enzymatic degradation products from XGA in these complex pectins demonstrated that the degradable xylogalacturonans from both sources have a similar xylose side chain distribution. The disaccharide β-D-Xyl-(1,3)-GalA was the predominant product from these substrates.

The number of enzymatic degradation products from xylogalacturonan present in apple and potato pectin was much lower than the number of products from a xylogalacturonan derived from Gum Tragacanth. This suggests a relatively uniform distribution of xylose in the degradable part of XGA from apple and potato pectin. In addition, dimeric side chains of xylose were observed in digests of XGA from both pectins, which apparently did not hinder the action of XGH. From this it is assumed that Xyl–Xyl as well as Xyl substituted GalA residues are accepted in subsite −1 of xylogalacturonan hydrolase.

Keywords: Apple modified hairy regions; Potato modified hairy regions; Gum Tragacanth; Pectin; *endo*-Xylogalacturonan hydrolase

1. Introduction

Pectinases play an important role in processing plant materials to food products, such as depectinization of fruit juices, maceration of vegetables and fruit, and extraction of vegetable oils (Benen, van Alebeek, Voragen, & Visser, 2003; Grassin & Fauquembergue, 1996; Heldt-Hansen et al., 1996; Thibault & Ralet, 2001). A whole array of pectinases has been isolated from various plants, bacteria and fungi, including *Aspergillus niger*. These enzymes can be divided into those acting on the 'smooth' regions and those acting on the ramified 'hairy' regions (HR) of pectin (Benen, Vincken, & van Alebeek, 2002; Vincken, Voragen, & Beldman, 2003; Voragen, Beldman, & Schols, 2001). The HR, as isolated from apple, consists of xylogalacturonan and rhamnogalacturonan. In addition, the latter pectic polysaccharide can also be ramified with long neutral sugar side-chains (Schols & Voragen, 2002; Vincken, Schols, et al., 2003).

Xylogalacturonan (XGA) exists in various cell walls of plants (Thibault & Ralet, 2001; Voragen et al., 2001) and

Abbreviations: MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectometry; HPSEC, high-performance size-exclusion chromatography; HPAEC, high-performance anion-exchange chromatography; PAD, pulsed amperometric detection; RI, refractive index; XGH, xylogalacturonan hydrolase; GH, glycoside hydrolase; MHR, modified hairy regions; HR, hairy regions; p-MHR-S, saponified potato modified hairy regions; a-MHR-S, saponified apple modified hairy regions; AUA, anhydro-uronic acid; RGH, rhamnogalacturonan hydrolase; SEC, size exclusion chromatography; a-MHR-S-frA, saponified apple modified hairy regions fraction A; a-MHR-S-frB, saponified apple modified hairy regions fraction B; MS^2, mass fragmentation of a selected (parent) ion; MS^3, mass fragmentation of the selected daughter ion from the parent ion.

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has been structurally analyzed in several plant derived materials such as Gum Tragacanth (Aspinall & Baillie, 1963), apple pectin (Schols, Bakx, Schipper, & Voragen, 1995), soy pectin (Nakamura, Furuta, Maeda, Takao, & Nagamatsu, 2002) and pea pectin (Le Goff, Renard, Bonnin, & Thibault, 2001). In these pectins XGA consist of an α-(1→4)-linked D-galacturonic acid chain, which is substituted at O-3 with β-D-xylose side chains (Thibault & Ralet, 2001; Vincken, Schols, et al., 2003). Oligomeric side chains of xylose are proposed to exist in XGA from apple, soy, and pea pectin and contain 1,4-linked xylose residues (Oechslin, Lutz, & Amado, 2003), 1,4- and 1,2-linked xylose residues (Nakamura et al., 2002), and 1,2- and 1,3-linked xylose residues (Le Goff et al., 2001), respectively, based on sugar linkage analysis.

As XGA contributes to membrane fouling in the ultra filtration process for fruit juice clarification (Herweijer et al., 2003; Van der Vlugt-Bergmans, Meeuwsen, Voragen, & van Ooyen, 2000), it is crucial that it is completely degraded during the preceding enzymatic treatment of the fruit pulp. Degradation of XGA is possible with *exo*-polygalacturonases (Beldman et al., 1996; Kester, Benen, & Visser, 1999). In addition, the enzyme xylogalacturonan hydrolase (XGH), discovered in *Aspergillus tubingensis*, is able to degrade XGA in an endo fashion (Van der Vlugt-Bergmans et al., 2000). The corresponding gene for XGH from *A. tubingensis* has been over-expressed in the *A. niger* "PlugBug" (Van Dijck, 1999) and the forthcoming enzyme was subsequently purified (Beldman, Vincken, Meeuwsen, Herweijer, & Voragen, 2003). This enzyme has been studied for its pH and temperature stability as well as its activity towards xylogalacturonans with different degrees of xylose substitution (Beldman et al., 2003). Additionally, the mode of action of this enzyme towards XGA from alkali and acid modified Gum Tragacanth has been investigated by analysis of the structure of the degradation products (Zandleven, Beldman, Bosveld, Benen, & Voragen, 2005). In this study, XGH is investigated with respect to its activity towards XGA present in apple and potato MHR. Also some aspects about the subsite model of XGH are discussed based on the structure of XGA oligosaccharides formed.

2. Experimental

2.1. Substrates

Saponified potato MHR (p-MHR-S) and saponified apple MHR (a-MHR-S) were prepared as described (Schols, Posthumus, & Voragen, 1990). Enzymatic liquefaction of apples for the preparation of a-MHR-S was performed with a commercial enzyme preparation (Ultra-SP; Novozymes, Copenhagen, Denmark). An amount of 7.5 g of this substrate was further treated with rhamnogalacturonan hydrolase (RGH) and fractionated by preparative size exclusion chromatography (SEC) in order to obtain a XGA enriched a-MHR-S substrate. This procedure was done as follows: a-MHR-S (5 mg/mL final concentration) was treated with purified RGH (Novozymes, Copenhagen, Denmark; Kofod et al., 1994; Schols et al., 1990) for 24 h at 40 °C in 50 mM NaOAc buffer (pH 5.6). The final enzyme concentration was 3.33 μg/mL. The enzyme was inactivated by heating the reaction mixture for 10 min at 100 °C. Subsequently, the RGH treated a-MHR-S was dialyzed against water. A yield of 6.15 g RGH treated a-MHR-S was obtained after freeze-drying. A portion of 6 g from this material was applied onto a Superdex 30 PG column (5.5 L, 70 × 10 cm) using millipore water as eluent with a flow rate of 25 ml/min. Two fractions, a-MHR-S-frA (pooled fractions 18–27) and a-MHR-S-frB (pooled fractions 27–41), respectively, were collected (Fig. 1) and subsequently freeze-dried. The fractions a-MHR-S-frA and a-MHR-S-frB gave a yield of 1.15 and 2.0 g, respectively.

2.2. Neutral sugar composition and uronic acid content

p-MHR-S and a-MHR-S-frB were analyzed for their neutral sugar composition by gas chromatography (Englyst & Cummings, 1984), using inositol as an intern standard. The samples were treated with 72% (w/w) H_2SO_4 for 1 h at 30 °C, followed by hydrolysis with 1 M H_2SO_4 for 3 h at 100 °C. Prior to analysis by gas chromatography, the released neutral sugars were converted to their alditol acetates.

The uronic acid content of p-MHR-S and a-MHR-S-frB was determined by an automated *m*-hydroxydiphenyl assay using an autoanalyser (Skalar Analytical BV, Breda, The Netherlands; Blumenkrantz & Asboe-Hansen, 1973; Thibault & Robin, 1975).

2.3. HPSEC analysis

High-performance size-exclusion chromatography was performed on three TSK columns (7.8 mm ID × 30 cm per column) in a series of TSKGel G4000 PWXL, TSKGel G3000 PWXL and TSK2500 PWXL, in combination with a PWX-guard column. All columns were from TosoHaas (Japan).

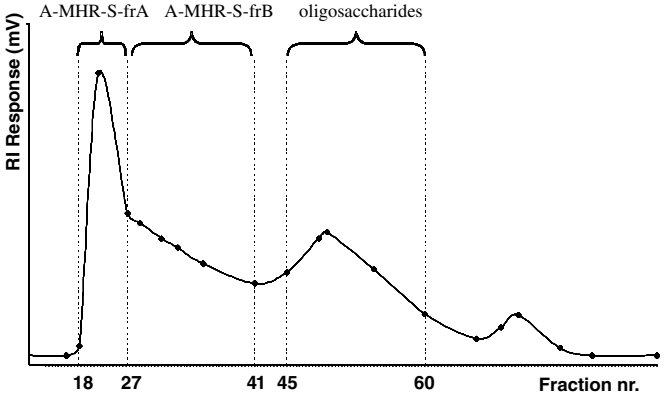


Fig. 1. Size-exclusion chromatography of RG-hydrolase treated a-MHR-S on Superdex 30 PG.

Elution took place at 30 °C using 0.2 M sodium nitrate at a flow rate of 0.8 mL/min. The eluate was monitored using a refractive index (RI) detector. The calibration was performed using pullulan standards with a molecular weight of 404, 112, 47.3, 22.8, 5.9 kDa and GalA (0.196 kDa).

2.4. HPAEC at pH 12

High-performance anion-exhange chromatography was performed on a Dionex system equipped with a CarboPac PA-1 column (4 mm ID × 250 mm) in combination with a CarboPac PA Guard column (3 mm ID × 25 mm) and pulsed amperometric detection (PAD). The elution (1 mL/min) of the oligosaccharides was performed by using a combination of two linear gradients, starting with 0–600 mM sodium acetate in 100 mM NaOH for 50 min, followed by 600–1000 mM sodium acetate in 100 mM NaOH for 5 min.

2.5. MALDI-TOF mass spectrometry

Prior to mass spectrometry analysis, samples were desalted by treatment with H^+-Dowex AG 50W X8 (Biorad, Hercules, CA), using a final concentration of 350 mg H^+-Dowex per mL digest. The desalted digests were mixed with a matrix solution (1 μL of sample in 1 μL of matrix) and applied on a MALDI sample plate. The matrix solution was prepared by dissolving 9 mg of 2,5-dihydroxybenzoic acid in a 1-mL mixture of acetonitrile:water (300 μL:700 μL).

Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) analysis was performed using an Ultraflex workstation (Bruker Daltonics, Hamburg, Germany) equipped with a nitrogen laser of 337 nm. The mass spectrometer was selected for positive ions, which are after a delayed extraction time of 200 ns, accelerated to a kinetic energy of 12 kV. The ions were detected in the reflector mode. External calibration of the mass spectrometer was performed using a mixture of maltodextrines (mass range 365–2309).

2.6. Electrospray mass spectrometry

Electrospray MS was performed on a LTQ Ion-trap (Thermo, San Jose, CA). Sample was running through a transferring capillary (100 μm ID) and a spraying capillary with an ID of 50 μm at a flow rate of 1 μL/min. MS analysis was carried out in the positive mode using a spray voltage of 4.5 kV and a capillary temperature of 200 °C. The capillary voltage and tube lens were set at 33 and 225 V, respectively. MS^2 and higher was executed using a window of 1 m/z and a relative collision energy of 20–30%.

2.7. Enzyme incubations

Purified XGH, prepared as described (Beldman et al., 2003), was used to degrade a-MHR-S-frA, a-MHR-S-frB and p-MHR-S (final concentration of 5 mg/mL). These substrates were dissolved in 50 mM NaOAc (pH 3.5) and incubated with XGH (final concentration of 0.35 μg/mL) for 20 h at 30 °C. The enzyme had a specific activity of 150 U/mg. Subsequently, the enzyme was inactivated by heating the reaction mixtures for 10 min at 100 °C. The digested substrates including their controls (i.e., a-MHR-S-frB and p-MHR-S, which were not treated with XGH) were desalted by treatment with H^+-Dowex AG 50W X8 (Biorad, Hercules, CA), using a final concentration of 350 mg H^+-Dowex per mL digest. The desalted samples were subsequently analyzed by HPSEC and HPAEC using a set of known XGA-oligosaccharides for identification (Zandleven et al., 2005).

2.8. Product analysis of XGH degraded a-MHR-S-frB and p-MHR-S at different time intervals

Oligosaccharide digests were prepared from a-MHR-S-frB and p-MHR-S as described above. At 0 min (blank), 30 min, 1 h, and 20 h, a sample was taken and incubated at 100 °C for 10 min to inactivate the enzyme. The obtained oligosaccharides were desalted and subsequently analyzed by HPAEC, and by MALDI-TOF MS or electrospray MS.

2.9. Fractionation of XGH degraded a-MHR-S-frB and p-MHR-S

A-MHR-S-frB and p-MHR-S were degraded by XGH using the same conditions as described in Section 2.7. These digests were fractionated by HPAEC on the Carbopac PA-1 column as described. Samples of the fractionated XGA oligosaccharides were desalted and analyzed by MALDI-TOF MS. Fractions containing $GalAXyl_2$ were pooled, desalted by H^+-Dowex AG 50W X8 (Biorad, Hercules, CA) and freeze-dried until further use.

2.10. Degradation of $GalAXyl_2$ by β-xylosidase

$GalAXyl_2$, as isolated from 25 mg of p-MHR-S and a-MHR-S-frB, respectively, was degraded by β-(1,4)-xylosidase (E.C.3.2.1.37; GH 3) from *Aspergillus awamori*. The conditions were as follows: the total yield of $GalAXyl_2$ was dissolved in 500 μL 50 mM NaOAC (pH 4.0) and incubated with β-(1,4)-xylosidase (final concentration of 0.85 μg/mL) for 20 h at 30 °C. The enzyme preparation had a specific activity of 1.45 U/mg.

Degradation of $GalAXyl_2$ was analyzed by HPAEC and MALDI-TOF MS, as described in Sections 2.4 and 2.5, respectively.

3. Results and discussion

3.1. Preparation of xylogalacturonan enriched fractions from a-MHR-S

In the current model for pectin, in particular for apple pectin, XGA is linked to rhamnogalacturonan I (Schols

et al., 1995). To facilitate the isolation of XGA, a-MHR-S was treated with RGH and subsequently fractionated by size-exclusion column chromatography. From this, XGA enriched fractions were obtained that were used for further degradation by XGH.

As shown in Fig. 1, column chromatography of RGH degraded a-MHR-S resulted in three distinct peaks, with a broad shoulder on the first peak. Fractions 18–27 and 27–41 were pooled and named a-MHR-S-frA and a-MHR-S-frB, respectively. As determined by HPSEC and HPAEC (results not shown), these fractions contained high molecular weight polysaccharides while fractions 45–60 contained oligosaccharides. The presence of high molecular weight XGA was further investigated in these polymer fractions by degradation with XGH and analysis of the products by HPLC. HPSEC and HPAEC analysis both demonstrated the formation of XGA oligosaccharides from a-MHR-S-frB (see Section 3.3) while only minor production of XGA oligosaccharides was observed from a-MHR-S-frA (results not shown). Apparently, this latter polymer fraction consists of RG-I structures with XGH-undegradable polysaccharides.

Based on the distinct formation of XGA oligosaccharides from a-MHR-S-frB it was decided to continue product analysis from this fraction only.

3.2. Sugar compositions of a-MHR-S-frB and p-MHR-S

The sugar compositions of a-MHR-S-frB and p-MHR-S are presented in Table 1. The amounts of xylose and galacturonic acid indicate that these substrates contain a significant amount of XGA. The arabinose content confirms the presence of long side chains of arabinan in rhamnogalacturonan I, in particular in the case for a-MHR-S-frB (Schols et al., 1990).

3.3. HPLC analysis of XGH degraded a-MHR-S-frB and p-MHR-S

The action of XGH was studied towards XGA in a-MHR-S-frB and p-MHR-S. Degradation of these substrates was followed by HPSEC and HPAEC as shown in Figs. 2 and 3, respectively.

From previous studies of RGH treated a-MHR-S (Schols et al., 1995) it is known that XGA, linked to rhamnogalacturonan, elutes in the first peak in the HPSEC chromatogram. This peak represents the high molecular weight fraction of RGH treated a-MHR-S-frB. Upon XGH treatment, this XGA-containing peak (which elutes around 19–22 min; Fig. 2), disappeared while in the case of XGH treatment of p-MHR-S only a partial decrease of the corresponding peak was observed. This shows that XGA degradation took place in both substrates. However, the partial decrease of the XGA containing peak of p-MHR-S can be ascribed to the presence of XGH-resistant parts of XGA and/or other high molecular weight polysaccharides of RG-I. As shown in Fig. 2, three peaks appeared at retention times around 30–33 min upon XGH treatment of a-MHR-S-frB and p-MHR-S. The latter peak is eluting in the size range of GalA (i.e., around 32.5 min), while the other two peaks represent XGA oligosaccharides.

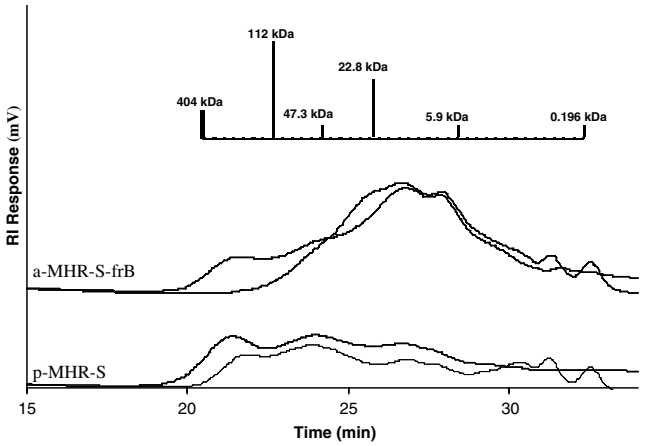


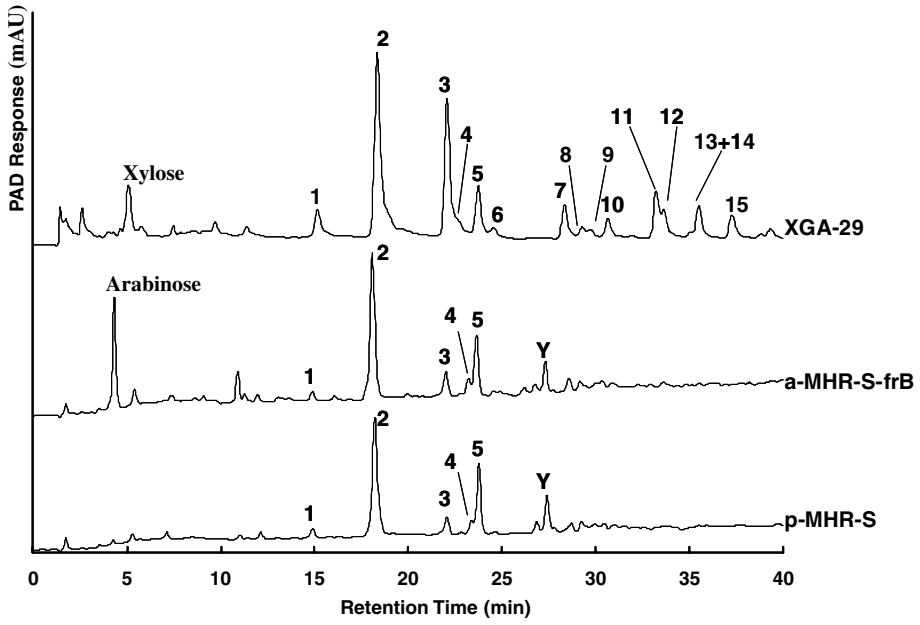
Fig. 2. HPSEC of a-MHR-S-frB and p-MHR-S, respectively, untreated (thin line) and treated for 20 h with XGH (thick line). The ruler shows the molecular weights of the Pullunan standards (404, 112, 47.3, 22.8, and 5.9 kDa) and GalA (0.196 kDa).

The formation of XGA oligosaccharides from a-MHR-S-frB and p-MHR-S was also analyzed by HPAEC. To identify these oligosaccharides, a mixture of XGA oligosaccharides with known structures, derived from Gum Tragacanth (XGA-29), was used (Zandleven et al., 2005; Fig. 3). As shown in Fig. 3, HPAEC analysis demonstrated the formation of XGA oligosaccharides from the XGH degradable parts of XGA in a-MHR-S-frB and p-MHR-S. A similar type of XGA oligosaccharide production was observed for both substrates. Compared to the large number of different oligosaccharides present in the Gum Tragacanth digest, only a few distinct XGA oligosaccharides were released from a-MHR-S-frB and p-MHR-S. The main product was the disaccharide GalAXyl, while a significant amount of $GalA_2Xyl_2$ and minor quantities of GalA, $GalA_2$ and $GalA_2Xyl$ were found. Other unidentified oligosaccharides,

Table 1
Sugar composition (mol%) of a-MHR-S-frB and p-MHR-S

Substrate	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	AUA	(w/w%)[a]	Xyl:GalA
a-MHR-S-frB	3	0	61	9	0	9	0	19	75	0.47
p-MHR-S	5	0	18	12	1	15	1	49	60	0.24

[a] Percentage of carbohydrate in the sample, based on dry weight.



1) GalA	○	4) $GalA_2$	●○	7) $GalA_3Xyl$	10) $GalA_3Xyl'$	13) $GalA_4$
2) GalAXyl	○ with X	5) $GalA_2Xyl_2$	●○ with X X	8) $GalA_3Xyl_2$	11) $GalA_4Xyl$	14) $GalA_4Xyl'$ + $GalA_5Xyl_2$
3) $GalA_2Xyl$	●○ with X	6) $GalA_2Xyl'$	●○ with X	9) $GalA_3$	12) $GalA_4Xyl_2$	15) $GalA_5Xyl$

Fig. 3. HPAEC of XGH degraded XGA-29 (from Gum Tragacanth; used as standard), a-MHR-S-frB, and p-MHR-S. The table summarizes the XGA oligosaccharides of which some have been structurally characterized (Zandleven et al., 2005). The accentuated XGA oligosaccharides (') are isomers with different distribution of xylose over the GalA backbone ●, GalA; ○, reducing GalA and Y = XGA oligosaccharide with unknown structure.

including an oligosaccharide eluting at 27.4 min (shown as peak Y) were also observed. Large XGA oligosaccharides as obtained from XGA-29 (which are shown as peaks 7–15; Fig. 3), were hardly produced from a-MHR-S-frB and p-MHR-S.

The appearance of unknown oligosaccharides in the a-MHR-S-frB and p-MHR-S digests, necessitated further identification by MALDI-TOF MS and is described in Section 3.4.

Free arabinose was also found in the a-MHR-S-frB digest. As the XGH preparation does not contain arabinofuranosidase, this arabinose is probably liberated by acid hydrolysis during the incubation temperature and inactivation step at 100 °C (pH 3.5) of the digestion mixture. Indeed a lower amount of free arabinose (about 60% reduction) was detected by HPAEC analysis of a-MHR-S-frB, which was not treated at 100 °C (results not shown). The formation of free arabinose was even further reduced (about 90% reduction) when a-MHR-S-frB was incubated at pH 6.0.

The susceptibility for hydrolysis of the arabinosyl linkages at a low pH has also been reported before (Beldman, Schols, Pitson, Searle-van Leeuwen, & Voragen, 1997). Also free arabinose was expected in the p-MHR-S digest, but could however not be detected by HPAEC analysis (results not shown). This is probably related to the lower arabinose content of this substrate (Table 1) as well as to structural differences of this arabinan (Beldman et al., 1997).

3.4. Product analysis of XGH degradable parts of XGA from a-MHR-frB and p-MHR-S at different time intervals

a-MHR-S-frB and p-MHR-S were degraded by XGH for different time intervals and analyzed by HPAEC (Fig. 4) and MALDI-TOF MS (Fig. 5) for the presence of XGA oligosaccharides.

Degradation of a-MHR-S-frB and p-MHR-S during 30 min and 1 h resulted mainly in the production of the disaccharide GalAXyl (Fig. 4), which was also reported before for XGH treatment of a-MHR-S (Beldman et al., 2003) and XGA-29 from Gum Tragacanth (Zandleven et al., 2005), respectively. In addition, a significant amount of $GalA_2Xyl_2$ was observed as degradation product from a-MHR-S-frB and p-MHR-S.

Comparing the HPAEC profiles of Figs. 3 and 4 for the corresponding samples of a-MHR-S-frB and p-MHR-S (20 h of treatment with XGH) only some minor differences

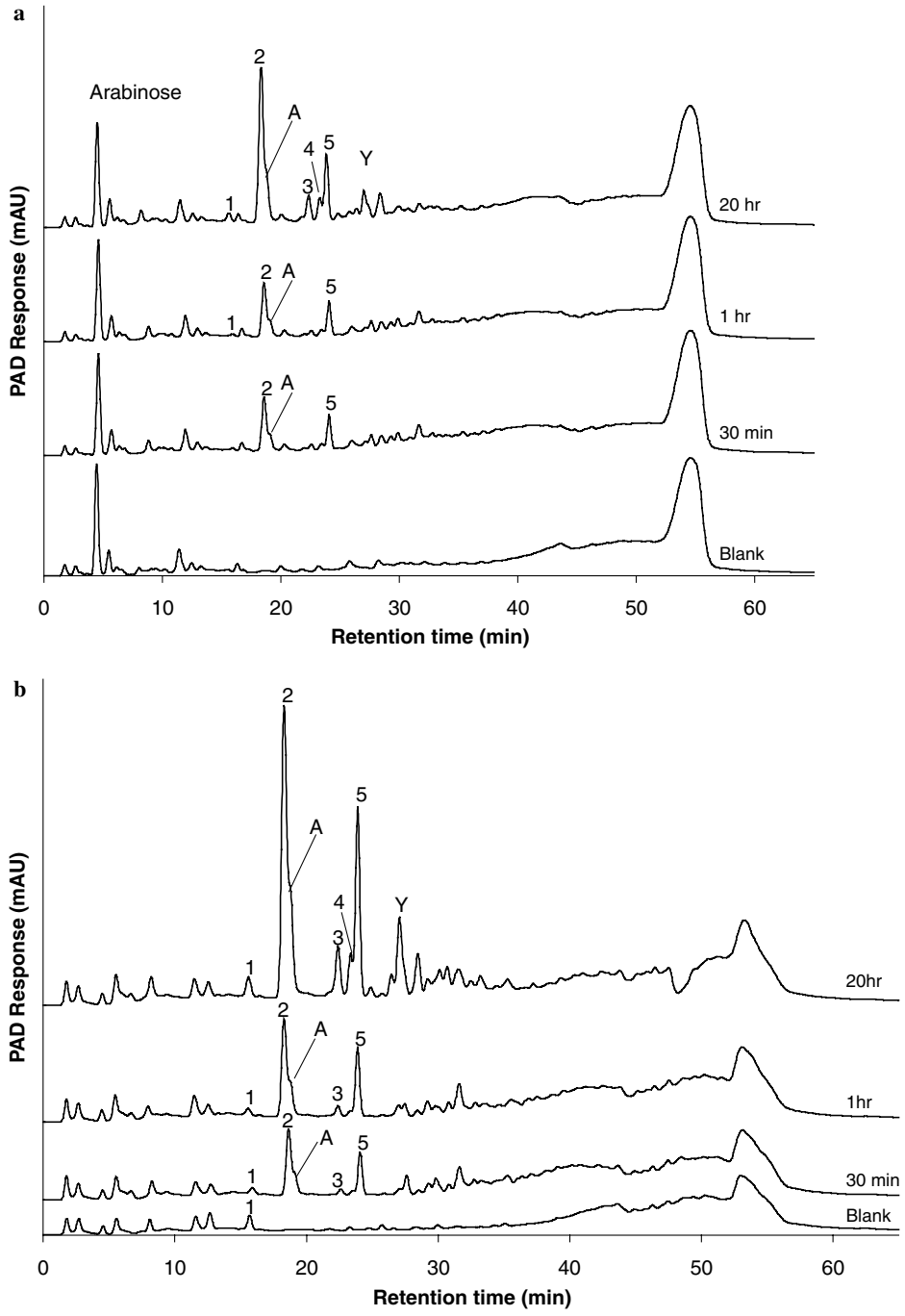


Fig. 4. HPAEC of (a) a-MHR-S-frB after treatment with XGH for 0 min (blank), 30 min, 1 h and 20 h; and (b) p-MHR-S after treatment with XGH for 0 min (blank), 30 min, 1 h and 20 h. All samples were treated at 100 °C for 10 min to inactivate the enzyme. Numbers refer to XGA oligosaccharides as shown in the table in Fig. 3. A = $GalAXyl_2$, Y = XGA oligosaccharide with unknown structure.

were observed, which falls within the range of experimental variance.

The HPAEC results were confirmed by MALDI-TOF MS analysis of the products from p-MHR-S (Fig. 5) and a-MHR-S-frB (results not shown) for the samples after 20 h of incubation. The MS spectra of both degraded substrates showed the main product GalAXyl, and significant amounts of $GalA_2Xyl_2$, $GalA_2$ and $GalA_2Xyl$. Two novel XGA oligosaccharides, $GalAXyl_2$, with a m/z of 481, and $GalA_2Xyl_3$, with a m/z of 789, were observed as products from both substrates. The formation of $GalAXyl_2$ and $GalA_2Xyl_3$ by XGH suggests the presence of dimeric side chains in XGA of these pectic polysaccharides from apple and potato. In order to confirm this, the structure of $GalAXyl_2$ and $GalA_2Xyl_3$ was investigated further (see Section 3.5). As previously hypothesized, XGH is a processive enzyme which prefers to act between two xylosylated GalA units, based on its

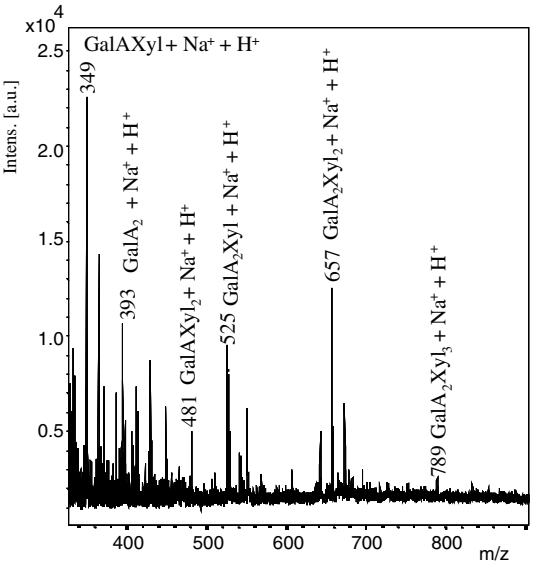


Fig. 5. Positive mode MALDI-TOF MS spectrum of p-MHR-S after treatment with XGH for 20 h. The *m*/*z* masses of the XGA oligosaccharides include H^+ and Na^+.

mode of action towards XGA from Gum Tragacanth (Zandleven et al., 2005). This report confirms this hypothesis, based on the predominant production of GalAXyl by XGH treatment of a-MHR-S-frB and p-MHR-S. Furthermore, these results indicate that a rather regular distribution of xylose side chains over the galacturonan back-bone exists in the degradable parts of XGA of both sources. This is based on the relatively little variation in oligosaccharide products in the XGH digests.

Degradation of XGA from Gum Tragacanth by XGH showed a large variety of XGA oligosaccharides (Zandleven et al., 2005), indicating a random distribution of xylose side chains. This is confirmed in the present study and can be explained by the preparation procedure of XGA from Gum Tragacanth, involving a mild TFA treatment (Beldman et al., 2003), which leads to a random release of xylose residues from the XGA substrate.

The oligosaccharide $GalAXyl_2$ was identified in the digests by MALDI-TOF MS, but could not yet be identified in the corresponding HPAEC chromatograms of these samples. For this, XGH-degraded p-MHR-S was fractionated by HPAEC and the presence of $GalAXyl_2$ in the fractions was analyzed by MALDI-TOF MS. It was found that $GalAXyl_2$ and GalAXyl co-elute during HPAEC analysis (results not shown). However, due to experimental variance, in some cases these oligosaccharides could partly be separated by HPAEC, as for instance shown in Fig. 4. In this figure $GalAXyl_2$ eluted slightly later than GalAXyl and is indicated as "shoulder" A. These results also demonstrate that the production of $GalAXyl_2$ from a-MHR-S-frB and p-MHR-S by XGH is significant. As shown in Fig. 4, the peak height for this oligosaccharide was almost half of the peak height for GalAXyl, which implies that a substantial amount of dimeric side chains of xylose is present in xylogalacturonans from apple and potato pectin. In line with these results, it is hypothesized that peak Y (Figs. 3 and 4) corresponds to $GalA_2Xyl_3$, based on the elution behavior of neighboring XGA oligosaccharides.

3.5. Structural investigation on $GalAXyl_2$ and $GalA_2Xyl_3$ from p-MHR-S and a-MHR-S-frB

The fine structure of $GalAXyl_2$ and $GalA_2Xyl_3$ in XGH digests of p-MHR-S and a-MHR-S-frB was studied by electrospray MS (Figs. 6 and 7). As shown in Fig. 6, the presence of $GalAXyl_2$ is confirmed for p-MHR-S, as demonstrated by the fragmentation of this oligosaccharide in MS^2 mode. The appearance of a fragment at *m*/*z* 305, corresponding to two linked xyloses, demonstrates the dimeric side-chain in $GalAXyl_2$. This fragment was observed at a higher abundance when MS^3 analysis was performed on a fragmentation product of $GalAXyl_2$ at *m*/*z* 463, which corresponded to $GalAXyl_2$ with the loss of OH^- and H^+ (Fig. 6; inserted panel).

An identical fragmentation spectrum was obtained for $GalAXyl_2$ that was formed by fragmentation of $GalA_2Xyl_3$ in the MS^2 mode (for the latter, see Fig. 7). This confirms that a dimeric side chain of xylose also exists in the XGA oligosaccharide $GalA_2Xyl_3$.

Similar results were acquired for $GalAXyl_2$ and $GalA_2Xyl_3$ present in the a-MHR-S-frB digest (results not shown), which points out that these dimeric side-chains of xylose are also present in XGA from apple pectin.

Based on linkage analysis, the existence of side chains longer than one xylose unit in XGA has been indicated for apple (Oechslin et al., 2003) and pea pectin (Le Goff et al., 2001). In this report, the occurrence of dimeric side chains of xylose in XGA in apple and potato pectin is demonstrated based on the structures of the oligosaccharides $GalAXyl_2$ and $GalA_2Xyl_3$.

It is generally accepted that the single terminal xylose is in the β-form and is linked at the O-3 position of GalA in XGA (Beldman et al., 2003; Le Goff et al., 2001; Schols et al., 1995; Vincken, Schols, et al., 2003). From this, we assume that the dimeric side chains in XGA from a-MHR-S-frB and p-MHR-S are also linked at this position.

The nature of the linkage between the xyloses in the dimeric side chains in XGA from apple and potato pectin is unknown. In order to obtain information about the anomeric form of the terminal xylose residue, we purified the oligosaccharide $GalAXyl_2$ from a p-MHR-S and a-MHR-S-frB digest to near homogeneity (>95%) by analytical HPAEC. Subsequently, it was treated with β-(1,4) xylosidase from *A. awamori* and analyzed by HPAEC and MALDI-TOF MS (results not shown). $GalAXyl_2$ from both sources was degraded by this enzyme to free xylose and GalAXyl, which implies that the two xyloses are β-linked. Linkage analysis of apple pectin (Schols et al.,

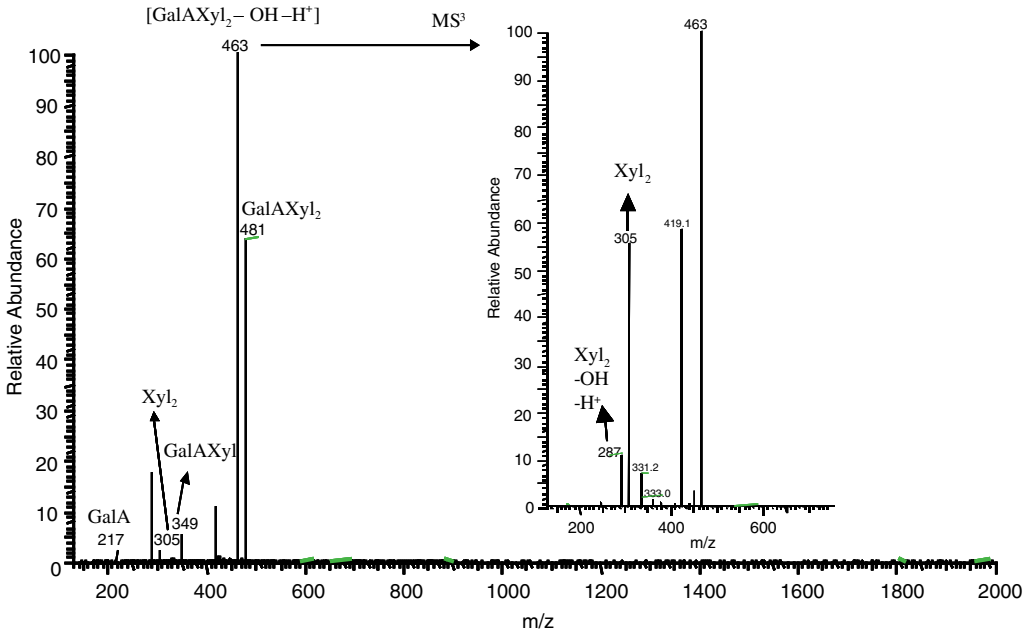


Fig. 6. MS^2 scan of $GalAXyl_2$ at m/z 481 including a MS^3 scan of a fragment of $GalAXyl_2$ at m/z 463 (inserted panel). The m/z value of $GalAXyl_2$ include the addition of an H^+ and Na^+. The fragment at m/z 463 represents $GalAXyl_2$ with the loss of OH^- and H^+.

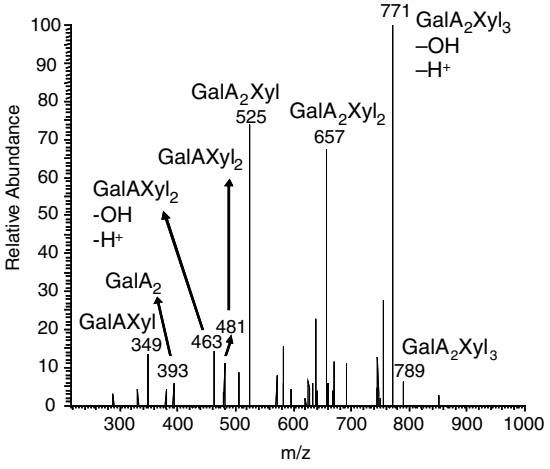


Fig. 7. MS^2 scan of $GalA_2Xyl_3$ at m/z 789. The m/z value of $GalA_2Xyl_3$ include the addition of an H^+ and Na^+.

1995) and cellulase degraded cellulosic residues of apples (Oechslin et al., 2003), respectively, show that 1,4-linked xylose residues prevail as non-terminal xylose in apple pectin. Schols et al. (1995) found that 90% of the total amount of non-terminal xylose residues exist as 1,4-linked xylose residues, whereas Oechslin et al. (2003) showed that exclusively 1,4-linked xylose residues are present. From these studies it is reasonable to assume that the dimeric side chains of xylose present in a-MHR-S-frB could also be 1,4-linked.

The fact that a-MHR-S-frB and p-MHR-S were successfully degraded by XGH with concomitant production of $GalAXyl_2$ and $GalA_2Xyl_3$ implies that the dimeric side chains in XGA from these sources did not hinder the action of the enzyme. We already showed that xylosylated GalA units can accommodate subsites −1 and +1 of XGH (Zandleven et al., 2005). This study indicates that GalA residues that have a side chain of two xylose moieties, can also fit in these subsites. This is illustrated in Fig. 8, which shows the formation of $GalAXyl_2$ from a specific part of a XGA molecule, in two consecutive steps. Based on this model, it can be speculated that the dimeric side chains of xylose in XGA from apple and potato pectin are probably pointed towards the outside of the binding cleft of XGH, when the enzyme–substrate complex is formed.

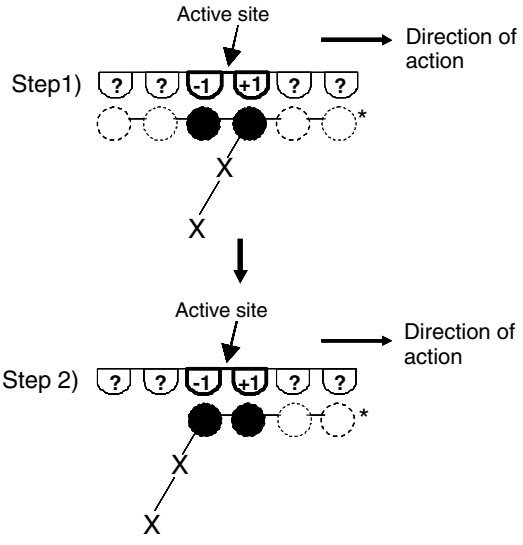


Fig. 8. Representation of the proposed action of XGH to produce $GalAXyl_2$ from XGA in apple and potato pectin. ●, GalA; X = xylose. The asterix (*) indicates the reducing end.

Conclusively, this study confirms the occurrence of XGA in apple and potato pectin by the use of XGH which specifically acts towards this polymer. The degradable part of XGA from both sources is similar in structure, contains a regular distribution of xylose side chains, and also contains dimeric side chains of xylose which are presumably β-1,4-linked in the case for apple pectin.

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