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Purification and characterisation of two exo-polygalacturonases from *Aspergillus niger* able to degrade xylogalacturonan and acetylated homogalacturonan

T. Sakamoto¹, E. Bonnin^{*}, B. Quemener, J.-F. Thibault

Unité de Recherche sur les Polysaccharides, leurs Organisations et Interactions, INRA, BP 71627, 44316 Nantes Cedex 03, France

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Abstract

Two exo-polygalacturonases (EC 3.2.1.67) were purified from a commercial *Aspergillus niger* enzyme preparation by ammonium sulfate precipitation, preparative electrofocusing, anion-exchange and size-exclusion chromatographies. The enzymes had molar masses of 82 kDa (exo-PG1) and 56 kDa (exo-PG2). Exo-PG1 was stable over wider pH and temperature ranges than exo-PG2. Addition of 0.01 mM HgCl₂ increased the exo-PG2 activity 3.4 times but did not affect exo-PG1. Analysis of the reaction products of (reduced) pentagalacturonate by high-performance anion-exchange chromatography revealed that both enzymes split the substrate from the non-reducing end in a multi-chain attack mode. Exo-PG1 had a broad specificity towards oligogalacturonates with different degrees of polymerisation, while digalacturonate was the most favorable substrate for exo-PG2. Both enzymes degraded xylogalacturonan from pea hull in an exo manner to produce galacturonic acid and Xyl–GalA disaccharide, as identified by electrospray ionization-ion trap mass spectrometry (ESI-ITMS). Moreover, exo-PGs split acetylated homogalacturonan in an exo manner, producing galacturonic acid and acetylated galacturonic acid, as shown by ESI-ITMS.

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1. Introduction

Pectins are acidic polysaccharides of high molar mass and one of the main components of the primary cell walls of dicotyledons. Structural analysis of pectins is important because of their function in the cell wall as a cementing agent [1], their role during ripening of fruit [2], in food processing [3], and as dietary fiber [4]. Pectins consist of homogalacturonic or “smooth” regions, and rhamnogalac-

turonic or “hairy” regions carrying neutral sugar side chains. Galacturonic acid (GalA) residues may be substituted by different compounds such as methanol, acetic acid, and xylose, in the “smooth” as well as in the “hairy” region. The carboxyl groups of pectin are partially esterified with methanol [5], and the hydroxyl groups at position 2 or/and 3 of GalA residues are sometimes acetylated [6,7]. The degrees of methylation (DM) or acetylation (DAc) are defined as the number of esterified carboxyl or hydroxyl groups per 100 GalA units. Xylogalacturonan, an α -1,4-D-galacturonan to which D-xylopyranose units are attached at position O-3 in the β -configuration as side chains, can be found in cell walls such as Japanese radish [8], carrot [9], soy [10], and apple [11]. In pea hull xylogalacturonan, the molar ratio of xylose to GalA is 0.72. GalA residues carry mainly single terminal xylose residues attached on O-3 and few short side chains of xylosyl residues linked on O-2 or sometimes on O-3 [12].

Enzymatic degradation of pectic polysaccharides is efficient for their structural analysis since enzymes have strict substrate specificities. Enzymes that degrade arabinan, gal-

Abbreviations: AHG, acetylated homogalacturonan; DAc, degree of acetylation; DM, degree of methylation; DP, degree of polymerisation; ESI-ITMS, electrospray ionization-ion trap mass spectrometry; GalA, galacturonic acid; HG, homogalacturonan; HPAEC, high-performance anion-exchange chromatography; HPLC, high-performance liquid chromatography; PG, polygalacturonase

^{*} Corresponding author. Tel.: +33-2-4067-5058; fax: +33-2-4067-5066.

E-mail addresses: sakamoto@biochem.osakafu-u.ac.jp (T. Sakamoto), bonnin@nantes.inra.fr. (E. Bonnin).

¹ Present address. Division of Applied Biological Chemistry, Graduate School of Agriculture and Biological Sciences, Osaka Prefecture University, Osaka 599-8531, Japan.

actan, and rhamnogalacturonan have been used for the elucidation of the structure of “hairy” fragments of pectins [13–15]. On the other hand, endo-polygalacturonase (endo-PG), pectin lyase, and exo-polygalacturonase (exo-PG) have been utilized in analysing methyl-esterification pattern in “smooth” regions or quantifying the amount of non-esterified GalA residues of homogalacturonic region [16–18]. Recently, exo-PGs produced by both *Aspergillus aculeatus* [10] and *Aspergillus tubingensis* [19] have been reported to be able to split the saponified soy and apple xylogalacturonan, respectively. Moreover, one enzyme termed endo-xylogalacturonan hydrolase found in *A. tubingensis* [20], was able to cleave a xylose-substituted GalA backbone but not homogalacturonan (HG). These enzymes would be useful for elucidation of the fine structure of xylogalacturonan.

Extensive degradation of pectic polysaccharides is also important for processing plant materials such as clarification of fruit juices [21]. Better understanding of characteristics of the enzymes involved in the degradation of pectin makes their use more effective in the biotechnological fields. Enzymes that cleave HG in an exo manner have been classified into two types depending on their reaction products. Exo-PGs (EC 3.2.1.67) and exo-polygalacturonosidases (EC 3.2.1.82) [22] release monomeric and dimeric GalA, respectively. Both types of enzymes belong to family 28 in a classification of glycosyl hydrolases based on amino acid sequence similarities [23]. So far, some *Aspergillus niger* exo-PGs have been purified from the mycelium extracts by Mill [24,25] and also from the culture filtrates by Hara et al. [26]. Kester and Visser [27] have found an exo-PG activity in a commercial pectinase preparation (Pectinase K2B 078) of *A. niger*. However, information on the properties and the reaction modes towards various substrates of the enzymes are not fully available. In this study, we purified two exo-PGs from a commercial *A. niger* enzyme preparation (Pectinex AR, Novozymes) and the characteristics of the enzymes were studied.

2. Materials and methods

2.1. Enzyme

The commercial enzyme preparation Pectinex AR from *A. niger* was kindly provided by Novozymes (Fontenay-sous-Bois, France).

2.2. Substrates

Polygalacturonic acid was purchased from Sigma. Sugar beet pectin was extracted from the extruded sugar beet pulp (Générale Sucrière, France) with water [28]. De-esterification of sugar beet pectin was carried out at pH 12 for 24 h at 4 °C. HG was obtained by hydrolysis of de-esterified beet pectin in 0.1 M HCl for 72 h at 80 °C [29]. Acetylated HG

(AHG) was the non-degradable sugar beet pectin after treatment with pectin methylesterases, rhamnogalacturonase, endo-arabinanase, and endo-galactanase [28]. β -1,4-galactan was obtained from potato pulp (Roquette Frères Company, Lestrem, France) and α -1,5-arabinan from sugar beet pulp. Both preparations were described previously [30]. Pea hull xylogalacturonan was isolated according to the method of Le Goff et al. [12]. Highly methylated pectin (Grinsted™ Pectin URS 1200; E81) was a kind gift of Danisco-Cultor (Denmark). Characteristics of polysaccharides are summarized in Table 1. Oligogalacturonates with different degrees of polymerisation (DPs) were obtained as described by van Alebeek et al. [32]. Reduced pentagalacturonate was prepared by reduction of reducing ends of the substrate with 10 mM NaBH₄ in 25 mM NaOH at 30 °C for 4 h [33].

2.3. Enzyme assay

A typical assay for PG activity was performed by measuring the release of reducing groups in a reaction mixture containing polygalacturonic acid (1 g/l) in 50 mM sodium acetate buffer, pH 4.0, and enzyme sample at 40 °C. Reducing sugars were measured by the method of Nelson [34] using GalA as the standard. One unit of enzyme activity was defined as the amount of enzyme that formed reducing groups corresponding to 1 μ mol of GalA in 1 min.

Discrimination between exo-PG and endo-PG activities after column chromatographies was done using high-performance anion-exchange chromatography (HPAEC). Enzyme was incubated with polygalacturonic acid under the standard conditions. An aliquot was withdrawn at intervals and boiled for 5 min to stop the reaction. Concentration of reducing sugars in the mixture was determined by the method of Nelson. A sample with a concentration of about 50 μ g/ml of reducing sugars was analysed by HPAEC and the ratio of peak areas of mono to digalacturonate was calculated. The enzyme fractions containing much exo-PG activity show higher values of this ratio than those of less amount of the activity.

Table 1
Sugar compositions of the substrates

Substrate	Sugar composition (mg/g)					DAc (%)	DM (%)
	Rha	Ara	Xyl	Gal	AUA		
Sugar beet pectin	30	213	3	53	414	49	51
De-esterified beet pectin	32	202	3	54	461	26	15
AHG	35	53	2	53	586	45	24
HG	2	0	0	0	804	<1	<3
E81 ^a	14	3	–	44	877	–	81
Xylogalacturonan ^b	–	–	282	–	413	3	0

AUA: anhydrouronic acid, DAc: degree of acetylation, DM: degree of methylation.

^a Data from the Ref. [31].

^b Data from the Ref. [12].

The sensitivity of the enzymes to HgCl_2 and GalA was examined by adding 0.01 and 0.32 mM, respectively, to the reaction mixture containing HG (2 g/l) in 50 mM sodium acetate–HCl buffer, pH 3.6. Amount of GalA released was quantified by HPAEC.

Hydrolysis of reduced pentagalacturonate was carried out by incubating the reaction mixture containing 1.25 mU of the enzyme and 0.1 ml of 0.1 mM substrate in 50 mM sodium acetate–HCl buffer, pH 3.6, at 40 °C for 15 min, and the products were analysed by HPAEC.

Activities on arabinan and galactan were tested by incubating 10 mU of the enzyme with 200 μl of the substrate (5 g/l) in 50 mM sodium acetate buffer, pH 4.0, and at 40 °C overnight followed by measurement of reducing sugars in the reaction mixture by the method of Nelson.

2.4. Purification of *exo*-PGs

Pectinex AR was dialysed against cold deionized water and 30 ml of the dialysate was used as the starting material. Solid ammonium sulfate was added to the enzyme solution to a 70% saturation. After 90 min of gentle stirring, the precipitate was recovered by centrifugation at $10,000\times g$ for 20 min, dissolved in deionized water and dialysed against water. Proteins were focused in a preparative Rotofor cell (Bio-Rad) in the presence of carrier ampholytes Bio-Lyte 3/5 (Bio-Rad). Activity of eluted enzymes was measured by the standard PG assay and enzymes were separated into four fractions (A1–A4 in Fig. 1). The A3 fraction from electrofocusing was collected and dialysed against 20 mM piperazine–HCl buffer, pH 6.0. The dialysate was loaded on a Mono Q HR 5/5 column (Amersham Pharmacia) equilibrated with the dialysis buffer using a Pharmacia fast protein liquid chromatography system. The bound proteins were eluted by a linear gradient of NaCl (from 0 to 0.2 M) at a flow rate of 0.5 ml/min and monitored by absorption at 280 nm. One milliliter fractions were collected and B2 fraction (Fig. 1) was pooled. After concentration, the enzyme solution was applied to a HiLoad 16/60 Superdex 75 column (Amersham Pharmacia) equilibrated with 20 mM sodium acetate buffer, pH 4.0, containing 0.1 M NaCl. The elution was carried out with the same buffer at a flow rate of 1 ml/min, and fractions of 1 ml were collected. C1 fraction (Fig. 1) was pooled, concentrated, and rechromatographed using the same column in the same conditions two more times. The enzyme fraction of C1 after the third Superdex 75 column chromatography was loaded to a Superose 12 HR 10/30 column (Amersham Pharmacia) equilibrated with 20 mM sodium acetate buffer, pH 4.0. The elution was carried out with the same buffer at a flow rate of 0.5 ml/min, and fractions of 0.5 ml were collected. Two *exo*-PGs separated by this column were independently rechromatographed using a Superose 12 HR 10/30 column with the above conditions. Protein homogeneity was evaluated by SDS-PAGE by the method of Laemmli [35] with a continuous 10–20% polyacrylamide gel.

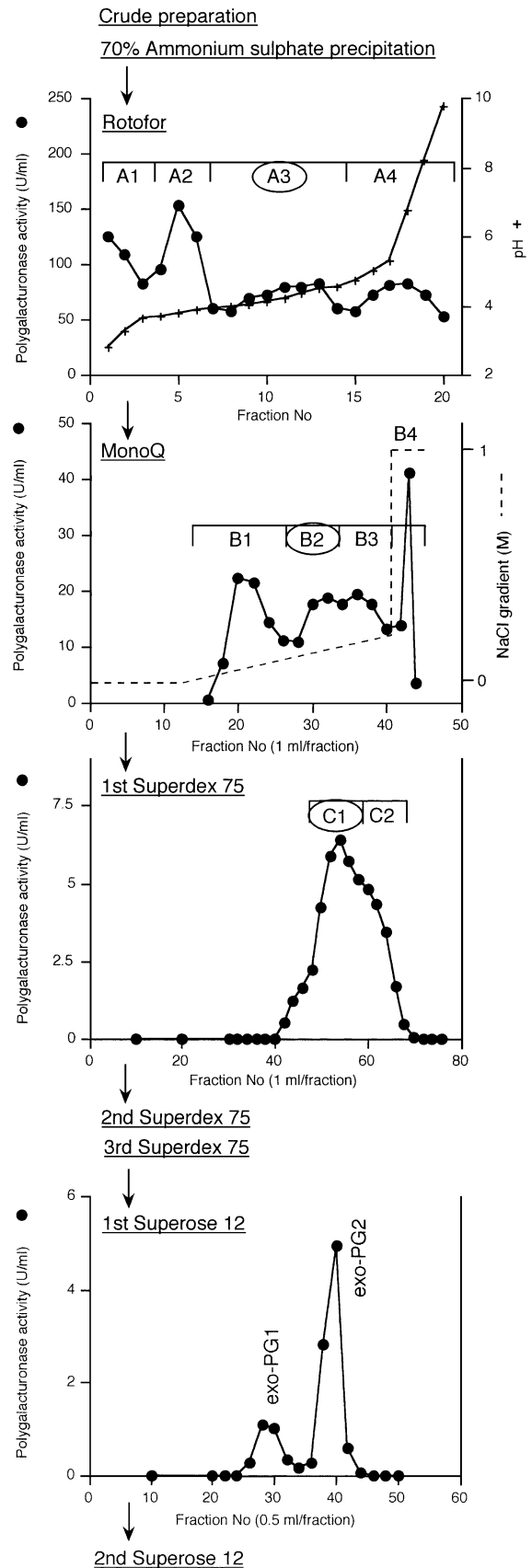


Fig. 1. Elution profiles of the *exo*-PGs on column chromatography during their purification.

2.5. Influence of pH and temperature

The enzyme reaction was performed at various pHs by using 50 mM sodium acetate–HCl buffer (pH 2.4–5.2) at 40 °C and at various temperatures (30–70 °C) in 50 mM sodium acetate buffer, pH 4.0. Temperature stability was evaluated by measuring the residual activity after 30 min of preincubation of the enzyme at temperatures between 40 and 70 °C in 50 mM sodium acetate buffer, pH 4.0. pH stability was studied by preincubating the enzyme at 40 °C for 5 h at various pHs, using 100 mM sodium acetate–HCl buffer (pH 1.8–5.2) and sodium phosphate buffer (pH 5.8–7.0). All the enzyme activities were assayed using HG (2 g/l) as the substrate.

2.6. Removal of carbohydrates from *exo*-PGs

For removal of carbohydrates *N*-linked to the *exo*-PGs, 2 µg of the enzymes were incubated with 15 mU of endoglycosidase H (Sigma) [36] in the buffer supplied by the manufacturer at 40 °C for 1 h. Furthermore, *N*-deglycosylated proteins were incubated in 0.1 M NaOH for 30 min at room temperature, which allowed *O*-deglycosylation of the enzymes.

2.7. Conditions of high-performance liquid chromatography (HPLC)

GalA and oligogalacturonates were separated by HPAEC using a CarboPac PA-1 column at the flow rate of 1 ml/min with a 25 ml linear gradient from 0.3 to 0.55 M sodium acetate in 0.1 M NaOH on a Dionex system. The effluent was monitored with a pulsed amperometric detection. The reaction products from xylogalacturonan were eluted at the same flow rate with a 45 ml linear gradient from 0.15 to 0.45 M sodium acetate in 0.1 M NaOH.

For separation of acetylated GalA, methanol, and acetic acid, reverse phase chromatography was carried out with a C18 Superspher column (Merck) using 4 mM H₂SO₄ as the solvent at a flow rate of 0.7 ml/min at 25 °C [37]. The effluent was monitored with a refractive index detector.

2.8. Isolation of the unknown products from xylogalacturonan and AHG

Exo-PG2 (0.1 U) was incubated with 0.7 ml of xylogalacturonan (5 g/l) in 50 mM sodium acetate–HCl buffer, pH 3.6, for 24 h at 40 °C and then boiled for 5 min to inactivate the enzyme. The reaction products were separated using a CarboPac PA-1 column under the conditions described above. The eluent containing the unknown product was passed through a Carbohydrate Membrane Desalter (Dionex) and collected.

For isolation of the unknown compounds released from AHG by digestion with *exo*-PG2, a reaction mixture containing 0.1 U of the enzyme and 1 ml of the substrate (20 g/l) in 20

mM sodium succinate buffer, pH 3.6, was incubated for 20 h at 40 °C. The mixture was loaded on a PD-10 column (Amersham Pharmacia) equilibrated with distilled water to remove residual polymers. The elution was carried out with distilled water, and fractions of 1 ml were collected. Unknown compound-containing fractions, which were eluted from 5 to 10 ml, were pooled, concentrated to dryness under reduced pressure and dissolved in 0.4 ml of distilled water. The unknown compounds were separated with a C18 Superspher column under the conditions described above. In case of separation of the sample used for electrospray ionization-trap mass spectrometry (ESI-ITMS) experiments, 100 mM acetic acid was used as the solvent for HPLC.

2.9. Conditions of ESI-ITMS

ESI-ITMS experiments were achieved on a Finnigan LCQ (San José, CA, USA) ion trap mass spectrometer using negative and positive electrospray as the ionization processes. Collected fraction of the purified sample was diluted by methanol (40% v/v) before its infusion at a flow rate of 3 ml/min into the ESI source. The 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/MS experiments, the various parameters (collision energy, activation qz, activation time) were adjusted in order to optimize signal and get maximal structural information from the ion of interest [38]. Before negative ESI-ITMS, 2 ml of the purified sample were passed through cation-exchange resin AG 50W-X8 (ammonium form; Bio-Rad).

2.10. Other analytical methods

The protein concentration was assayed either by the method of Bradford [39] with bovine serum albumin as the standard or by measurement of absorbance at 280 nm. Uronic acids were quantified by the automated 3-hydroxybiphenyl method [40]. The neutral sugar content was determined by gas liquid chromatography after hydrolysis with sulfuric acid and alditol acetates derivation [41]. Acetyl and methoxyl groups were liberated from pectins by saponification in 0.5 M NaOH for 1 h at 4 °C and quantified by HPLC using a C18 Superspher column [37]. Concentration of Xyl–GalA was determined by HPAEC using the isolated disaccharide as the standard. The coefficient factor of Xyl–GalA to digalacturonate in our conditions of HPAEC was 1.005.

3. Results

3.1. Purification of the enzymes

We used the commercial preparation Pectinex AR for isolation of two *exo*-PGs since the presence of the enzyme

activity in the preparation has been suggested by our previous paper [42]. Fig. 1 shows some elution patterns of the exo-PGs during their purification. After electrofocusing, PG activity was detected in all fractions and enzymes were separated into four fractions. Presence of exo-PG activity in the four fractions was evaluated by calculating the ratio of concentrations of mono to digalacturonate in the reaction products of polygalacturonic acid with each enzyme fraction using HPAEC, showing that A3 fraction had the highest value. Chromatography of the exo-PG-rich fraction on a Mono Q column separated PG activity as four peaks and HPAEC analysis showed that the exo-PG activity mainly existed in B2 fraction. High galactanase activity was also found in this fraction (data not shown). The exo-PG-active fraction was then applied to a size-exclusion chromatography using a HiLoad Superdex 75 column, which partially separated into three fractions, namely galactanase (just before C1), exo-PG (C1), endo-PG (C2). Galactanase and endo-PG activities could be eliminated by repeating chromatographies three times with the same column. After the third Superdex 75 chromatography, the exo-PG fraction did not produce digalacturonate under the experimental conditions. However, it gave two protein bands of 56 and 82 kDa on SDS-PAGE, suggesting that there were two different exo-PGs, which were separated by a Superose 12 column chromatography. Both enzyme fractions produced only GalA when incubated with polygalacturonic acid as the substrate. Therefore, these enzymes were confirmed to be exo-PGs. The enzymes eluted former and latter were designated exo-PG1 and exo-PG2, respectively. The purification procedure for exo-PGs is summarized in Table 2. The yield of enzyme activity was very low. Since crude enzyme preparation of *A. niger* predominantly contains endo-PG activities, most of the initial PG activity may depend on the action of these enzymes or on their synergistic activities with exo-PGs. Also, the method used for detection of exo-PG containing fractions was not specific for exo enzymes.

Table 2
Purification of exo-PGs from *A. niger*

Procedure	Protein (mg)	Activity (U)	Specific activity (U/mg)	Yield in activity (%)
Crude preparation	837	16,200	19.4	100
70% ammonium sulfate	573	13,000	22.7	80.2
Preparative electrofocusing	124	2250	18.1	13.9
Mono Q	30.5	427	14.0	2.6
1st Superdex 75	9.53	194	20.4	1.2
2nd Superdex 75	5.54	162	29.2	1.0
3rd Superdex 75	3.48	99.4	28.6	0.6
1st Superose 12				
exo-PG1	0.504	6.64	13.2	0.04
exo-PG2	1.71	55.3	32.3	0.34
2nd Superose 12				
exo-PG1	0.24	3.23	13.5	0.02
exo-PG2	1.07	33.6	31.4	0.21

Although the purified enzyme preparation of exo-PG2 gave a single protein band on SDS-PAGE, it contained a little quantity of contaminating pectin methylesterase activity. To study substrate specificities of exo-PGs, it was necessary to eliminate the activity. Exo-PG2 and pectin methylesterase were separated using a Mono Q HR 5/5 column with a gradient from 0.1 to 0.25 M NaCl in 20 mM piperazine–HCl buffer, pH 6.0.

3.2. General properties of the exo-PGs

SDS-PAGE analysis of the purified enzymes showed a single protein band at a molar mass of 82 and 56 kDa for exo-PG1 and exo-PG2, respectively, suggesting that the two exo-PGs were different proteins. The molar masses of the deglycosylated exo-PG1 and exo-PG2 after treatment of an endoglycosidase H decreased to 71 and 43 kDa, respectively, indicating that both proteins contained *N*-linked carbohydrates. Moreover, exo-PG1 and exo-PG2 lost 50% and 61% of their activities on HG after removal of their *N*-linked carbohydrates, respectively. The treatment of *O*-deglycosylation of the enzymes gave no effect on their molar masses.

Influence of pH and temperature on the enzymes was examined. Optimum activities occurred at pH 3.4–3.8 for exo-PG1 and at 3.4–4.2 for exo-PG2, respectively. Both enzymes had their highest activities at 60 °C. The two exo-PGs showed quite different stabilities. More than 70% of the initial exo-PG1 activity remained after 5 h of incubation at pHs from 2.8 to 6.3. On the other hand, exo-PG2 was stable at only pHs from 2.8 to 4.8 in 5 h incubation at 40 °C (Fig. 2a). After incubation of the enzymes at pH 4.0 and 60 °C for 30 min, about 75% and 25% of the initial activity of exo-PG1 and exo-PG2 remained (Fig. 2b). Thus, exo-PG1 was stable over a wider pH range and over a wider temperature range than exo-PG2.

Addition of 0.01 mM HgCl₂ increased exo-PG2 activity by 3.4 times and had no effect on exo-PG1 activity. *A. niger* exo-PGs reported by Mill [24] and Hara et al. [26] were also enhanced 11 and 1.4 times in the presence of 0.001 and 0.02 mM HgCl₂, respectively. Therefore, exo-PG2 was deduced to be similar to the above two enzymes.

The enzyme activities (87% and 76%) of exo-PG1 and -PG2 remained in the presence of 0.32 mM GalA. Both enzymes seemed to be less sensitive to GalA than the *A. tubingensis* exo-PG, which was inhibited by GalA with *K*_i=0.3 mM at pH 4.2 [43].

3.3. Action pattern of the exo-PGs

Generally, enzymes degrade polymer chains in either multi-chain or single-chain attack mode. The product formation at the different reaction times was monitored with HPAEC using pentagalacturonate as the substrate. An example for exo-PG1 is shown in Fig. 3. GalA and tetragalacturonate were accumulated with similar concentrations

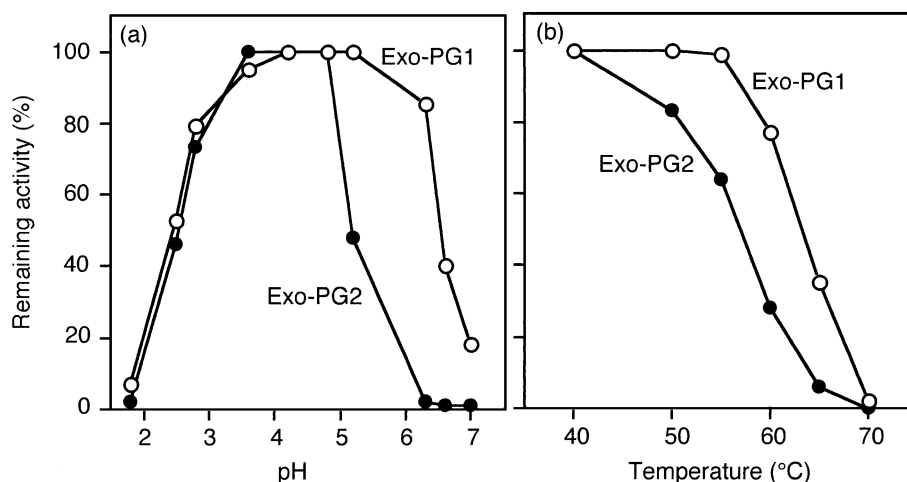


Fig. 2. Effect of pH (a) and temperature (b) on enzyme stability.

and little amounts of di- and trigalacturonate were detected during the early stage of the enzyme reaction. The product formation for both enzymes was typical of a multi-chain attack [44].

To determine whether the enzymes cleave the substrates at the reducing end or at the non-reducing end, the initial reaction products were analysed by HPAEC using reduced pentagalacturonate as a substrate. GalA was detected accompanied with reduced oligogalacturonates and no reduced GalA was found in the reaction mixture. These results indicated that the enzymes split the substrate at the non-reducing end. Both exo-PGs rapidly degraded reduced pentagalacturonate to GalA and reduced digalacturonate.

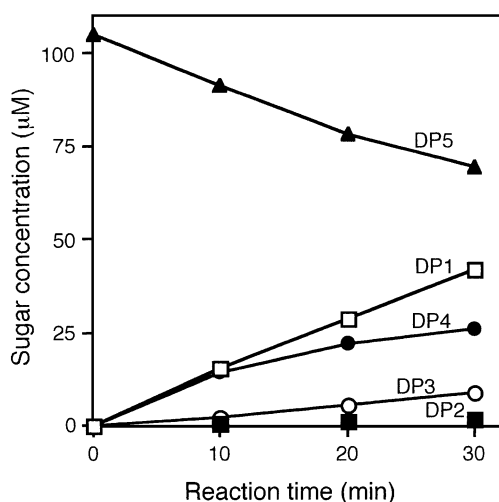


Fig. 3. Analysis of the enzymatic products of pentagalacturonate with exo-PG1. A reaction mixture containing 0.5 mU of the enzyme and 0.2 ml of 0.1 mM pentagalacturonate in 50 mM sodium acetate–HCl buffer, pH 3.6, was incubated at 40 °C at the times shown. Aliquots were taken at intervals and boiled for 5 min to inactivate the enzyme followed by quantification of the reaction products with HPAEC. DP1 to DP5 represent GalA to pentagalacturonate, respectively.

Little amount of reduced digalacturonate was cleaved by exo-PG2 after 3 h of reaction. On the opposite, the *A. tubingensis* exo-PG was unable to split reduced digalacturonate [43].

3.4. Enzyme activity towards oligogalacturonates and various plant cell wall polysaccharides

Table 3 shows the specificities of the purified exo-PGs on galacturonates with different DPs at a 0.1 mM concentration. Digalacturonate was the best substrate for the action of exo-PG2 which seemed to prefer oligogalacturonates to HG. On the other hand, exo-PG1 had a broad specificity towards galacturonates of different DPs compared to exo-PG2 and was active on the polymer.

Degradation of various plant cell wall polysaccharides by exo-PGs was also investigated (Table 4). Both exo-PGs did not show activity towards α -1,5-arabinan and β -1,4-galactan. The enzymes exhibited the highest activity towards HG. Sugar beet pectin was the poorest substrate for both enzymes. The activities on de-esterified beet pectin were higher than those on sugar beet pectin in each

Table 3
Enzyme activity of the exo-PGs towards galacturonates with different DPs

Substrate	Relative activity	
	Exo-PG1	Exo-PG2
DP2	100	100
DP3	86	40
DP4	72	16
DP5	63	13
HG ^a	42	5

^a Average DP of HG was 149. The enzyme was incubated with 100 μ l of 0.1 mM the substrate in 50 mM sodium acetate–HCl buffer, pH 3.6, at 40 °C for 10 min. Concentration of GalA released was determined by HPAEC. Using 1.5 mU of exo-PG1 or 0.25 mU of exo-PG2 for the enzyme reactions, respectively.

Table 4
Degradation of various substrates by exo-PGs

	Amount of GalA released ($\mu\text{g/ml}$) ^a	
	Exo-PG1	Exo-PG2
Sugar beet pectin	1	1
De-esterified beet pectin	38	65
AHG	23	38
HG	106	186
E81	8	19
Xylogalacturonan	14	8

A reaction mixture containing 35 μl of enzyme (1 mU) and 5 μl of the substrate (4 g/l) in 50 mM sodium acetate–HCl buffer, pH 3.6, was incubated at 40 °C for 16 h.

^a Concentration of monomeric GalA in the reaction mixture was determined by HPAEC.

enzyme reaction. These substrates had similar quantities of “hairy” regions but different DM and DAc. The differences of substrate specificity seemed to be due to DM or DAc. Highly methylated pectin was also considerably resistant to the enzymes, indicating that methyl ester groups interfered the enzyme activities. An *A. niger* exo-PG has been reported to be able to release free GalA from the non-reducing terminus only when the following GalA is not methyl-esterified [17]. AHG could be degraded to some extent by the enzymes although most of GalA residues in this substrate are esterified with acetate or residual methanol. This result suggests that the exo-PGs released acetylated GalA. Enzyme activities of the exo-PGs on xylogalacturonan was low. HPAEC profiles of the reaction products of the substrate with each enzyme revealed that both enzymes released GalA as the main product accompanied with an unknown compound. Fig. 4 shows an example for exo-PG2.

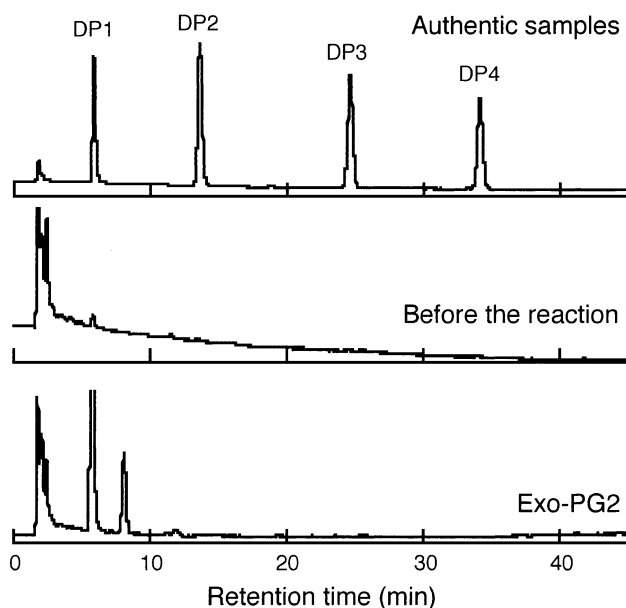


Fig. 4. HPAEC analysis of the enzymatic products of pea hull xylogalacturonan obtained with exo-PG2. Authentic samples DP1 to DP4 represent GalA to tetragalacturonate, respectively.

To study the mode of action of the exo-PG2 on xylogalacturonan, we determined the structure of the unknown product. It was isolated on a CarboPac PA-1 column and analysed by both positive and negative ESI-ITMS and by acid hydrolysis. In positive ESI-ITMS, two major ions with molar masses of 349 and 675 were observed, which were assigned to be the sodiated molecule $[\text{M}+\text{Na}]^+$ and $[2\text{M}+\text{Na}]^+$, respectively. Negative ESI-ITMS gave an intensive peak at 325 corresponding to the deprotonated $[\text{M}-\text{H}]^-$. These results indicated that the molar mass of the compound was 326. Moreover, acid hydrolysis of the compound and its alditol acetate derivation gave a peak corresponding to xylose in gas liquid chromatography. It could then be concluded that the unknown product was the disaccharide (Xyl–GalA) of xylose ($\text{Mw}=150$) and GalA ($\text{Mw}=194$). Furthermore, positive and negative ESI-MS/MS gave intensive daughter ions at 217 ($194+23$) and 175 ($194-18-1$) when molecular ions of Xyl–GalA were selected to be trapped, respectively. Cleavages of glycosidic linkages occur preferentially at the non-reducing and reducing side of the glycosidic oxygen in positive and negative ESI-MS/MS, respectively [45,46]. Therefore, it can be concluded that the compound was Xyl1→GalA. Considering the structure of xylogalacturonan [12], it is likely that this disaccharide is Xyl1→3GalA. These results demonstrated that both enzymes degraded xylogalacturonan in an exo manner, producing GalA and Xyl–GalA. Progression of products released by exo-PG2 on xylogalacturonan is shown in Fig. 5. During the early stage of the reaction, the enzyme

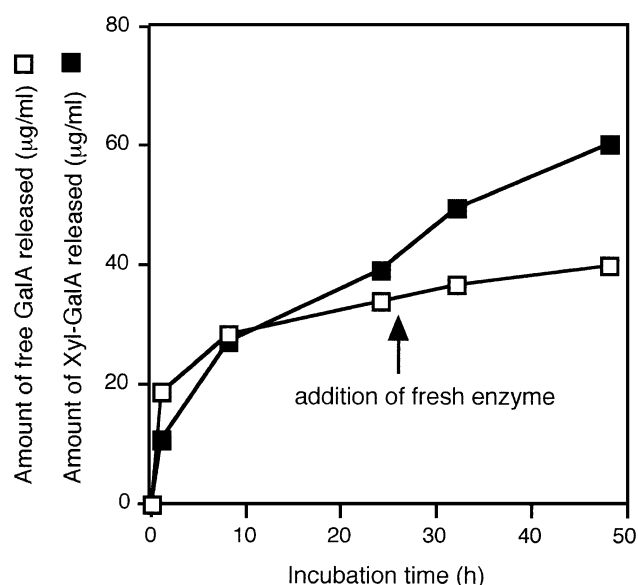


Fig. 5. Progression of products released by exo-PG2 on pea hull xylogalacturonan. A reaction mixture containing 0.3 ml of xylogalacturonan (1 g/l) in 50 mM sodium acetate–HCl buffer, pH 3.6, and 60 μl of the enzyme (50 mU) was incubated at 40 °C at the times shown. The fresh enzyme (25 mU) was added to the mixture at 24 h after the reaction started. The products released into the mixture were quantified by HPAEC. Total GalA and xylose contents were determined by both automated 3-hydroxybiphenyl method [38] and gas liquid chromatography [39].

released GalA more easily than the disaccharide. When the incubation was prolonged, the enzyme gradually produced the disaccharide. This data suggested that the enzyme had higher affinity for the non-substituted GalA residues than for those substituted with xylose. Our previous results about the fine structure of xylogalacturonan demonstrated that 28%, 53%, and 19% of the total GalA residues were free, substituted with a single xylose, and with dixylosides, respectively [12]. Taking into account the percentages and sugar content of the substrate (Table 1), concentrations of GalA, Xyl–GalA, and Xyl–Xyl–GalA units in the reaction mixture calculated to be 96, 319, and 163 mg/ml, respectively. Exo-PG2 released 40.1 $\mu\text{g/ml}$ of GalA and 60.2 $\mu\text{g/ml}$ of Xyl–GalA after 48 h of reaction, demonstrating that 42% and 19% of free GalA residues and Xyl–GalA units were released and that 22% of total glycosidic linkages between GalA were cleaved. The high percentage of free GalA released may indicate that xylogalacturonan had short HG regions without xylose substitution. During degradation of xylogalacturonan, GalA and Xyl–GalA were predominantly formed and little quantities of two other compounds, which have not been identified, were also produced. Even though 19% of the total GalA is substituted by dixylosides, there was no intensive peak corresponding to Xyl–Xyl–GalA. This suggested that substitution with dixylosides inhibited or at least decreased the activity of exo-PG2.

As mentioned above, exo-PGs seemed to degrade AHG extracted from sugar beet pectin, which is characterised by a high DAc at *O*-2 and/or *O*-3 of the GalA in the backbone. Taking into account the ability of exo-PGs to split xylogalacturonan and its chemical structure, our interest was focused on the degradation of AHG by these enzymes. All the analysis of enzymatic reaction products were performed until here using HPAEC under strong alkaline condition. Even if acetylated GalA is released by the enzymatic action, it is saponified during separation of reaction products in this condition. Partially methylated GalA oligomers can be separated on HPAEC at weak acidic conditions [18]. However, analysis of exo-PG2 digests of AHG with HPAEC under weak acidic condition gave only one peak eluting at the same retention time as GalA, showing no separation of acetylated and free GalA (data not shown). To our knowledge, no previous report described the separation of acetylated GalA from free GalA. To confirm the release of acetylated GalA with exo-PGs, we tried to separate acetylated and free GalA by reverse phase chromatography. Reaction products of AHG with two exo-PGs were analysed using a Superspher column, which gave an unknown compound in addition to GalA. An example for exo-PG2 is shown in Fig. 6. The compound was then purified with the same column and treated with 0.1 M sodium hydroxide for 2 h at room temperature. The unknown compound disappeared and GalA and acetic acid appeared, which strongly suggested that GalA was esterified with acetic acid. Then, the unknown product was analysed by ESI-ITMS, which gave a molar mass of 259 in positive ESI-ITMS and 235 in

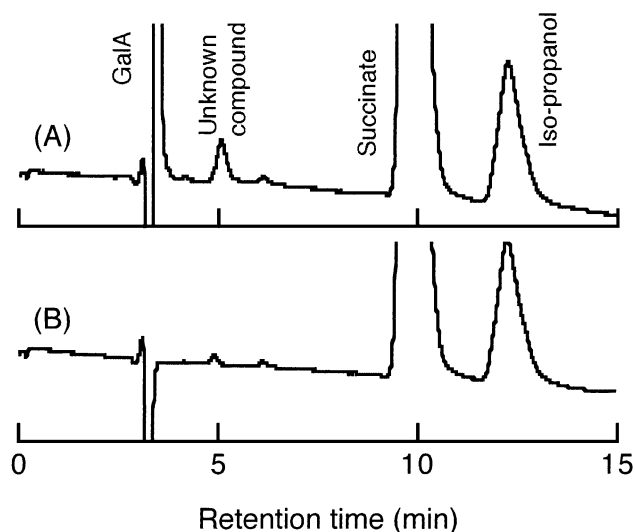


Fig. 6. HPLC analysis of the products released by treating AHG with exo-PG2 using a C18 Superspher column. (A) exo-PG2 digestion, (B) without enzyme. The reaction mixture containing 50 mU of the enzyme and 500 μl of the substrate (3 g/l) in 20 mM sodium succinate buffer, pH 3.6, was incubated at 40 $^{\circ}\text{C}$ for 20 h followed by boiling for 5 min and treatment of isopropanol for 1 h at 4 $^{\circ}\text{C}$ to precipitate residual polymers. The supernatant was analysed by a C18 Superspher column.

negative ESI-ITMS, respectively. From these results, it can be concluded that the unknown compound was acetylated GalA.

4. Discussion

This work deals with characterisation of two exo-PGs purified from a commercial *A. niger* enzyme preparation (Pectinex AR). The pectinase preparations produced by *A. niger* contain various kinds of PGs. The endolytic enzymes are dominant in these preparations [27], which makes difficult the purification of exo-PGs. We used HPAEC to distinguish between exo-PG and endo-PG activities during their purification and to characterise the purified enzymes. HPAEC is a powerful tool for analysis of the activities of glycanases [47,48]. Lerouge et al. [49] have used this technique to investigate the activities of an endo-arabinanase and an arabinosidase.

The properties of the two purified exo-PGs were quite different in terms of molar mass, pH and thermal stabilities, sensitivity to Hg^{2+} , and substrate specificities towards oligogalacturonates. Hara et al. [26] reported that *A. niger* produced two distinct exo-PGs in the culture broth and that one of the enzymes (exo-PG I) was activated by Hg^{2+} . The enzyme had a molar mass of 66,000, pH optimum 3.8 and stability 2.5 to 5.0, and temperature optimum 60 $^{\circ}\text{C}$ and stability up to 50 $^{\circ}\text{C}$. These properties are in good agreement with those described here for exo-PG2. The other enzyme reported by them (exo-PG II), which is not affected by Hg^{2+} , was much different from exo-PG1 in molar mass

(63,000) and pH optimum (4.5). Mill [24,25] also reported that pH optimum of the Hg^{2+} -sensitive exo-PG from *A. niger* was lower than that of the other Hg^{2+} -non-sensitive exo-PG. However, exo-PG1 was not found to have higher pH optimum than exo-PG2. Therefore, exo-PG1 might be different from *A. niger* enzymes reported previously. The *A. tubingensis* exo-PG is an enzyme with molar mass of 78,000, broad specificity towards oligogalacturonates, and non-sensitive to Hg^{2+} [43]. Taking into account these properties, exo-PG1 seems to be similar to this enzyme.

Both exo-PGs isolated here degraded pea hull xylogalacturonan in an exo manner to produce GalA and Xyl–GalA, which was identified by analyses of HPAEC, ESI-ITMS, and acid hydrolysis. These enzymes would be useful for structural analysis of plant cell walls as well as processing of xylogalacturonan-rich materials.

Degradation of AHG was also a new feature of *A. niger* exo-PGs. GalA and acetylated GalA as the reaction products could be separated by using a C18 column. To our knowledge, it is the first report on the degradation of AHG by an exo-PG. Exo-PGs could be applied for degradation of sugar beet pectin, which has a high DAC, in combination with other pectolytic enzymes and also for analysis of acetylation pattern in HG.

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