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Protopectinase-T: a rhamnogalacturonase able to solubilize protopectin from sugar beet

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Abstract

Protopectinase-T (PPase-T), isolated from the culture broth of *Trametes sanguinea*, was able to release pectic substances from sugar beet by degrading rhamnogalacturonan. The smallest polysaccharide (SPS) that can serve as a substrate for PPase-T was prepared from sugar beet pulp by extraction with NaOH and digestion with α-L-arabinofuranosidase, α-L-arabinase, and β-(1 → 4)-D-galactanase. The reaction products of SPS with PPase-T were isolated by chromatography on DEAE-Toyopearl 650M and Toyopearl HW40-S columns, and analyzed by labeling the reducing ends with [^{3}H]$NaBH_4$ and by ^{13}C NMR spectroscopy. The results indicated that PPase-T cleaved galactopyranosyluronic–rhamnopyranosyl linkages in SPS. Moreover, it was confirmed that the chemical structure of SPS contains a backbone based on units of [→ 4)-α-D-Gal*p*A-(1 → 2)-α-L-Rha*p*-(1 →].

1. Introduction

Pectic polysaccharides are the most abundant class of carbohydrates in many plant primary cell walls [1,2]. The dry weight percentages that are typically reported for dicot primary cell walls are 35% pectin, 20–30% cellulose, 25% hemicellulose, and up to 20% glycoprotein. While pectic polysaccharides are localized in the middle lamella and are responsible for cellular cohesion, pectins have also been immunolocalized throughout the primary cell wall [3] with unknown function. Protopectin is defined as that residual material left after extraction with water, buffers, and chelators from plant tissues [4]. A significant proportion of pectic polysaccharides in most plants are soluble in aqueous solution. The features

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of the partial insolubility of pectin are complicated, and include (*1*) bonding of pectin molecules to other cell wall constituents, such as hemicellulose or cellulose, (*2*) binding of pectin molecules with polyvalent ions, such as Ca^{2+}, $Fe^{2+,3+}$, and Mg^{2+}, (*3*) salt bridging between the carboxyl groups of pectin molecules and the basic groups of protein, and (*4*) mechanical entwining of the pectin molecules with each other and with other cell wall constituents [5].

An enzyme that liberates water-soluble pectic substances from protopectin by restricted hydrolysis was named protopectinase [6]. A protopectinase from a yeast was reported in 1978, followed by other microbial protopectinases [7–11]. These enzymes catalyze the hydrolysis of the polygalacturonic acid region in protopectin, liberating water-soluble pectic substances. We have isolated two protopectinases that do not degrade the polygalacturonic acid region in protopectin, and they are tentatively called protopectinase-C (PPase-C) and -T (PPase-T). PPase-C was isolated from *Bacillus subtilis* IFO 3134 [12]. PPase-T, purified to homogeneity by ion-exchange, hydrophobic-interaction, and gel-permeation chromatographies, was isolated from the culture broth of *Trametes sanguinea* IFO 6490 (unpublished data).

This paper deals with the determination of the mechanism involved in pectin solubilization from sugar beet by PPase-T.

2. Experimental

Plant materials and substrates.—Sugar beet pulp remaining after extraction of the sugar was obtained from Tienen Sugar Co. (Tienen, Belgium). α-(1 → 5)-L-Arabinan was prepared from sugar beet pulp by the method of Tagawa and Kaji [13]. Arabinogalactan from soybeans was isolated by the method of Morita [14]. Xylan from oat spelts was obtained from Tokyo Kasei TCI (Tokyo, Japan). Glycosidic-nitrophenol compounds, polygalacturonic acid, and arabinogalactan (from larch wood) were purchased from Sigma Chemical Co. (St. Louis, MO). Pectins from lemon and apple were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and pectin from sugar beet was kindly provided by Professor E.J. Vandamme (University of Ghent, Belgium). Methyl-esterified polygalacturonate was prepared from polygalacturonic acid with MeOH–HCl by the method of Jansen and Jang [15]. Oligogalacturonates were prepared as follows: polygalacturonic acid (1 g) was partially hydrolyzed with 100 U of endo-polygalacturonase in 100 mL of 100 mM acetate buffer, pH 5.0, at 37°C for 2 h. After denaturing the enzyme at 100°C for 5 min, the mixture was concentrated in vacuo and centrifuged. Trimers and tetramers of galacturonic acid in the supernatant were isolated by chromatography on a Dowex 1 × 8 (200–400 mesh) column.

Extraction of pectic substances from plant tissues.—Pectic substances were sequentially extracted from plant tissues by the methods of Pilnik and Voragen [16], Barbier and Thibault [17], and Rombouts and Thibault [18]. Sugar beet pulp (100 g) was suspended in 95% EtOH, boiled at 80°C for 10 min, and ground with a blender. The insoluble residue in alcohol was washed with 95% EtOH until the

eluate became colorless. It was then soaked in 1 L of distilled water at room temperature for 1 h and the slurry was filtered. The filtrate was concentrated to 1/10 of its original volume in vacuo and 4 vol of EtOH were added. The precipitate formed was collected by decantation, dissolved in 10 mL of distilled water, and lyophilized. Water-soluble pectic substances were obtained (900 mg) and they were called WSP. The water insoluble residue of the WSP preparation was soaked in 1 L of 1% sodium hexametaphosphate (SHMP) solution, adjusted to pH 5.0, and kept at room temperature for 1 h. The slurry was filtered and the filtrate was treated in the same way as the WSP preparation, giving 1.2 g of HMP. The residue was treated in the same manner with 50 mM HCl at 80°C, and then with 50 mM NaOH at 4°C, yielding 7.3 g of HP and 14.1 g of OHP, respectively.

Preparation of alkali-soluble pectin (ASP).—Sugar beet pulp (50 g) was mixed with 3 L of 0.1 N NaOH, and the mixture was heated at 100°C for 1 h. The slurry was filtered through gauze and the filtrate was centrifuged. Acetone (3 vol) was added to the resulting clear solution. The precipitate formed was dissolved in distilled water. The solution was treated batchwise with Dowex-50W (H^+ form) to remove cations associated with pectic substances. The effluent was loaded onto a DEAE-Cellulofine AH column (6 × 28 cm; Chisso Corp., Tokyo) equilibrated with 50 mM acetate buffer, pH 5.0, and washed with the same buffer. The bound polysaccharides were eluted with 1 M acetate buffer, pH 5.0, and 10-mL fractions were collected. PPase-T degradation activity towards each fraction was assayed, and the fractions that served as substrates were pooled, concentrated in vacuo, dialyzed against distilled water, precipitated by the addition of EtOH (3 vol), and finally lyophilized, giving ASP.

Preparation of the smallest polysaccharide (SPS) that can serve as a substrate for PPase-T.—ASP was completely dearabinosylated by a mixture of α-L-arabinofuranosidase and α-L-arabinase in 20 mM acetate buffer, pH 5.0, and the mixture was chromatographed by gel filtration with a Superose 12 column. The fractions containing polysaccharides that could be degraded by PPase-T were pooled, yielding ASP-A. ASP-A was treated by β-(1 → 4)-D-galactanase in 20 mM acetate buffer, pH 5.0, and purified by chromatography on the same column. We named this fraction SPS.

Enzyme preparations.—α-L-Arabinofuranosidase [19] and α-L-arabinase were prepared from culture filtrates of *Aspergillus niger* K1 strain (kindly supplied by Professor A. Kaji, Kagawa University, Japan). Pure β-(1 → 4)-D-galactanase [20] was kindly provided by Dr. Y. Tominaga (Osaka Municipal Technical Research Institute, Japan). The endo-polygalacturonase was purified from a culture of *Trichosporon penicillatum* SNO-3 by the method of Sakai and Okushima [8].

Enzyme assays.—Pectin-releasing activity was calculated from the amount of pectic substances solubilized from cell wall fraction residues as determined by the carbazole–H_2SO_4 method [21]. The mixture (1 mL) contained 20 mg of cell wall fraction residue, 950 μL of 100 mM acetate buffer, pH 5.0, and 50 μL of an appropriate enzyme dilution, and was allowed to react for 60 min at 37°C. One unit of pectin-releasing activity towards the residue was defined as the enzyme activity that liberates soluble pectic substances corresponding to 1 μmol of

D-galacturonic acid per mL of mixture in the above mentioned conditions in 1 min. Different cell wall fraction residues of the sequential steps during pectin extraction from sugar beet pulp were used as substrates in assays for pectin-releasing activity.

Degrading activity of PPase-T towards water soluble polysaccharides was assayed by measurement of reducing groups liberated by the method of Somogyi [22]. In this case, several polysaccharides were used as substrates according to the purpose of the experiment. Mixtures containing 300 μL of 0.1% solution of the corresponding soluble polysaccharide in 100 mM acetate buffer, pH 5.0, and 10 μL of an appropriate enzyme dilution in the same buffer were allowed to react for 60 min at 37°C. One unit of degrading activity towards water soluble polysaccharides was defined as the enzyme activity that liberates reducing groups corresponding to 1 μmol of D-galacturonic acid per mL of mixture in the above mentioned conditions in 1 min. Degrading activity of PPase-T towards various nitrophenyl glycosides was spectrophotometrically assayed by measurement at 420 nm of nitrophenolate ion liberated [19].

Gel permeation chromatography.—(*i*) Chromatography with a fast protein liquid chromatography apparatus (Pharmacia LKB Biotechnology AB, Upsala, Sweden) equipped with a Superose 12 HR 10/30 column involved injection of 500 μL of a 10% solution of pectic substances onto the column equilibrated with 20 mM acetate buffer, pH 5.0, containing 100 mM NaCl. Fractions (1 mL) were collected at a flow rate of 0.75 mL/min. (*ii*) Chromatography on a Toyopearl HW-40S column (1 × 60 cm; Tosoh Corp., Tokyo) involved loading 1 mL of a 1% solution of pectic substances onto the column and eluting with distilled water at 30 mL/h. Fractions (1 mL) were collected.

Ion-exchange chromatography.—A 1% solution of pectic substances (15 mL) was loaded onto a DEAE-Toyopearl 650M column (2 × 16 cm; Tosoh) equilibrated with 50 mM acetate buffer, pH 5.0. After washing the column with 100 mL of the same buffer, the bound substances were eluted with a linear gradient of acetate buffer (200 mL, 50 to 1000 mM, pH 5.0) at a flow rate of 30 mL/h, and fractions (5 mL) were collected.

High-pressure liquid chromatography (HPLC).—For analysis of sugar composition, HPLC was done with Shodex KS-801 and KS-802 columns (Showa Denko, Tokyo) using distilled water as the solvent at a flow rate of 0.6 mL/min at 80°C. The effluent was monitored with a refractive index detector (Showa Denko). Sugars with reducing groups labeled with tritium, were chromatographed with a KS-802 column with distilled water as the solvent at a flow rate of 0.7 mL/min at room temperature. Radioactivity was detected by a radioisotope detector (model 171, Beckman Instruments Inc., Fullerton, CA).

NMR spectroscopy.—^{13}C NMR spectra were measured with a Jeol JNM-GX 270 NMR spectrometer (67.5 MHz) with a solution of saccharides in D_2O (30 mg/0.5 mL, pD 5.2) in a 5-mm tube at 23°C. The chemical shifts were referenced to the signal for sodium 4,4-dimethyl-4-silapentane-1-sulfonate (2.217 ppm).

Labeling of sugars with [^{3}H]NaBH$_4$.—Reducing-end sugars were labeled with tritium by treatment with [^{3}H]$NaBH_4$ in 25 mM NaOH according to the method of Takasaki and Kobata [23]. The polysaccharides labeled with tritium were

Table 1
Sugar content and pectin-releasing activity of PPase-T on sugar beet pulp cell wall fraction residues remaining after treatment with various extraction solutions [a]

Extraction solution [b]	Amount of polysaccharides released [c] (mg/10 mg cell wall fraction residue)			Pectin-releasing activity [d] (U/mL)
	Galacturonic acid	Neutral sugar	Total	
Ethanol	1.64	0.90	2.54	0.03
Water	1.58	0.85	2.43	0.10
SHMP	1.40	0.88	2.28	0.33
HCl	0.74	0.58	1.32	3.17
NaOH	0.25	0.28	0.53	2.50

[a] A detailed description of each extraction step can be found in the Experimental section. [b] Cell wall fraction residues after sequential treatment with each extraction solution. [c] Polysaccharides were extracted with 1 N HCl at 100°C for 4 h and quantified. Total polysaccharide is the sum of galacturonic acid and neutral sugar content. [d] Pectin-releasing activity was assayed as described in the Experimental section.

hydrolyzed with 1 N HCl at 110°C for 2 h, and the resulting monosaccharides labeled with tritium were identified by HPLC. Standard sugars were also labeled in the same manner.

Sugar analysis.—Galacturonic acid was determined by the method of Blumenkrantz and Asboe-Hansen [24]. Neutral sugars were determined by the phenol–H_2SO_4 method [25] (using D-galacturonic acid and D-galactose as standards) as the difference between the total absorbance (which corresponds to both neutral and acidic sugars) and the one corresponding to the galacturonic acid previously quantified. The composition of neutral sugars was also analyzed by HPLC after hydrolysis with 1 N HCl at 110°C for 2 h.

3. Results and discussion

Enzyme activities of PPase-T towards various cell wall fraction residues and pectins obtained from sugar beet pulp.—The pectin-releasing activity of PPase-T towards various cell wall fraction residues from sugar beet pulp and their sugar content are summarized in Table 1. The enzyme showed strong activity towards cell wall fraction residues remaining after treatment with HCl and NaOH, but had little activity towards the residues treated with EtOH, water, and SHMP solution. Table 2 summarizes the sugar composition of the sequentially extracted pectic substances from beet pulp and the degrading activity of PPase-T towards these pectins. The enzyme catalyzed the hydrolysis of HP and OHP, but not WSP or HMP. The galacturonic acid contents of HP or OHP were lower than those of WSP or HMP. In the PPase-T reaction, a greater amount of pectic substances was released from cell wall fraction residues after treatment with HCl or NaOH than from the residues obtained with EtOH, water, or SHMP solution, even though in the latter case the residual fractions used as substrates contained more galactur-

Table 2
Sugar composition [a] (mol%) and degrading activity of PPase-T on different pectin fractions obtained from sugar beet pulp [b]

Pectin fraction [c]	GalA	Rha	Gal	Ara	Degrading activity [d] (U/mL)
WSP	68	2	7	22	n.d.
HMP	80	1	4	13	n.d.
HP	54	6	15	22	0.28
OHP	56	8	24	10	0.75

[a] Small amounts of sugars were neglected. [b] A detailed description of each extraction step can be found in the Experimental section. [c] WSP, water soluble pectin; HMP, sodium hexametaphosphate soluble pectin; HP, HCl soluble pectin; OHP, NaOH soluble pectin. [d] Degrading activity was assayed as described in the Experimental section; n.d., not detected.

onic acid (soluble in 1 N HCl at 100°C) than did the former fractions. This suggested that PPase-T might act on the rhamnogalacturonan regions or hairy regions of pectin, which contain many neutral sugar side-chains [26,27], rather than homogalacturonan regions.

Enzyme activities of PPase-T towards various pectin-related substrates.—Pectic substances prepared from sugar beet pulp have been studied in detail [18,26–29]. It was found that their hairy regions contain mainly galacturonic acid, arabinose, galactose, and rhamnose and a small amount of fucose, glucose, mannose, and xylose. The degrading activities of PPase-T on some pectin-related substrates are shown in Table 3. The enzyme catalyzed the hydrolysis of pectin, but not poly-

Table 3
Degrading activity of PPase-T on various substrates [a]

Substrate (origin)	Degrading activity
Polygalacturonate (orange)	None
Oligogalacturonate (orange)	None
Methyl-esterified polygalacturonate	None
Pectin (sugar beet)	Detected
Pectin (lemon)	Detected
Pectin (apple)	Detected
Arabinan (sugar beet)	None
Arabinogalactan (soybean)	None
Arabinogalactan (larch wood)	None
Xylan (oat spelts)	None
o-Nitrophenyl-β-D-galactopyranoside	None
p-Nitrophenyl-α-L-rhamnopyranoside	None
p-Nitrophenyl-α-L-arabinofuranoside	None
p-Nitrophenyl-β-D-fucopyranoside	None
p-Nitrophenyl-α-L-fucopyranoside	None
p-Nitrophenyl-α,β-D-mannopyranoside	None
p-Nitrophenyl-α,β-D-glucopyranoside	None

[a] The mixtures contained 0.1% substrate, 10 μg of PPase-T, and 20 μmol of acetate buffer, pH 5.0, in a total volume of 1 mL. Reactions were conducted at 37°C for 20 h.

galacturonic acid, methyl-esterified polygalacturonate, oligogalacturonate, or the other substrates tested.

Enzymatic hydrolysis of ASP.—As depicted in Table 2, PPase-T showed the highest activity on pectic substances extracted with NaOH. Alkali-soluble pectin (ASP) that could be degraded by PPase-T was prepared from sugar beet pulp by extraction under hot, alkaline conditions as described in the Experimental section. ASP was assumed to contain the hairy regions of pectin as main components, because the methyl-esterified homogalacturonan region is unstable in the alkaline treatment, being depolymerized into oligogalacturonides by transeliminative degradation. ASP was sequentially degraded, first by a mixture of α-L-arabinofuranosidase and α-L-arabinase, and then by β-(1 → 4)-D-galactanase, producing ASP-A and SPS, respectively. After each step, the mixture was loaded onto a Superose 12 column to separate undegradable polysaccharides (ASP-A and SPS) from low molecular weight products (Fig. 1). SPS was treated with an endo-polygalacturonase to reduce its molecular size, but this substrate was resistant to the enzyme. The cleavage of SPS by PPase-T yielded the lowest molecular weight undegradable product (SPST). Fig. 1 (a, b, and c) shows the degrading activity of PPase-T on ASP, ASP-A, and SPS. The sugar compositions of ASP, ASP-A, SPS, and SPST are shown in Table 4 (SPST-1 and SPST-2 are described below). After the first step (treatment by α-L-arabinofuranosidase and α-L-arabinase), no arabinose could be detected. Galactose, however, still remained after galactanase treatment in the second step. The remaining galactose fragments may have been too small to be cleaved further by a galactanase. The monosaccharide compositions of SPS and SPST were identical.

Identification of reducing ends of reaction products of SPS treated with PPase-T.—For identification of the reducing-end sugar residues formed in the PPase-T reaction toward SPS, the following experiment was performed. A mixture containing SPS and PPase-T was incubated in 100 mM acetate buffer, pH 5.0, at 45°C for 1 h, and the newly formed reducing-end sugars were labeled with tritium by treatment with [^{3}H]$NaBH_4$. Polysaccharides labeled with tritium were analyzed by HPLC after hydrolysis with HCl. HPLC chromatograms of the reducing-end sugars are shown in Fig. 2. Comparing the elution patterns before and after PPase-T digestion, it can be seen that two peaks (a at 8.6 and d at 14.2 min) increased in the latter case. These peaks were identified by coelution with standards as derivatives of D-galacturonic acid. The size of the other peaks did not change significantly following PPase-T digestion, which indicates they were derived from reducing-end sugars of the substrate (SPS). The peaks a and d in Fig. 2(A) appeared when a galacturonic acid standard was prepared under the experimental conditions used. Peaks a and d were identified as galactonic acid and as galactono-(1 → 4)-lactone from their standards, respectively. Additional support for the identity of galactono-(1 → 4)-lactone was provided by saponifying the sample shown in Fig. 2(C) with 1 N NaOH at room temperature. After 3 h reaction, peak d was converted into peak a and the amounts of peaks b and c remained unchanged. The reduction of galacturonic acid with $NaBH_4$ produces galactonic acid, which is known to lactonize under hot, acidic conditions to form galactono-(1 → 4)-lactone [30].

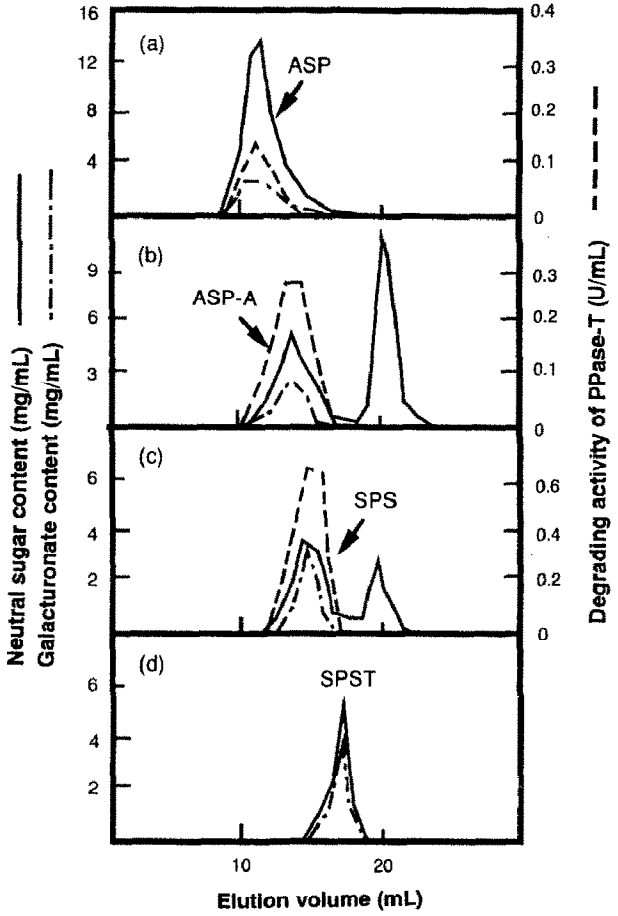

Fig. 1. Elution patterns of digests of pectic substances degraded by various enzymes on Superose 12 for the isolation of SPS. (a) ASP. (b) Reaction products of ASP with arabinofuranosidase and arabinase. (c) Reaction products of ASP-A with galactanase. (d) Reaction products of SPS with PPase-T.

Table 4
Sugar composition [a] (mol%) of different sugar beet pectin fractions sequentially degraded by various enzymes [b]

Pectin fraction	GalA	Rha	Gal	Ara
ASP	8	5	15	72
ASP-A	27	17	56	–
SPS	43	31	26	–
SPST	43	31	26	–
SPST-1	41	38	21	–
SPST-2	44	25	31	–

[a] Small amounts of sugars were neglected. [b] A detailed description of the different pectin fractions can be found in the text.

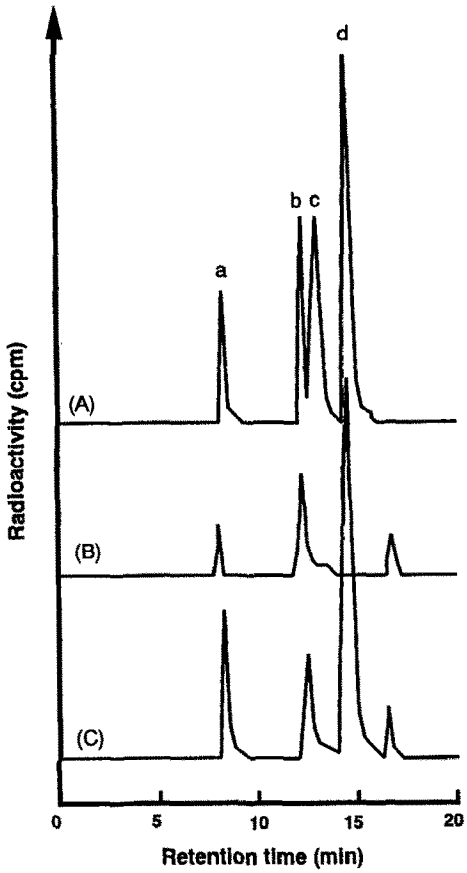

Fig. 2. Analysis of the HCl hydrolyzates of [^{3}H] $NaBH_4$ reduced product of SPS with PPase-T. (A) Standard [a, galactonic acid; b, rhamnitol; c, galactitol; d, galactono-(1 → 4)-lactone]. (B) Before the enzyme reaction. (C) After the enzyme reaction.

These data indicated that the polysaccharides formed by PPase-T have galacturonic acid at their reducing ends.

Isolation and characterization of reaction products of SPS and PPase-T.—For checking the nonreducing-end sugar residues in products of the PPase-T reaction, the reaction products of the PPase-T digest of SPS were isolated. SPS was hydrolyzed by PPase-T until no more reaction products were formed, and the hydrolyzates were chromatographed on a DEAE-Toyopearl ion-exchange column (Fig. 3). The unbound and bound fractions, which were named SPST-1 and SPST-2, respectively, were pooled and subsequently chromatographed on a Toyopearl HW40-S gel-permeation column (Fig. 4). Figure 4 shows that the molecular size of SPST-1 (ca. 1000) was smaller than that of SPST-2 (ca. 3000). Analysis of sugar composition of both fractions revealed that SPST-2 had a higher galactose content and a lower rhamnose content than SPST-1 (Table 4).

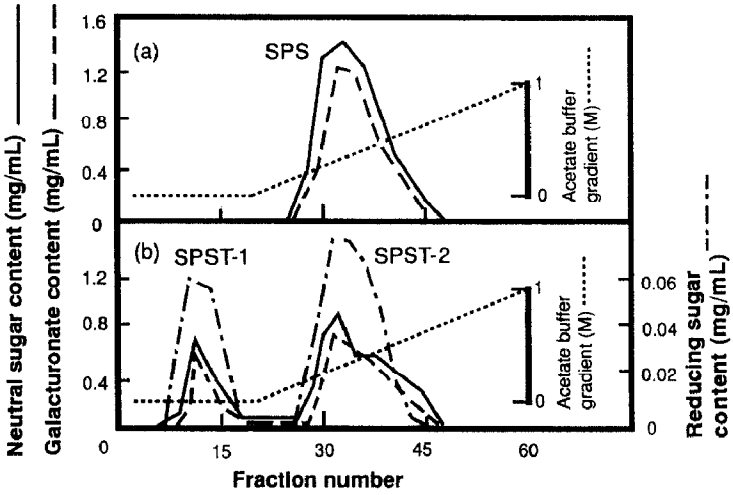

Fig. 3. Chromatography of reaction products of SPS with PPase-T on DEAE-Toyopearl (a) before and (b) after the reaction.

The ^{13}C NMR spectra of SPS and SPST-1 were compared to those published by Colquhoun et al. [31] (Fig. 5 and Table 5). The ^{13}C NMR spectrum of SPST-1 was almost identical to that of oligosaccharide fraction D (an oligosaccharide mixture obtained by enzymatic degradation of the modified hairy regions of apple pectin with a rhamnogalacturonase [32]). The ^{13}C NMR chemical shifts described here were up to 2 ppm larger than those reported by Colquhoun et al. [31]. This discrepancy was due to the use of different internal standards.

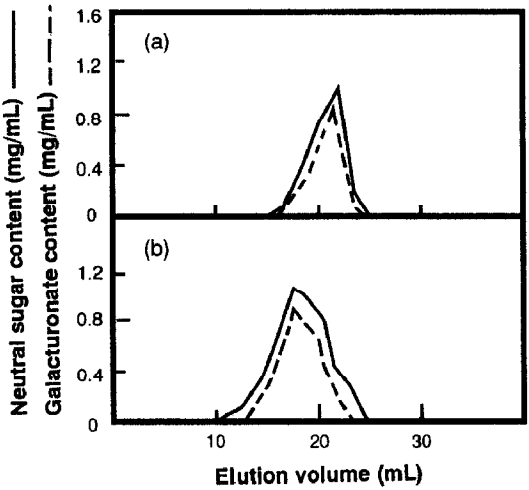

Fig. 4. Chromatography of SPST-1 and -2 on Toyopearl HW 40-S (a, SPST-1; b, SPST-2).

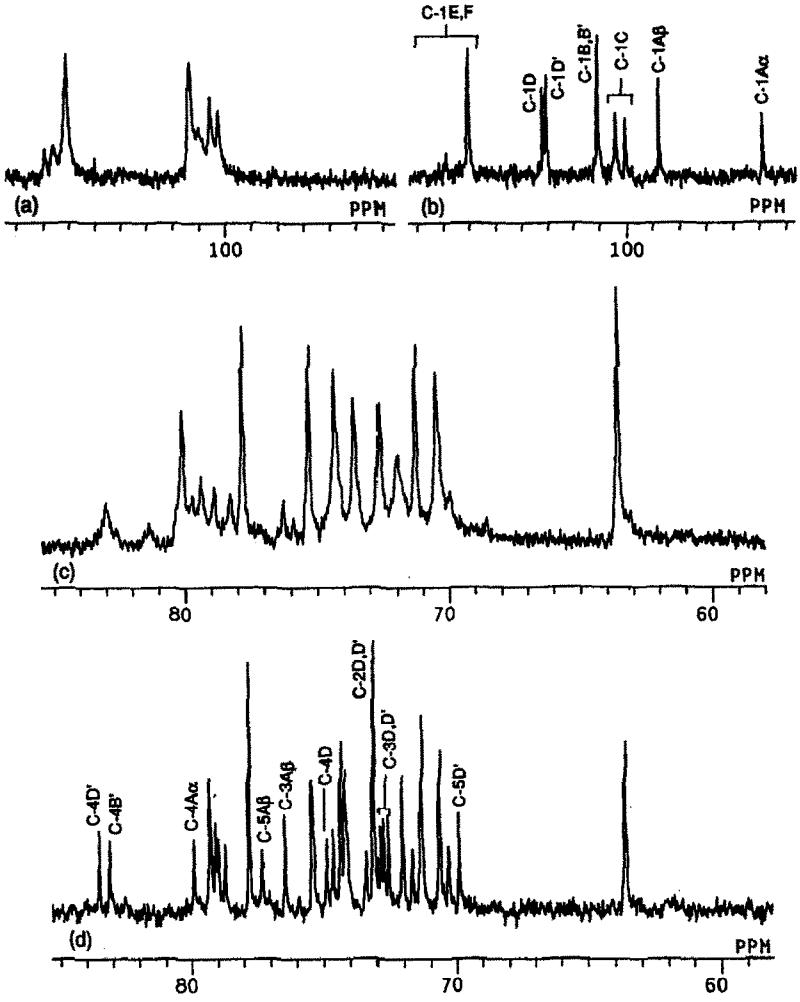

Fig. 5. ^{13}C NMR spectra of SPS and SPST-1. (a) C-1 region for SPS. (b) C-1 region for SPST-1. (c) C-2/6 region for SPS, excluding C-6 of GalA and Rha. (d) C-2/6 region for SPST-1, excluding C-6 of GalA and Rha; B' and D' represent Rha units substituted for Gal.

The resonances at 94.9 ppm (αC-1), 98.8 ppm (βC-1), 76.5 ppm (βC-3), 79.9 ppm (αC-4), and 77.3 ppm (βC-5) were assigned to galacturonic acid residues on the reducing ends of SPST-1 (residue A in Fig. 6). These findings provided further evidence that PPase-T produced saccharides with galacturonic acid at their reducing ends. The two peaks at 103.1 and 103.2 ppm arose in the region from 94 to 108 ppm for C-1 resonances in SPST-1. These peaks were almost identical to those of α-rhamnopyranose on the nonreducing ends of oligosaccharide fraction D reported by Colquhoun et al. [31]. The other peaks, at 73.1, 72.9, 74.9, 83.5, and 69.9 ppm, which were assigned to C-2D, C-2D', C-4D, C-4D', and C-5D' in Fig. 6, were also

Table 5
Chemical shifts (ppm) of the ^{13}C resonances for SPST-1

Unit	C-1	C-2	C-3	C-4	C-5	C-6
Gal*p*A (r.e. [a])						
αA	94.9	70.6		79.9	73.4	177.5
βA	98.8		76.5		77.3	176.8
α-Rha*p*						
B	101.1	78.7	72.1	74.6	71.7	19.3
B′ [b]	101.1	79.3	72.1	83.1	70.3	19.5
α-Gal*p*A						
C	100.1	70.6		79.0	74.2	177.5
	100.4			79.1		
α-Rha*p* (n.r.e. [c])						
D	103.2	73.1	72.8	74.9	71.4	19.4
D′ [d]	103.1	72.9	72.6	83.5	69.9	19.6
β-Gal*p*						
E,F	106.0	74.4	75.4	71.3	77.8	63.6
			75.4			

[a] r.e., Reducing end. [b] B′, Gal-substituted Rha at position 4. [c] n.r.e., Nonreducing end. [d] D′: Gal-substituted terminal Rha at position 4.

present in SPST-1. From these results, the nonreducing end sugar residues of SPST-1 were identified as α-rhamnopyranoses. PPase-T hydrolyzed galactopyranosyluronic-rhamnopyranosyl linkages in SPS to release SPST-1 and SPST-2. These results showed that PPase-T had a reaction mechanism similar to the rhamnogalacturonase from *Aspergillus aculeatus* reported by Schols et al. [32].

The resonances of C-1 and C-4 in the (1 → 4)-linked α-Gal*p*A units in SPST-1 were 1.5 ppm lower than those of the same units in oligogalacturonates. This fact indicates that the two neighboring sugars of the (1 → 4)-linked Gal*p*A residues in SPST-1 were not α-D-Gal*p*A but were α-L-Rha*p* residues [31]. Integrations of the essential signals for the C-1 resonances of SPST-1 were summarized in Table 6. The ratio of integration for splitting/internal of Gal*p*A and that of Rha*p* were both 1.34. These results suggest that SPST-1 is likely a tetrameric backbone with some dimeric contamination. Moreover, the ratio of integration for Rha*p*/Gal*p* was 2.6, which indicated that a galactosyl side group was attached at the rate of two to three rhamnose residues. The data in Table 4 also support this SPST-1 structure, since there are 1.8 times as much rhamnose as galactose. From these results, we can postulate four different structures for SPST-1 (Fig. 6). Namely, SPST-1 molecules with zero, one (either at the B′ or at the D′ unit), or two Rha*p* units substituted for Gal*p* residues could exist. The structures with only one Gal*p*

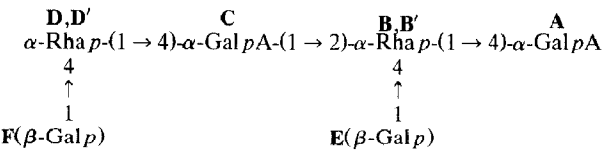

Fig. 6. The postulated structure of SPST-1. Molecules with zero, one (either the B′ or the D′ unit), or two Rha*p* units substituted for Gal*p* residues are proposed.

Table 6
Integration for the anomeric region of SPST-1

Unit	Chemical shift (ppm)	Integral
α-Gal*p*A (r.e. [a])	94.9	22.8
β-Gal*p*A (r.e. [a])	98.8	36.1
α-Gal*p*A	100.1, 100.4	43.8
α-Rha*p*	101.1	52.0
α-Rha*p* (n.r.e. [b])	103.1, 103.2	69.9
β-Gal*p*	106.0	47.2

[a] r.e., Reducing end. [b] n.r.e., Nonreducing end.

residue attached to the Rha*p* residue (B′ or D′) represent the best match with the data presented here. Both rhamnogalacturonan I [33,34] isolated from sycamore cells, and modified hairy regions [35] from apple pectin have a structure based on the unit [→ 4)-α-D-Gal*p*A-(1 → 2)-α-L-Rha*p*-(1 →]. Similarly, ASP prepared from sugar beet pulp seemed to have a backbone structure based on the unit [→ 4)-α-D-Gal*p*A-(1 → 2)-α-L-Rha*p*-(1 →] based on monosaccharide composition (Table 4) and NMR data.

The degrading activity of PPase-T towards ASP, ASP-A, SPS, SPST-1, and SPST-2 with substrate concentrations adjusted to a constant galacturonic acid content (0.07%) was 0.40, 0.55, 0.60, 0, and 0.02 U/mL, respectively. The degrading activity of PPase-T towards SPST-1 and SPST-2 was not or rarely detected, since SPST-1 and SPST-2 are essentially limit products. Therefore, the reason that PPase-T exhibits no activity with SPST-1 and SPST-2 might be that they have been depolymerized to a size too small to bind to the PPase-T active site.

Arabinan chains in the hairy fragment of sugar-beet pectin were tentatively proposed to be mainly attached directly to rhamnose residues of rhamnogalacturonan [29]. In our experiments, however, treatment of ASP with β-(1 → 4)-galactanase removed a high proportion of arabinan chains from ASP together with galactose oligomers (data not shown). This indicated that most arabinan chains were linked to rhamnogalacturonan through an interposed β-(1 → 4)-galactan chain. We are unable to explain these different results on the structure of the sugar-beet pectin hairy region.

The highest pectin-releasing activity was obtained with cell wall fraction residues treated with HCl for sugar beet pulp (Table 1) and with SHMP for lemon peel (data not shown). This might suggest that there are some differences between sugar beet and lemon pectin structure.

Although the characteristics of PPase-T have not been published yet, we found that it is a protein with an apparent molecular weight of 55 000 by SDS-PAGE, and has an isoelectric point around pH 8.1. The enzyme was stable from pH 3 to 5 and up to 50°C. The pH and temperature optima for the enzyme were 4.0 and 50°C, respectively. Schols et al. [32] reported that their rhamnogalacturonase had a similar molecular weight (51 000), pH optimum (3–4), and temperature optimum (40–50°C). It is not known if this rhamnogalacturonase [32] exhibits pectin-releasing activity. When the isoelectric point of the rhamnogalacturonase and other

characteristics (amino acid composition, inhibitors of the enzyme, etc.) of both enzymes are known, it will be easier to compare these enzymes. Nevertheless, given the present data, we conclude that both enzymes have very similar features.

PPase-T catalyzes the degradation of protopectin, releasing soluble pectin. The molecular weight of pectin released by PPase-T from lemon peel was estimated by gel permeation chromatography to be ca. 130 000. It was higher than that of pectin extracted by chemical methods (70 000–100 000). Thus, PPase-T may be a useful tool for the industrial production of pectins as well as for basic studies of structures of pectic substances in plant materials.

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