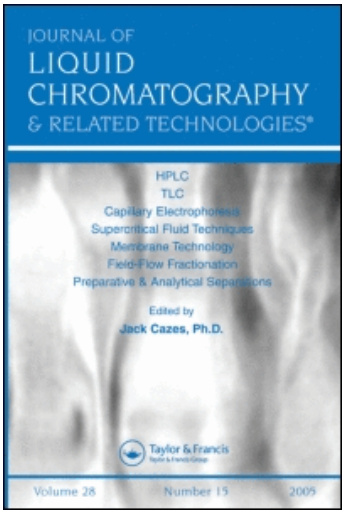

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Enzymic-HPLC Determination of the Amount and Distribution of the Galacturonan Region in Pectate Molecules

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ENZYMIC-HPLC DETERMINATION OF THE AMOUNT AND DISTRIBUTION OF THE GALACTURONAN REGION IN PECTATE MOLECULES

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ABSTRACT

Degradation percentages of pectates by exopolygalacturonate lyase, exopolygalacturonase (exo-PG), and exo-PG and endopolygalacturonase in combination can be accurately measured by our HPLC method. This enzymic-HPLC method was successfully used for the structural analysis of pectic substances, which are usually composed of two distinct regions of linear galacturonan (GN) and branched rhamnogalacturonan (RG). The GN regions were classified for convenience into four categories and the relative amounts of each GN region were represented by α~δ, *viz.*, (α) GN chains having a nonreducing end, (β) those having a reducing end, (γ) those having both reducing and nonreducing ends, and no neutral sugar residues, and (δ) those interposed between two RG regions; in addition, (ε) the amount of the residual galacturonate, mainly comprised in the RG regions. The procedure for the determination of α~ε was described in detail, and the results of application to several pectate preparations were reported.

INTRODUCTION

Pectic substances in the primary cell walls of higher plants are usually composed of two distinct polysaccharide regions, linear galacturonan (GN) and branched rhamnogalacturonan (RG).[1-4] The rhamnose-rich RG region carries a variety of neutral sugar side chains consisting of arabinose, galactose, xylose, sometimes fucose, mannose, and glucose. The GN regions can be differentiated into three or four types by their location in the pectate molecules, *viz.*, (1) the GN chains located at the reducing end side, (2) those at the nonreducing end side of the pectate molecules, and (3) those interposed between two RG regions. In addition, (4) an appreciable amount of the GN molecule, which has no RG region, would probably be contained in some pectate preparations such as those obtained from ripening fruits.[5,6] The galacturonate component of pectic preparations is assayed either colorimetrically using specific reagents[7-10] or by high performance liquid chromatography (HPLC).[11] However, the existing methods are not directly applicable to the determination of the GN distribution in pectate molecules.

In our previous work,[12] we have shown that an HPLC method is highly reliable and accurate for measurement of the degradation percentages of pectates by exopolygalacturonate lyase (exo-PGL, EC 4.2.2.9), exopolygalacturonase (exo-PG, EC 3.2.1.67), or by a combined action of exo-PG and endopolygalacturonase (endo-PG, EC 3.2.1.15). The present paper deals with the further development of the enzymic-HPLC method for the determination of the amount and distribution of the GN region in pectate molecules.

MATERIALS

Enzymes.

Enzyme preparations were obtained in manners similar to those described before; exo-PGL,[13] exo-PG,[14] and endo-PG[15] were isolated from *Erwinia carotovora* subsp. *carotovora* IFO 13921, carrot roots, and

Kluyveromyces fragilis, respectively. Driselase solution was prepared as previously described.[11]

Pectic acids.

Pectate samples were prepared as previously described[11] from strawberry (*Fragaria* x *ananassa* DUCH. cv. Toyonoka) fruits during two stages of maturity (small green, I; red ripe, II), and citrus peels of Banpeiyu (*Citrus shrandis* OSBECK) and Natsudaidai (*Citrus natsudaidai* HAYATA). Strawberry fruits were obtained from Hiroshima Prefectural Agricultural Experiment Station and citrus fruits were bought from a local store. Lemon pectate was prepared from commercial lemon pectin (Nacalai Tesque, Japan).

Limit-pectates and RG.

Exo-PGL[16] and exo-PG[14,17] attack the reducing and nonreducing chain end of pectate molecules, respectively,and their actions stop as an anomalous linkage other than the α-1,4 linked galacturonate is approached. Therefore, the prolonged action of exo-PGL or exo-PG on pectate results in the formation of a "limit-pectate" without the GN chain at the side of the reducing or nonreducing end, respectively; the former limit-pectate was named PGL-limit-PA and the latter PG-limit-PA. PGL-limit-PA was prepared from the lemon pectate (sodium salt) as follows: A reaction mixture (200 ml) containing 1.0% pectate, 50 mM Tris-hydrochloric acid buffer (pH 8.5 at 35°C), about 20 units of exo-PGL, and a small amount of toluene was incubated in a cellophane tube (VT 802, Nacalai Tesque) at 35°C for 3 days. During the incubation, the reaction mixture was dialyzed against several changes of the same Tris buffer to remove the degradation products (4,5-unsaturated digalacturonate), followed by continued dialysis against 20 mM sodium acetate buffer, pH 4.0, at 4°C for 2 days. The enzyme in the inner liquid was then removed by passage through a column (2 x 10 cm) of Amberlite CG-50 equilibrated with the same acetate buffer. The effluent, containing PGL-

limit-PA, was concentrated to about 40 ml under reduced pressure, and mixed with 3 volumes of 99% ethanol. The precipitated PGL-limit-PA was washed successively with 80 and 99% ethanol, and then with acetone before drying at room temperature. PG-limit-PA was also prepared from the lemon pectate using a similar procedure; exo-PG in 50 mM sodium acetate buffer, pH 4.5, was used instead of exo-PGL in the Tris buffer.

RG was prepared from the lemon pectate by exhaustive hydrolysis with the purified endo-PG as previously described.[11] The hydrolysis products of low molecular weight, removed into the dialysate, were also used as an oligogalacturonate preparation in this experiment. The dialysate was concentrated under reduced pressure and the oligogalacturonates were precipitated with ethanol. The precipitates were successively washed with 99% ethanol and acetone, and finally dried in a desiccator.

Tetragalacturonate, 4,5-unsaturated digalacturonate, and galacturonate.

Tetragalacturonate was separated from the foregoing oligogalacturonate preparation by anion-exchange chromatography on a Dowex 1-x8 column.[18] 4,5-Unsaturated digalacturonate was prepared from the previous degradation products of lemon pectate by the exo-PGL; the dialysates were combined and, after treating with Bio-Rad AG1-x2, neutralized with 1 M sodium hydroxide, and concentrated to about 10 ml under reduced pressure. 4,5-Unsaturated digalacturonate (sodium salt) in the concentrate was precipitated with 10 volumes of 99% ethanol. The precipitates were collected and successively washed with 99% ethanol and ether, and finally dried in a desiccator. Reagent grade α-D-galacturonate (monohydrate) was purchased from Nacalai Tesque.

METHODS

Degradation of pectates by pectinases to the final stage.

The reaction mixtures (total volume 0.5 ml) containing the pectic sample (0.05% as free galacturonate), glycerol (0.1%, internal standard

for HPLC), sodium acetate buffer (50 mM, pH 5.0, for exo- and endo-PG) or Tris-hydrochloric acid buffer (50 mM, pH 8.5 at 35°C, for exo-PGL), and enzyme (50 mU) were incubated at 35°C for 48 hr; for the combined action of exo- and endo-PG, 100 mU/ml of each enzyme was used. In the cases of the degradation by exo-PGL and exo-PG in sequence, the reaction mixtures containing 0.1% pectate, 0.1% glycerol, 20 mM Tris buffer (pH 8.5 at 35°C), and exo-PGL (100 mU/ml) were incubated at 35°C for 48 hr. An aliquot of 0.25 ml was then removed from the reaction mixtures and added to a solution (0.25 ml) containing exo-PG (25 mU) and sodium acetate buffer (100 mM, pH 5.0), and the resulting mixture was reincubated at 35°C for 48 hr. The sample solutions for HPLC analysis were prepared as follows: The reaction mixtures incubated were filtered with a Millipore Molcut II GC (1×10^4 Da exclusion limit for globular protein) and the filtrates (10 or 20 μl) were used for injection.

Analytical methods.

Galacturonate contents of pectic solutions were measured by the carbazole method of Galambos;[7] the pectates in the solutions were completely prehydrolyzed to monomeric units by Driselase, because the conjugated galacturonate residues in pectate give a higher color intensity than free galacturonate with the carbazole reagent.[10] One unit of pectinase activities is defined as the amount of the enzyme which releases 1 μmole of reducing group per min. The HPLC conditions were the same as those previously described:[12] column, Shodex SUGAR SH1821, 8 x 300 mm; precolumn, Shodex SUGAR SH1011P, 6 x 50 mm; mobile phase, 0.005 N sulfuric acid; column temperature, 40°C; flow rate, 1.0 ml/min; chromatograph, Jasco TRI Rotar-VI; detector, Jasco RID-300; recorder, SIC Chromatocorder 11.

Designation of the GN region in pectate molecules.

The GN regions present in pectate molecules or in a pectate preparation were classified for convenience into four categories and the

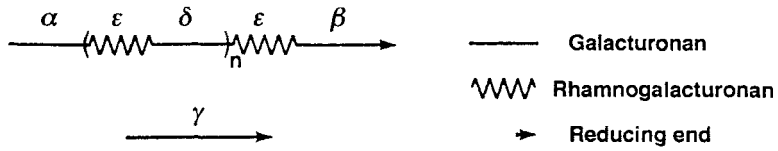

FIGURE 1. Two models for the estimation of GN regions in pectate molecules.

relative amount of each region was represented by α~δ as shown in Fig. 1, *viz.*, (α) the GN chain having a nonreducing end, (β) the GN chain having a reducing end, (γ) the linear GN molecule having both reducing and nonreducing ends and containing no neutral sugar residues, and (δ) the GN chain interposed between two RG regions. Furthermore, the residual galacturonate residues, which are mainly comprised in the RG regions, are sorted into the ε category.

RESULTS AND DISCUSSION

Enzymic-HPLC measurement of the GN content in model mixtures containing both homogeneous and heterogeneous pectate molecules

Table 1 shows the results of enzymic-HPLC analysis of the model mixtures containing tetragalacturonate and PG- or PGL-limit-PA, the former tetramer being used as a substitute for the linear homogalaturonan molecule (γ shown in Fig. 1). The reason for substituting the limit-PAs for heterogeneous pectate molecules is to completely remove any linear homogalacturonan molecules from the pectate sample, because such homogalacturonan molecules might be contained in the original pectate preparation. The results in Table 1 indicate virtually complete recoveries of the added tetragalactuonate. Accordingly, the present enzymic-HPLC method is clearly applicable to the determination of the linear homogalacturonan content (γ) in a pectate preparation. The figures of 371 and 382 in columns A and C of Table 1 correspond to the contents in GN region α and β, respectively, of the original lemon pectate (see also

TABLE 1
Degradation of Mixtures of Limit-PAs and Tetragalacturonate by Exo-PGL and Exo-PG

Substrate	Degradation product (μg/ml)	
	Exo-PGL	Exo-PG
(A) PGL-limit-PA	0	371
(B) PGL-limit-PA and tetramer	506	864
(B) - (A)	506 (101.2)	493 (98.6)
(C) PG-limit-PA	382	0
(D) PG-limit-PA and tetramer	890	490
(D) - (C)	508 (101.6)	490 (98.0)

The figures in parentheses represent the percent recoveries of tetramer (tetragalacturonate) added. The values represent the average of triplicate analyses.

Table 3). As an example, the HPLC-chromatograms of the degradation products of the reaction mixtures containing PGL-limit-PA are shown in Fig. 2. Degradation of tetragalacturonate by exo-PGL gives an equivalent amount of digalacturonate and unsaturated digalacturonate, because only the central glycosidic bond of the tetragalacturonate is susceptible to this enzyme action. Under the present HPLC conditions, both dimers did not separate and emerged as a single peak at the retention time of about 7 min (Fig. 2-B). They also gave approximately the same response during refractometric monitoring (data not shown).

Enzymic-HPLC determination of the relative values of α~ε for pectate preparations

Table 2 shows the susceptibilities of the GN regions (α~δ) to the action of each pectinase. The values of α~ε for a pectate preparation are readily obtainable by a combination of the procedures of either (1), (3), and (5) or alternatively (2), (4), and (5) in Table 2. For example, the

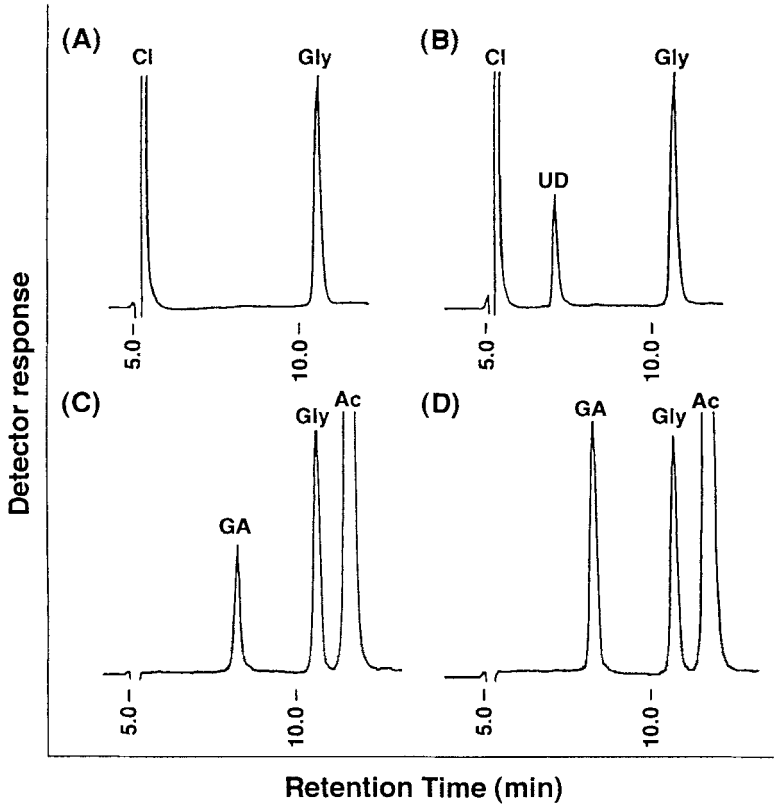

FIGURE 2. HPLC chromatograms of the degradation products of PGL-limit-PA and a mixture of PGL-limit-PA and tetragalacturonate by exo-PGL and exo-PG.
Reaction mixtures: PGL-limit-PA, 0.05% (A~D); tetragalacturonate, 0.05% (B and D); 50 mM Tris-hydrochloric acid buffer of pH 8.5 (A and B); 50 mM acetate buffer of pH 5.0 (C and D); enzyme concentration, 100 mU/ml of exo-PGL (A and B) or exo-PG (C and D); glycerol, 0.1%; incubation, at 35°C for 48 hr. Peak identity; GA, galacturonate; UD, 4,5-unsaturated digalacturonate; Gly, glycerol; Ac, acetate; Cl, chlorine.

TABLE 2
GN Regions Susceptible to the Enzyme Actions

Enzyme	GN region
(1) Exo-PG	$(\alpha + \beta)$
(2) Exo-PGL	$(\beta + \gamma)$
(3) Exo-PGL Exo-PG	$(\beta + \gamma)$ and α
(4) Exo-PG Exo-PGL	$(\alpha + \gamma)$ and β
(5) Exo- and Endo-PG	$(\alpha + \beta + \gamma + \delta)$

See Fig. 1.

degradation percentage using procedure (1) (exo-PG in separation) gives the value of $(\alpha + \gamma)$, and those using procedure (3) (exo-PGL and exo-PG in sequence) provide the values of $(\beta + \gamma)$ and α. Similarly, the procedure (5) (exo- and endo-PG in combination) gives the total value of $(\alpha + \beta + \gamma + \delta)$. Thus, each value of α~δ can be easily determined using these four degradation percentages. The galacturonate content (ε) in the RG regions is given by subtracting the value of $(\alpha + \beta + \gamma + \delta)$ from 100. Figure 3 shows an example of the HPLC chromatograms of the degradation products of lemon pectate obtained by the combination of procedures (1), (3), and (5). The galacturonate peaks in Fig. 3-A and 3-B give the values of $(\alpha + \gamma)$ and $(\alpha + \beta + \gamma + \delta)$, respectively, and the unsaturated digalacturonate and galacturonate peaks in Fig. 3-C correspond to the values of $(\beta + \gamma)$ and α, respectively.

Table 3 presents some results of the application of this enzymic-HPLC method to the determination of the values (α~ε) in pectic preparations. As can be seen, the δ value of Natsudaidai pectate was zero. This indicates that Natsudaidai pectate has only one RG region in the heterogeneous molecule, *viz.*, the numerical value of "n" is zero in the heterogeneous molecule model (see Fig. 1); the α and β values suggest that the RG region locates in the middle of the pectate molecule. Lemon pectate probably has similar structural features to those of Natsudaidai pectate, because its δ value is very low and all of the other values also are in fair agreement with those for Natsudaidai pectate.

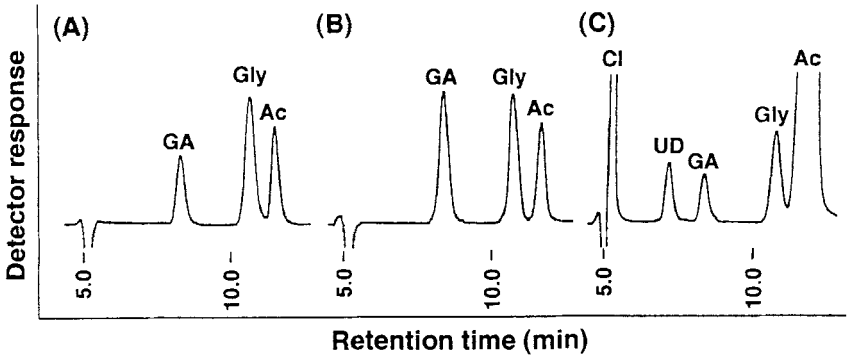

FIGURE 3. HPLC chromatograms of the degradation products of lemon pectate by exo-PG (A), exo- and endo-PG in combination (B), and exo-PGL and exo-PG in sequence (C).
See Table 2 and Fig. 2.

TABLE 3
α~ε Values for Several Kinds of Pectates

Origin of pectates	α	β	γ	δ	ε
Commercial lemon pectin	38.8	42.1	7.6	2.3	9.2
Natsudaidai peel	36.0	48.3	7.1	0.0	8.6
Banpeiyu peel	27.1	35.8	17.1	11.3	8.4
Unripe-strawberry fruit	13.4	15.2	2.9	52.1	17.4
Ripe-strawberry fruit	21.6	16.7	24.9	22.4	14.4
RG from lemon pectin	1.4	1.7	0.0	0.0	96.9

The values represent the average of triplicate analyses.

The pectate of unripe-strawberry fruit gave a high δ value (52.1%) and a low γ value (1.9%), in contrast to those of the citrus pectates. On the other hand, the strawberry pectate at the ripe stage showed a large increase in the γ value and a large decrease in the δ value. These results suggest that the increased free galacturonan molecules (γ) in the ripe-strawberry pectate were produced by enzymatic cleavage at the inner galacturonan region (δ) of the unripe-strawberry pectate during ripening. This formation of free galacturonan is probably due to the action of an endo-PG. This view is strengthened by our finding that activities of both

exo- and endo-PGs were detected in the same Toyonoka strawberry fruits, although their activities were very weak.[19] As for the formation of galacturonan in ripe-strawberry fruits, Knee *et al.*[20] also have been observed that free water-soluble (in the presence of EDTA) galacturonan in the strawberry wall increases greatly during ripening.

In the case of RG, the values for α and β were very low and those for γ and δ were zero (Table 3). The RG was prepared from lemon pectate by exhaustive hydrolysis with the *Kluyveromyces* endo-PG. Therefore, the RG cannot comprise any GN regions corresponding to γ or δ. Meanwhile, each of endo-PGs requires at least two or more hydrolyzable galacturonidic bonds to exhibit its activity.[21] Thus, small amounts of the galacturonate residues corresponding to α (1.4%) and β (1.7%) remained insusceptible to the endo-PG action at both chain ends of the RG molecules.

The present HPLC method has already been proved to be sufficiently accurate for measurement of the degradation percentages of pectate by pectinases; under the conditions similar to those of the present experiments, the coefficients of variation of the degradation limits of lemon pectate were less than 2.0% for each of the exo-PGL and exo-PG, and for the combined action of exo- and endo-PG.[12] From the results obtained in this study, we concluded that the present enzymic-HPLC method is useful for the analysis of the structural features of pectic substances, although there is still room for further improvement in this method. The values of α~ε for a pectate preparation should give useful information on the distribution of GN regions and galacturonate residues in pectate molecules.

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