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ISOLATION AND CHARACTERIZATION OF CELL-WALL PECTIC SUBSTANCES FROM POTATO TUBER

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Key Word Index—*Solanum tuberosum*; Solanaceae; potato; cell walls; pectic substances; methylation analysis.

Abstract—Exhaustive treatment of potato tuber tissues by purified endo-polygalacturonase from *Aspergillus japonicus* solubilized 95% of the total uronides of the tissue. The galacturonides released from the tissue were separated into four fractions (3.2, 6.8, 3.5 and 86.5% in order of decreasing MW) by gel filtration on Sephadex G-100 and Bio-Gel A-5 m columns. The three fractions of highest MW were further purified by DEAE-Sephadex column chromatography. They all contained neutral sugars and a small amount of protein. Methylation analysis indicated that two high MW fractions contained almost the same glycosyl linkages; main linkages in descending order of concentration were 4-linked galactose, 5-linked arabinose, 4-linked galacturonic acid, and 2,4-linked rhamnose. The intermediate MW fraction was a very complex polysaccharide which contained 3,4-linked galacturonic acid, terminal, 3- and 3,4-linked rhamnose, and branched arabinosyl and galactosyl residues. The four fractions were formed even in an early stage of the enzymatic attack, in which only 15% of the total tissue uronides were solubilized.

INTRODUCTION

Pectic substances are important constituents of the cell wall of higher plants. They play a role in cell-wall cementing in the middle lamella and can be regarded as filler substances within the primary cell wall. Therefore, the degradation of cell-wall pectic substances by pectolytic enzymes causes cell separation [1], release of cell-wall-bound enzymes [2], and leakage of electrolytes from protoplasts which results in cell death [3].

Structural studies of cell-wall pectic substances may be important for the understanding of the dynamic aspects of the plant cell wall. Albersheim and co-workers [4–6] isolated and characterized pectic substances from suspension-cultured sycamore cells which possessed mainly primary cell walls. The middle lamella, the cementing layer between adjacent primary cell walls, has been considered to consist principally of pectic substances. However, no information is yet available regarding the structure of middle lamella pectic substances.

Exhaustive treatment of potato tubers by purified endo-polygalacturonase (PG) of *Aspergillus japonicus* solubilized about 95% of the total uronides of the tissue. This indicates that the galacturonides released by the enzyme may be derived from the middle lamella and the primary cell wall. The present paper describes the isolation and characterization of galacturonide-containing substances released from potato tuber tissues by the enzyme.

RESULTS

Isolation of galacturonides released from potato tuber tissues by PG

PG of *A. japonicus* released higher amounts of galacturonides from potato tuber tissues than pectin lyase from the same fungus [7]. Exhaustive extraction of the potato tuber pectic substances was carried out by subjecting the tissue three successive times to the action of the PG. The first and the second PG treatments released 85.6 and 7.9%, respectively, of the total uronides. The third treatment, however, released only 1.4% of the uronides. Thus, PG of *A. japonicus* solubilized 94.9% of the uronides of the tissue.

The soluble products of the three treatments were combined and lyophilized. The lyophilized sample was dissolved in buffer and chromatographed on Sephadex G-100. The galacturonides released from the tissue were separated into high MW (10% of total), intermediate MW (3.5%) and low MW (86.5%) galacturonides (Fig. 1).

The high MW fractions (fractions 8–12) were bulked and concentrated by ultrafiltration. The concentrated solution was applied to a Bio-Gel A-5 m column. One-third of the galacturonides was eluted at void volume and the remainder was partially retained. The former accounted for 3.2% of the total galacturonides released, and the latter accounted for 6.8%. The excluded and partially retained galacturonides were collected separately, concentrated by ultrafiltration, dialysed, and applied to a DEAE-Sephadex A-50 column. As shown in Fig. 2, the neutral sugars and protein of both fractions were co-eluted with galacturonides, indicating that these components may be attached to the galacturonides.

Intermediate MW galacturonides which were eluted in fractions 16–20 on Sephadex G-100 column were also applied to a DEAE-Sephadex A-50 column. Most protein components and about one-third of the neutral sugars were not adsorbed on the column but all of the galacturonides were adsorbed (Fig. 3). NaCl gradient elution gave two peaks the major one (fractions 31–36) of which was collected.

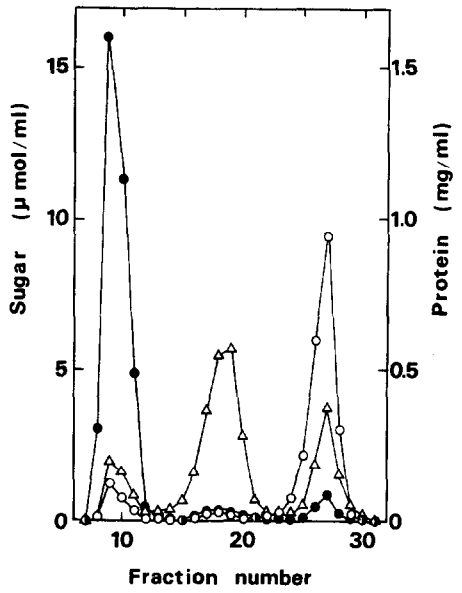

Fig. 1. Gel filtration of products solubilized from potato tuber tissues by PG. The lyophilized sample was dissolved in 2 ml 0.1 M NH_4OAc buffer (pH 4.5), and the insoluble material was removed by centrifugation at 10000 *g* for 10 min. The solution was placed on a Sephadex G-100 column (1.9 × 98 cm) and eluted with 0.1 M NH_4OAc buffer (pH 4,5) at a flow rate of 20 ml/hr. Fractions (10 ml) were collected and each fraction was assayed for galacturonides (○) by the *m*-hydroxydiphenyl method, for neutral sugars (●) by the anthrone method, and for proteins (△) by the Lowry method. Blue dextran, trypsin inhibitor from soya bean (MW 21 500), and monogalacturonic acid eluted from this column in fractions 9, 18, and 27, respectively.

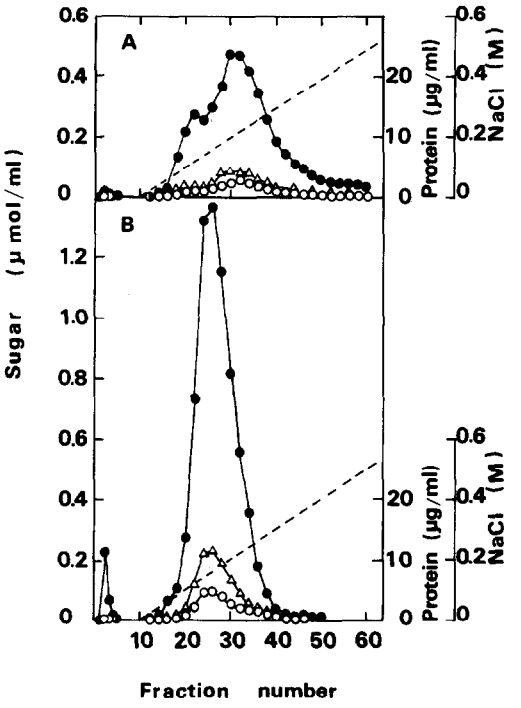

Fig. 2. DEAE-Sephadex column chromatography of high MW galacturonides obtained by Bio-Gel A-5 m column. Excluded fractions (A) and partially retained fractions (B) obtained by gel filtration of Bio-Gel A-5 m column (1.9 × 98 cm) were concentrated by ultrafiltration, dialysed against 20 mM NaOAc buffer (pH 6.0) and applied to a DEAE-Sephadex A-50 column (0.9 × 25 cm) that was eluted with 20 mM NaOAc buffer (pH 6.0) followed by a linear 0.0–0.6 M NaCl gradient (-----) in the same buffer. The eluate was collected in 10 ml fractions. The fractions were assayed for galacturonides (○) by the *m*-hydroxydiphenyl method, for neutral sugars (●) by the anthrone method and for proteins (△) by the Lowry method.

Characterization of the four fractions isolated

The galacturonides released from potato tuber tissues were separated into four fractions (fractions I–IV in order of decreasing MW) by gel filtration on Sephadex G-100 and Bio-Gel A-5 m columns.

Fraction I was excluded from Bio-Gel A-5 m, which indicates that its MW was more than 5×10^6. On the other hand, fraction II was partially retained in a Bio-Gel A-5 m column but was completely excluded from a Bio-Gel A-1.5 m column. Fraction III corresponded with the elution volume of trypsin inhibitor from soya bean, suggesting that its MW was about 2×10^4. Fraction IV was shown to be a mixture of mono-, di-, and tri-galacturonic acid by TLC [8]. As demonstrated previously [7], the formation of oligogalacturonides was due to the combined action of potato tuber cell wall-bound pectinesterase and PG added.

The compositions of fractions I, II and III are given in Table 1. Each fraction contained a small amount of protein. Knee reported that association between polyuronide and protein resulted from aggregation of the components during the drying process [9]. But these fractions obtained without a drying process also contained protein [S. Ishii, unpublished work].

There were few differences between fractions I and II in uronide and protein content and sugar composition. Fraction III, however, was different from I and II in several respects. It contained a higher percentage of galacturonides with a higher degree of esterification than fractions I and II. The molar ratio of galacturonic acid: rhamnose: arabinose: galactose of fraction III was 2:1:3:1, while those of fractions I and II were about 1:1:2:8.

The glycosyl linkage compositions of fractions I, II, and III are given in Table 2. The composition of fraction I was quite similar to that of fraction II. The major component was 4-linked galactose followed by 5-linked arabinose. These two residues comprised about 75% of total sugar residues in fractions I and II. The galacturonosyl residues were 4-linked and terminal with a molar ratio of about 45:1. Most of rhamnosyl residues in fractions I and II were 2,4-linked.

On the other hand, few 2,4-linked rhamnosyl residues were detected in fraction III. The rhamnosyl residues were composed of terminal, 2-linked, 3-linked, and 3,4-linked residues in the molar ratio 1:2:2:1. Fraction III was distinctly different from fractions I and II in that it contained considerable amounts of branched galacturonic acid (3,4-linked). In contrast to fractions I and II, 68% of the total arabinosyl and 55% of the galactosyl residues were branched. Fraction III was also characterized by the presence of terminal arabinopyranosyl residues.

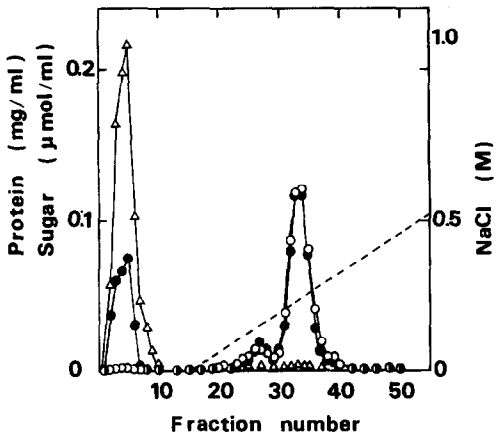

Fig. 3. DEAE-Sephadex column chromatography of intermediate MW galacturonides obtained by Sephadex G-100 column. Fractions 16–20 in Fig. 1 were collected and lyophilized. The lyophilized material was dissolved in 3 ml 20 mM NaOAc/ buffer (pH 6.0) and applied to a DEAE-Sephadex A-50 column (0.9 × 25 cm) that had been equilibrated with 20 mM NaOAc buffer (pH 6.0). The column was washed with 100 ml 20 mM NaOAc buffer (pH 6.0), and then eluted with a linear 0.0–0.8 M NaCl gradient (-----) in the same buffer. The eluate was collected in 10 ml fractions. The fractions were assayed for galacturonides (○) by the *m*-hydroxydiphenyl method, for neutral sugars (●) by the anthrone method, and for proteins (△) by the Lowry method.

It is not known why total branched glycosyl residues were higher than total terminal glycosyl residues in all fractions.

The monosaccharide compositions of these fractions estimated from the results of methylation analysis were in fairly good agreement with the results of the colorimetric determination of each sugar (Table 1) with the exception of the galacturonic acid and galactose in fraction III.

Galacturonides released from the tissues in an early stage of PG attack

It is probable that the attack of the middle lamella by enzymes may precede that of the primary cell wall [10]. Therefore, a substantial part of galacturonides released in an early stage of PG attack is supposed to be derived from the middle lamella.

After a 10 min incubation, 15.2% of the total tissue uronides were solubilized from potato tuber tissues by PG. After removal of the tissues, the soluble fraction was further incubated at 30° for 3 hr in order to degrade exhaustively the galacturonide linkages of soluble products. This fraction was concentrated and fractionated by gel filtration on Sephadex G-100 and Bio-Gel A-5 m columns. The elution patterns were quite similar to those obtained by exhaustive PG treatment, although there were quantitative differences. The galacturonides released consisted of 2.0% of fraction I, 3.0% of fraction II, 3.3% of fraction III, and 91.7% of fraction IV. Thus, every fraction was formed even in an early stage of PG attack, although the proportions were slightly different. Neutral sugar and protein content and sugar linkage compositions of these fractions were essentially the same as those obtained by exhaustive PG treatment.

DISCUSSION

It is quite difficult to distinguish between pectic substances in the middle lamella and those in the primary cell wall. There are few differences between the galacturonide-containing substances released in an early stage of PG attack and those released by exhaustive PG treatment. It is suggested that a substantial part of the former may be derived from the middle lamella and the latter is a mixture derived from the middle lamella and the primary cell wall.

Most of the galacturonides in cell-wall pectic substances were converted by PG into mono-, di-, and trigalacturonic acids. This indicates that most of the galacturonosyl residues occur as unbranched α-1,4-linked polymers of homogalacturonan regions of pectic substances or pure galacturonan.

There were few differences between fraction I and II except for their MW. Methylation analysis showed that arabinosyl and galactosyl residues in fractions I and II may be present as linear chains of 5-linked arabinan and 4-linked galactan. It seems probable that these chains may be attached at C-4 of most of the rhamnosyl residues of the rhamnogalacturonans, the backbone chains of fractions I

Table 1. Compositions of fractions I, II and III isolated from soluble products released from potato tuber tissues by PG

	Fraction I	Fraction II	Fraction III
Composition		(% dry wt)	
Uronide	7.4	8.1	32.6
Neutral sugar	68.8	80.2	63.2
Protein	2.6	2.5	1.6
Degree of esterification		(% of uronide)	
	5.9	3.1	30.5
Sugar composition		(% total sugar)	
Galacturonic acid	9.7	9.2	34.0
Rhamnose	7.3	7.5	14.8
Arabinose	14.3	16.5	38.0
Galactose	68.6	66.8	13.2

Table 2. Glycosyl linkage compositions of fractions I, II and III isolated from soluble products released from potato tuber tissues by PG

Sugar, mode of linkage	Fraction I	Fraction II	Fraction III
Galacturonic acid			
Terminal	0.2	0.2	6.1
4-linked	8.6	9.2	8.1
3,4-linked	0	0	6.8
Rhamnose			
Terminal	0	0	2.8
2-linked	0.7	1.0	5.2
3-linked	0	0	5.3
2,4-linked	6.8	7.1	0.5
3,4-linked	0	0	2.9
Arabinose			
Terminal (furanose)	1.4	2.6	9.9
Terminal (pyranose)	0	0	1.5
5-linked	12.1	10.3	0.7
2,5-linked	0	0	13.0
3,5-linked	0	0	13.2
Galactose			
Terminal	1.4	2.2	7.4
4-linked	66.4	63.2	3.3
2,4-linked	1.0	1.1	8.3
3,4-linked	1.4	3.1	5.0

and II. These fractions are structurally similar to rhamnogalacturonan I isolated from the cell wall of suspension-cultured sycamore [4, 6], except that rhamnosyl residues of rhamnogalacturonan I were 2- and 2,4-linked with a molar ratio of 1:1.

In contrast to fractions I and II, fraction III was highly branched and, therefore, had a complex structure. It contained branched galacturonic acid (3,4-linked), 3- and 3,4-linked rhamnose, terminal arabinopyranose, and 2,5-linked arabinose which were not detected in $CHCl_3$–MeOH-soluble fraction of methylated potato tuber cell wall [11].

Rhamnogalacturonan II, newly isolated as a complex polysaccharide from suspension-cultured sycamore cell wall by PG treatment [5], contained 3,4-linked galacturonic acid, 3-linked rhamnose and 3,4-linked rhamnose. But fraction III did not contain apiose, methyl xylose, fucose, and glucuronic acid as detected in rhamnogalacturonan II. It seems possible that this may be due to the difference between suspension-cultured cells and plant tissues.

EXPERIMENTAL

Treatment of potato tuber tissues by PG. Fresh tissues of potato tuber var. Danshaku were cut into small pieces (25 × 15 × 0.5 mm), washed several times with 0.1 M NH_4OAc buffer (pH 4.5), and allowed to stand in the same buffer at 30° for 4 hr. The tissues were then repeatedly washed with 50 mM NH_4OAc buffer (pH 4.5) until the washings no longer contained sugars detectable by the PhOH–H_2SO_4 method [12]. After removal of excess buffer soln with filter paper, 100 pieces of the tissue were placed in a 150 ml flask containing 50 ml 50 mM NH_4OAc buffer (pH 4.5) and purified PG of *A. japonicus* [13]. After 10 min the reaction mixtures containing 1000 units of PG were filtered through Toyo No. 5C paper and the filtrate was incubated for 3 hr. For exhaustive treatment after 7 hr of incubation the reaction mixtures containing 2000 units of PG were filtered through the filter paper and the residual tissues were repeatedly washed with buffer. The tissues were then suspended in 40 ml of the buffer and were incubated with the addition of 1000 units of PG for another 7 hr. The solubilized material was removed from the tissue by the filtration and washing. The third treatment was carried out in the above manner with another 1000 units of PG.

Analysis of components released. The galacturonide content was estimated by the *m*-hydroxydiphenyl method of ref. [14]. The degree of esterification was estimated by the method of ref. [15]. Total uronides of potato tuber tissues were determined by the method of ref. [16].

The following colorimetric assays were used for the determination of neutral sugars: the anthrone method for estimation of galactoside content [17], the Dische and Shettles method for estimation of rhamnoside content [17], and the Dische method for estimation of arabinoside content [17]. The galactoside content was corrected for rhamnosyl residues which give a positive reaction in the anthrone method.

Protein content was determined by the Lowry method [18] using BSA as a standard.

Analysis of glycosyl linkage compositions. Pectic substances were de-esterified in 1 ml of 0.05 M NaOH at 0° for 90 min prior to the reduction. The galacturonosyl residues were reduced by the carbodiimide method of ref. [19] using $NaBD_4$ instead of $NaBH_4$. The samples were methylated by the method of ref. [20] as modified by Lindberg [21]. The hydrolysis of the methylated polysaccharides was accomplished by heating at 100° for 2 hr in 85% $H \cdot CO_2H$ followed by heating at 100° for 12 hr in 0.25 M H_2SO_4. The methylated sugars were reduced with $NaBH_4$, acetylated, and analysed by GLC. The stainless steel column (2 m × 3 mm) was packed with Chromosorb W AW-DMCS (80–100 mesh) coated with silicone OV-225 (3%). The column oven temp. was raised linearly from 150 to 200° at a rate of 1°/min. Identification of the partially methylated alditol acetates was made according to their retention time on GLC and their MS data given by Björndal *et al.* [22]. The galacturonosyl linkages

were determined by the ratio of dideuterium-labelled fragment ions to unlabelled fragment ions obtained by MS analysis.

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