Enzymatic Maceration of Plant Tissues by Endo-Pectin Lyase and Endo-Polygalacturonase from Aspergillus japonicus

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ABSTRACT

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Aspergillus japonicus produces endo-pectin lyase and endo-polygalacturonase as the main pectin depolymerases. The two enzymes differed in ability to macerate tissues of different plant species. Endo-pectin lyase most rapidly macerated onion tissue, while endo-polygalacturonase was most effective on radish tissue. Potato tissues were similarly macerated by the two enzymes. Susceptibility of plant tissues to maceration by the enzymes may depend largely on the enzyme specificity but not enzyme concentration. There were also differences between the two enzymes in the effect of pH and calcium ions on macerating activity. The two enzymes apparently differed in ability to release pectic substances from plant cell walls. The pectic substances extractable with 0.05 N NaOH, which are commonly referred to as protopectins, were accessible to endo-pectin lyase, while those extractable with 0.5% ammonium oxalate were accessible to endo-polygalacturonase. The relationship between the susceptibility of plant tissue to maceration by the two enzymes and the susceptibility of each pectic substance in the cell wall to attack by the enzymes may suggest the type of pectic substance that is important in cell wall cementing.

Additional key words: fractional extraction of pectic substances, gel filtration.

Enzymatic maceration of plant tissues is an important factor in plant pathogenesis (2, 33), in fruit ripening, and leaf abscission (26). In some diseases, maceration is directly correlated with cellular death (9, 10, 24, 25, 31). Endo-polygalacturonase (endo-PG, EC 3.2.1.15) and endo-pectate lyase (endo-PAL, EC 4.2.2.1) that split α-1,4 glycosidic linkages in pectic acid at random, can macerate plant tissues (1, 5, 11, 24, 28, 35), and consequently the intercellular cement between plant cells was thought to be largely low-methoxyl pectic substances. Enzymes that attack pectin but not pectic acid [e.g., endo-pectin lyase (endo-PL, EC 4.2.2.3)] also macerate plant tissues (3, 4, 34). Thus, high-methoxyl pectins may also play a role in cell wall cementing (14, 20). Relatively few comparative studies have been made of enzymatic tissue maceration by endo-PL, endo-PG, and endo-PAL.

During maceration, breakdown of insoluble pectins in cell walls leads to the loss of tissue coherence and separation of individual cells. Cell wall pectins can be separated into: low-methoxyl pectin extractable with ammonium oxalate and EDTA, and high-methoxyl pectin extractable with NaOH (26). The latter is commonly referred to as protopectin.

Aspergillus japonicus, a nonpathogenic fungus, produces endo-PL and endo-PG as the main pectolytic enzymes (16), and both are capable of macerating various plant tissues (15, 17).

In this paper, comparative studies on enzymatic maceration by endo-PL and endo-PG are reported using purified enzymes from *A. japonicus*.

MATERIALS AND METHODS

Plant materials.—Fresh tissues of potato tuber (*Solanum tuberosum* L.), onion (*Allium cepa* L.) and radish root (*Raphanus sativus* var. *raphanistroides*) were cut into small pieces about 5 × 5 × 5 mm, washed several times with water, and the excess water was removed with filter paper. For plant cell wall preparations, the fresh tissues (1 kg) were rapidly sliced and boiled in 4 liters of 96% (v/v) ethanol for 20 minutes to kill the cells. The boiled tissues were filtered through a layer of cheesecloth, and fragmented in 75% (v/v) ethanol with a blender. The fragmented tissues were filtered on a Büchner funnel, washed with 75% (v/v) ethanol, and pressed almost dry. The cell wall residues were suspended in cold water and stirred at room temperature for 1 hour, and then filtered on a Büchner funnel. The residues were extracted twice more with cold water and then were washed successively with ethanol and ether, and dried in vacuo at room temperature.

In the case of potato tissue, before the extraction with cold water the cell wall residues were suspended in water and treated with crystallized α-amylase from *Bacillus subtilis* (Seikagaku Kogyo Co., Ltd.) at 55 C for 2 hours. White powders of potato (20.7 g), onion (10.7 g) and radish (4.4 g) were obtained, and they were regarded as plant cell walls.

Enzymes.—Purified endo-PL and endo-PG were obtained from the culture medium of *A. japonicus* 1744 ATCC 20236 by methods described previously (15, 17). Both purified enzymes were homogeneous on ultracentrifugation and disk electrophoresis, and were free from other enzymes. The purified endo-PL and endo-PG had a specific activity of 582 and 3,467 units/mg of protein, respectively. One unit of endo-PL was defined as the amount of enzyme that causes an increase in absorbance of 1.0/minute at 235 nm. The reaction mixture contained 1 ml of 1% pectin N.F. (Sunkist Growers Inc.), 1 ml of 0.1 M McIlvaine buffer (pH 6.0), and 0.5 ml of enzyme at 40 C (17). In the case of endo-PG, one unit of activity was defined as the amount of enzyme that reduces the viscosity of the reaction mixture 50% in 1 minute at 40 C (15). The reaction mixture contained 3 ml of 1% sodium polypectate (Sunkist Growers Inc.), 3 ml of 0.1 M McIlvaine buffer (pH 4.5), and 1 ml of enzyme.

Assay of macerating activity.—Cell separation and galacturonide release were used to estimate macerating activity in this study. Cell separating activity was determined by measuring the volume of single cells released from plant tissues. Tissue pieces (7 g wet weight) were placed in a 150-ml Erlenmeyer flask containing 25 ml of 0.1 M McIlvaine buffer, pH 4.5 (or 0.1 M acetate buffer, pH 4.5), 10 mg of bovine serum albumin, purified enzyme, and water to a final volume of 50 ml. The addition of bovine serum albumin was necessary for both enzymes to prevent their inactivation at low protein concentrations as described previously (15, 17). The flasks were shaken on a rotary shaker at 160 rpm and 40 C. At intervals, the reaction mixtures were filtered through a 0.86-mm (24-mesh) sieve, and 5 ml of filtrate was placed in a graduated test tube. Almost all the cells that passed through the sieve were single cells as confirmed by microscopic examination. The volume (ml) of single cells was measured when the cells had sedimented to an unchanged level (about 2 hours at room temperature).

Release of galacturonides from insoluble pectic substances was determined using plant cell walls as the substrate. The cell walls (50 mg) were placed in a 150-ml Erlenmeyer flask containing 50 ml of 0.1 M acetate buffer (pH 5.0), 10 mg of bovine serum albumin, and purified enzyme. The flasks were shaken on a rotary shaker at 120 rpm and 40 C. At intervals, the reaction mixtures were filtered through Toyo No. 5C paper, and the galacturonide content in the filtrates was determined by the carbazole method (22).

Analysis of pectic substances.—The galacturonide content of cell wall preparations was estimated by determining anhydrogalacturonic acid by the carbazole method (22). Neutral sugars were calculated as the difference between the total sugar estimated by the phenol-sulfuric method (7) and the galacturonic acid estimated by the carbazole method, and was expressed as mg of anhydroarabinose. Total pectic substances of plant cell walls were determined by the method of McCready

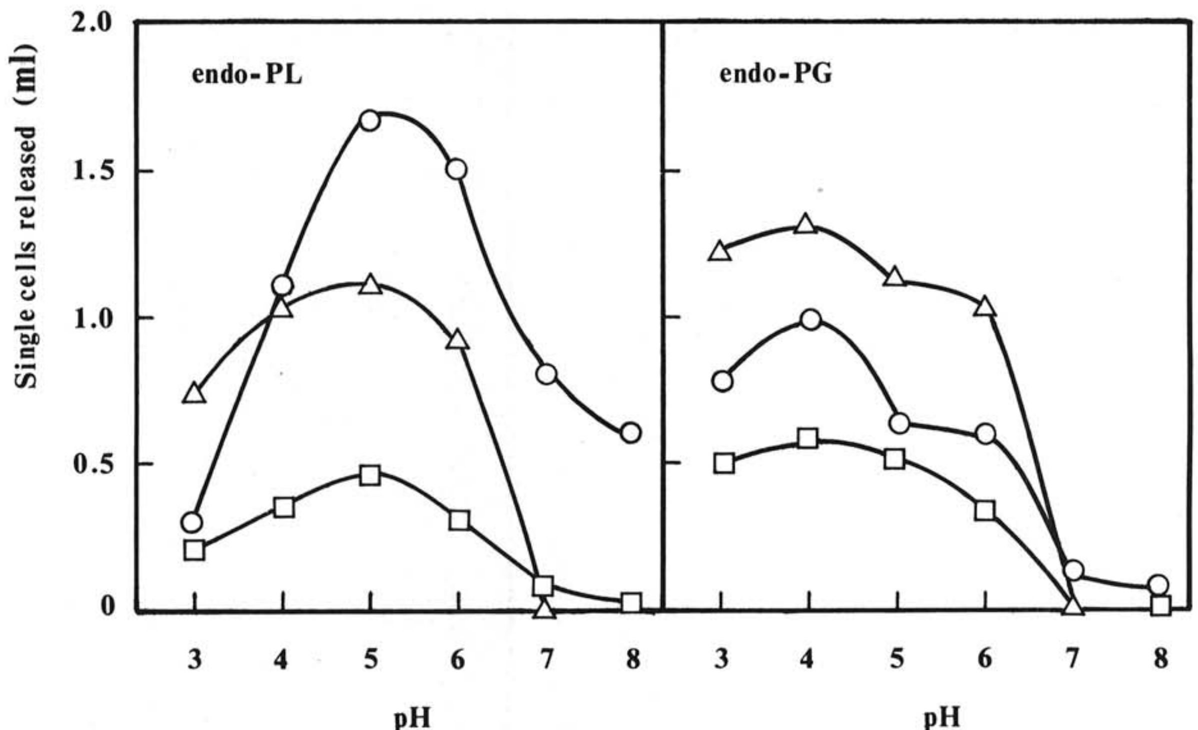

Fig. 1. Effect of pH on cell separating activity by endo-PL and endo-PG. Activity was measured in reaction mixtures containing 7 g of plant tissue, 25 ml of 0.1 M McIlvaine buffer of various pH values, 10 mg of bovine serum albumin, 0.1 mg of purified enzyme, and water to a final volume of 50 ml. They were shaken on a rotary shaker at 160 rpm and 40 C for 2 hours. The volume (ml) of single cells released from potato (□), onion (O) and radish (△) tissues was measured as described in "Materials and Methods".

and McComb (23). Fractional extraction of pectic substances in plant cell walls was carried out according to the method of Shewfelt (27).

Paper chromatography.—Paper chromatography of monosaccharides was carried out on Toyo No. 51A paper with an ethyl acetate-pyridine-water (10:4:3) solvent system. The sugars were detected with aniline hydrogen phthalate reagent (32). Before chromatography, polysaccharides were hydrolyzed by 1.0 N H_2SO_4 at 100 C for 6 hours and the hydrolyzates were treated with barium carbonate and Dowex 50W (19).

Gel filtration.—Gel filtration was carried out on a Sephadex G-100 column (1.9 × 98 cm) equilibrated with 0.2 M NaCl. Samples were applied in 1.0 ml to the column, and the elution was carried out with 0.2 M NaCl at a flow rate of 16 ml/hour. The soluble fraction containing pectic substances released from plant cell wall was concentrated to one-tenth of its original volume and subjected to gel filtration. Blue dextran, dextran T-40, dextran T-10, and monogalacturonic acid were used as reference saccharides. The sugar content in the eluent was determined by the phenol-sulfuric method (7).

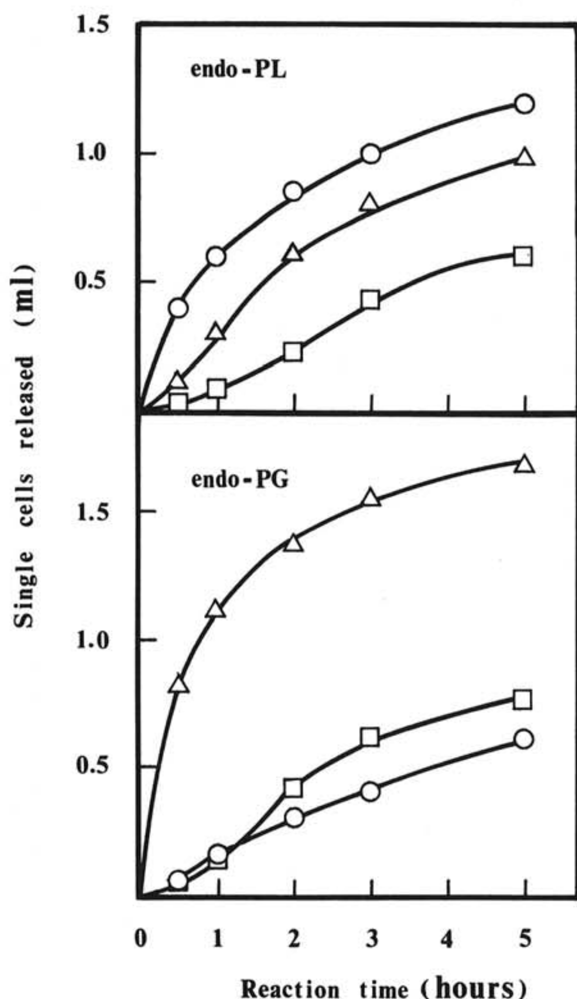

Fig. 2. Enzymatic cell separation of plant tissues by endo-PL and endo-PG. Reaction mixtures containing 7 g of plant tissue, 25 ml of 0.1 M McIlvaine buffer (pH 4.5), 10 mg of bovine serum albumin, 0.1 mg of purified enzyme and water to a final volume of 50 ml were shaken on a rotary shaker at 160 rpm and 40 C. At intervals, the volume (ml) of single cells released from potato (□), onion (O) and radish (Δ) tissues was measured as described in "Materials and Methods".

RESULTS

Cell separation of plant tissues.—1) *Effect of pH on cell separating activity.*—The influence of pH on cell separating activity by endo-PL and endo-PG was determined in McIlvaine buffer because activity of both enzymes in this buffer was much higher than in acetate or Tris-acetate buffers. The pH optimum for activity of endo-PL was about 5.0 in all plant tissues (Fig. 1), although the optimum was between 6.0 and 7.0 if soluble pectin was used as the substrate (17). On the other hand, the pH optimum for cell separating activity by endo-PG was about 4.0. This is in good agreement with the optimum of endo-PG with a pectic acid substrate (15). Cell separating activity of both enzymes was significantly lower at pH 7.0 and relatively few cells were released from the tissues above 7.0, except in the case of onion tissue incubated with endo-PL. No cell separation occurred in these plant tissues without enzyme.

2) *Susceptibility of plant tissue to enzymatic cell separation by endo-PL and endo-PG.*—As previously reported (15, 17), endo-PL and endo-PG from *A. japonicus* macerate plant tissues at different rates. Rates of tissue maceration by endo-PL (0.1 mg, 58.2 units) and endo-PG (0.1 mg, 346.7 units) were determined at pH 4.5, an intermediate point between the optima for the two enzymes. Endo-PL most rapidly macerated onion tissue, while endo-PG was most effective on radish tissue (Fig. 2). Potato tissues were similarly macerated by the two enzymes.

The susceptibility of plant tissues to enzymatic maceration was little affected by enzyme concentration. For example, endo-PL (5 μg, 2.9 units) macerated onion tissue more rapidly than endo-PG (0.1 mg, 346.7 units) did, and a similar result was obtained in the case of radish tissue. Thus, susceptibility of plant tissues to cell separation by endo-PL and endo-PG may depend largely on the enzyme specificity but not enzyme concentration. In other experiments (S. Ishii, *unpublished*) cabbage and spinach tissues were relatively susceptible to maceration by endo-PL, and carrot and cucumber tissues were susceptible to maceration by endo-PG.

3) *Effect of calcium ions and ethylenediaminetetraacetic acid on cell separating activity.*—Maceration of plant tissues by pectolytic enzymes is affected by metal ions, especially Ca^{++}, and metal chelating reagents (12, 21, 35). Maceration by most endo-PAL's is stimulated by Ca^{++} and inhibited by ethylenediaminetetraacetic acid (EDTA), probably because the enzyme requires Ca^{++} for activity (6, 9, 13).

The effect of Ca^{++} and EDTA on cell separation of plant tissues by endo-PL and endo-PG from *A. japonicus* was then examined, although the activity of neither enzyme was affected on pectic substrates (15, 17). The maceration of plant tissues by endo-PG was neither inhibited nor stimulated by Ca^{++} and EDTA (Table 1), as contrasted

TABLE 1. Effect of Ca^{++} and ethylenediaminetetraacetic acid (EDTA) on cell separating activity[a] of endo-pectin lyase (endo-PL) and endo-polygalacturonase (endo-PG)

Plant tissue	Reaction time (hours)	Volume of single cells released by enzyme[b] (ml)					
		endo-PL			endo-PG		
		Control	$CaCl_2$	EDTA	Control	$CaCl_2$	EDTA
Potato	1	0.05	0.01	0.03	0.08	0.10	0.08
	3	0.27	0.02	0.16	0.34	0.37	0.34
	5	0.39	0.08	0.29	0.52	0.60	0.55
Onion	1	0.30	0.31	0.30	0.11	0.11	0.12
	3	0.89	0.81	0.82	0.21	0.20	0.22
	5	1.22	1.13	1.12	0.49	0.48	0.51
Radish	1	0.09	0.01	0.04	0.11	0.10	0.14
	3	0.40	0.03	0.27	0.70	0.70	0.69
	5	0.67	0.12	0.51	1.00	1.03	1.03

[a]Activity was measured in acetate buffer with or without 10^{-3} M $CaCl_2$ and EDTA. Reaction mixtures were the same as in Fig. 2 except that 0.1 M acetate buffer (pH 4.5) was used instead of 0.1 M McIlvaine buffer (pH 4.5).

[b]At intervals, the volume (ml) of single cells released from plant tissue was measured as described in "Materials and Methods".

with the results of McClendon and Somers (21) and Zaitlin and Coltrin (35). In the case of endo-PL, cell separation of onion tissue was not affected by Ca^{++} and EDTA, but activity on potato and radish tissues was strongly inhibited by Ca^{++} and moderately so by EDTA.

4) *Combined action of endo-PL and endo-PG on cell separation of plant tissues.*—The results obtained above revealed differences in enzymatic maceration by endo-PL and endo-PG. Their joint action in cell separation was determined by adding 0.1 mg of endo-PL (58.2 units) and endo-PG (346.7 units) to the reaction mixture individually and in combination. With all plant tissues there was no synergistic action between these enzymes (Table 2).

TABLE 2. Effect of combination of endo-pectin lyase (endo-PL) and endo-polygalacturonase (endo-PG) on rate of cell separation of plant tissue[a]

Plant tissue	Enzyme[b]	Volume[c] of single cells separated after a reaction time (hours) of:		
		0.5	1	3
Potato	endo-PL	0.01	0.08	0.36
	endo-PG	0.02	0.10	0.57
	endo-PL + endo-PG	0.03	0.08	0.43
Onion	endo-PL	0.30	0.50	1.06
	endo-PG	0.02	0.10	0.29
	endo-PL + endo-PG	0.33	0.49	1.01
Radish	endo-PL	0.05	0.17	0.58
	endo-PG	0.42	0.85	1.31
	endo-PL + endo-PG	0.51	0.90	1.21

[a]Reaction mixtures were the same as in Fig. 2.

[b]Enzyme (0.1 mg) of endo-PL and endo-PG was added to the reaction mixture individually and in combination.

[c]The volume (ml) of single cells released from plant tissue was measured as described in "Materials and Methods".

Elarosi (8) found a synergistic relationship in maceration of potato tissue between the enzymes from *Rhizoctonia solani* and *Fusarium solani*, but not between the enzymes from *R. solani* and *Cylindrocarpon radicicola*. He suggested that stimulation between two macerating enzymes may be due to differences in mode of reaction or the sites of attack. The result obtained with endo-PL and endo-PG from *A. japonicus* suggest that the mode of action of the two enzymes in maceration of plant tissues may be regarded as nearly the same.

Release of pectic substances from plant cell walls.—1) *Effect of pH on release of galacturonides from plant cell walls by endo-PL and endo-PG.* —An acetate buffer system (0.1 M sodium acetate-acetic acid buffer for pH 3 to 6) and 0.1 M Tris-acetate buffer for pH 7 and 8 was used for the determination of galacturonide releasing activity, because a considerable amount of galacturonide was released without enzyme in the presence of McIlvaine buffer. In acetate buffer, however, less galacturonide was released without enzyme. The pH optima of galacturonide releasing activity of endo-PL and endo-PG were about 6.0 and 5.0, respectively (Fig. 3). Both values were respectively higher than the pH optima of cell separating activity by these enzymes. Similarly the pH-activity curves of galacturonide releasing activity were different from those of cell separating activity. For example, endo-PL released galacturonide from radish cell wall at neutral and alkaline pH's almost as effectively as at optimal pH, although no cell separating activity was observed in the tissue by the enzyme at the same pH's.

2) *Release of galacturonide from plant cell walls as a function of time.*—In a time course experiment galacturonide was released from plant cell walls in the absence of enzyme (Fig. 4). Endo-PL released galacturonide from onion and potato cell walls more rapidly than endo-PG, while the rate and extent of release from radish cell walls by the two enzymes was similar. This indicates that galacturonide releasing activity of the enzymes does not correspond with the ability of the enzymes to separate cells in plant tissues.

Enzymatic release of galacturonide from cell walls

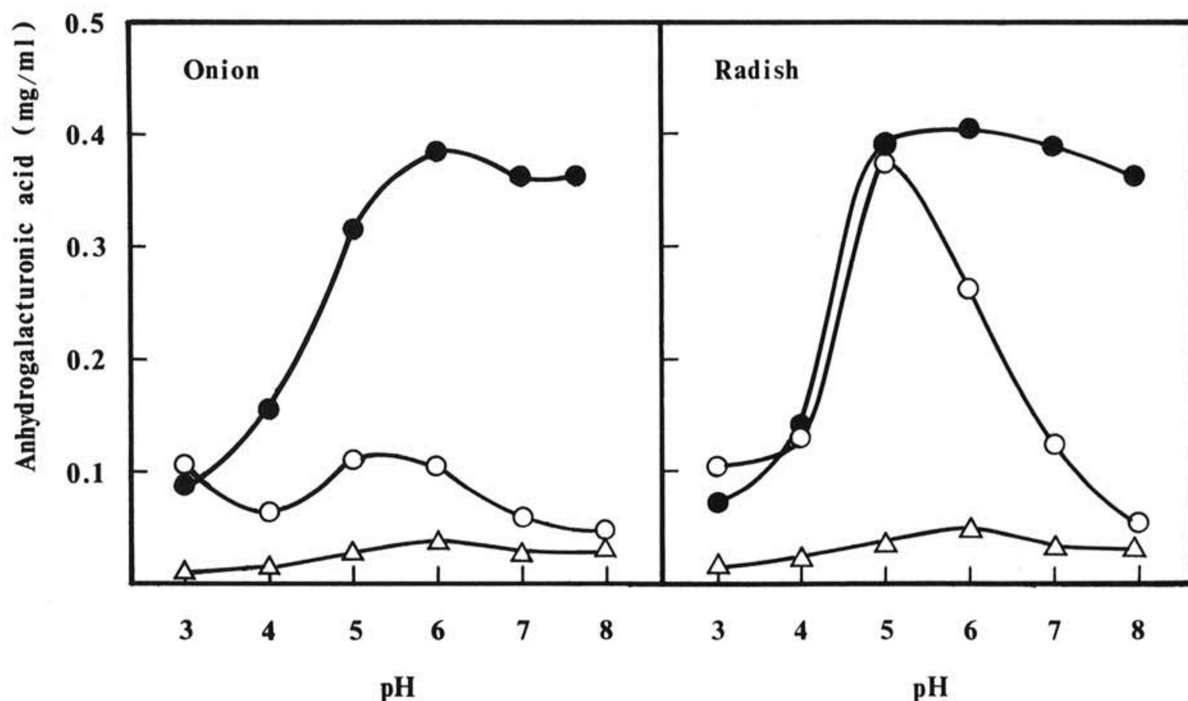

Fig. 3. Effect of pH on galacturonides releasing activity by endo-PL and endo-PG. Reaction mixtures containing 10 mg of plant cell wall, 5 ml of 0.1 M acetate buffer of various pH values, and 2 mg of bovine serum albumin. They were incubated with the addition of 1 μg of endo-PL (●) and endo-PG (O) or without enzyme (△) at 40 C. After 1 hour reaction mixtures were filtered through Toyo No. 5C paper, and galacturonides released from plant cell wall were determined by the carbazole method.

TABLE 3. Amounts of pectic substances and neutral sugars released from plant cell walls by endo-pectin lyase (endo-PL) or endo-polygalacturonase (endo-PG)

Plant tissue	Enzyme[a] treatment	Soluble fraction[b]: Total pectic substances (mg)	Soluble fraction[b]: Solubilization (%)	Soluble fraction[b]: Neutral[d] sugars (mg)	Residue[c]: Weight (mg)	Residue[c]: Pectic substances[e] (mg)
Potato	Control	1.26	15.4	4.06	91	6.94
	endo-PL	5.90	71.9	10.02	75	2.30
	endo-PG	4.30	52.4	5.40	86	3.90
Onion	Control	3.13	14.4	0	97	18.57
	endo-PL	16.21	74.6	9.24	70	5.49
	endo-PG	6.66	30.6	0.20	94	15.04
Radish	Control	3.91	13.3	0.05	92	25.47
	endo-PL	18.57	63.1	2.11	69	10.81
	endo-PG	19.78	67.3	1.85	69	9.60

[a]Each plant cell wall (100 mg) in 50 ml of 0.1 M acetate buffer (pH 5.0) was incubated with 30 μg of endo-PL and endo-PG or without enzyme at 40 C.
[b]After 1 hour reaction mixtures were filtered through Toyo No. 5C paper, and the residue was repeatedly washed with water. A combined solution of the filtrate and the washing was called as soluble fraction.
[c]The residue was successively washed with ethanol and ether, and dried in vacuo.
[d]Neutral sugars were calculated as the difference between the total sugars estimated by the phenol-sulfuric method and pectic substances estimated by the carbazole method, and were expressed an anhydroarabinose.
[e]Pectic substances in the residue were estimated as the difference between total pectic substances of plant cell wall and pectic substances released.

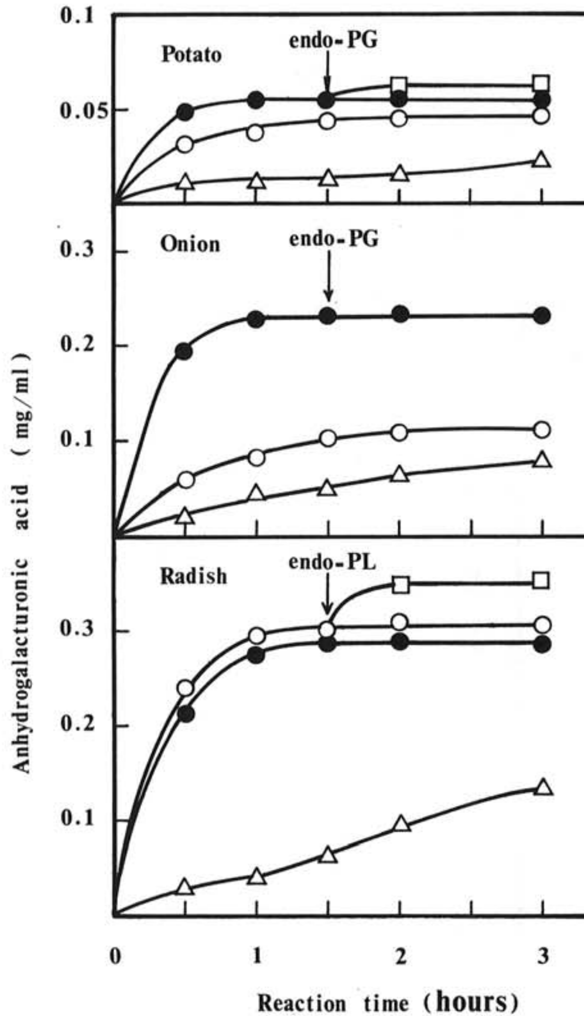

Fig. 4. Time course of release of galacturonides from plant cell walls. Reaction mixtures contained 50 mg of plant cell wall, 50 ml of 0.1 M acetate buffer (pH 5.0), and 10 mg of bovine serum albumin. They were shaken on a rotary shaker at 120 rpm and 40 C with the addition of 10 μg of endo-PL (•) and endo-PG (O) or without enzyme (Δ). After 1.5 hours reaction mixtures of potato and onion cell walls with endo-PL and radish cell wall with endo-PG were divided into two parts, and one (□) was added 5 μg of the other enzyme. At intervals, reaction mixtures were withdrawn and filtered through Toyo No. 5C paper, and galacturonides released from plant cell walls were determined by the carbazole method.

reached a maximum within 1.5 hours. If endo-PL and endo-PG could attack different pectic substances in cell walls, the addition of the other enzyme to the reaction mixture may be expected to release additional galacturonide. Thus, after 1.5 hours of incubation the other enzyme was added to the reaction mixture. The addition of endo-PG failed to increase the release of additional galacturonide from onion cell wall attacked by endo-PL (Fig. 4). However, in the cases of potato and radish cell walls initially incubated with endo-PL and endo-PG, respectively, addition of the other enzyme released additional galacturonide but a lesser amount than expected. This result suggested that the two enzymes did not attack completely different pectic substances in the cell walls.

In all cases, Ca^{++} had no effect on the rate and extent of enzymatic release of galacturonide from cell walls, although the ion strongly inhibited the cell separation from potato and radish tissues by endo-PL.

3) *Analysis of pectic substances released from plant cell walls.*—Total pectic substances in cell walls of potato, onion and radish were 8.2, 21.7 and 29.4%, respectively (Table 3). Endo-PL released about two-thirds of the polyuronides in potato and onion cell walls, while endo-PG released only 50 and 30% respectively from these cell walls. From radish cell walls, endo-PG converted 63-67% of the insoluble pectic substances to the soluble form. The soluble fractions from controls contained 13-15% of the total pectic substances probably released by the acetate buffer at pH 5.0.

Negligible amounts of neutral sugars were detected in the soluble fractions from controls, but with potato cell walls, 4.1 mg of neutral sugar was released. Paper chromatographic analysis of this fraction after hydrolysis gave only glucose, suggesting that this arose from starch that was not completely eliminated by the α-amylase treatment. In addition to glucose, soluble fractions of potato and radish cell walls treated with enzymes contained arabinose and galactose. The amount of neutral sugars released by endo-PL from potato walls was much higher than that released by endo-PG, but with radish walls the amounts were similar. A great deal of neutral sugar was released from onion cell wall by the action of endo-PL and this consisted of only galactose with a trace of rhamnose.

When the soluble fractions were concentrated to one-fifth their original volumes and ethanol was added (75%, v/v) the ratio of ethanol-soluble uronides to insoluble uronides differed between enzyme treatments. A large portion of the uronide (70-80%) released from cell walls by endo-PL was present in the ethanol-soluble fraction, but the material released by endo-PG made up only 10-15% of this fraction. The uronide released without enzyme and the neutral sugars released by enzymes were always recovered in the ethanol-insoluble fraction. These results suggest that the endo-PL converted pectic substances in plant cell walls into smaller products than did the endo-PG. Gel filtration of radish cell wall incubated with endo-PL and endo-PG showed about the same amount of pectic substances and neutral sugars (Fig. 5). The main component of the soluble fraction produced by endo-PL (fraction No. 49) contained oligomer, but no monomer and a small amount of substances with molecular weights between 10,000 and 40,000. The sample produced by endo-PG contained a relatively large amount of high molecular weight substances. Both samples contained polysaccharides with molecular weights of more than 40,000 that appeared almost at the void volume. Neutral sugars were detected only in these high molecular weight polysaccharides. The ratios of neutral sugars to galacturonic acid in these polysaccharides produced by endo-PL and endo-PG were estimated to be 2.6 and 1.4, respectively.

Similar elution patterns were obtained with soluble

TABLE 4. Fractional extraction of pectic substances in plant cell wall with or without enzyme treatment

Plant tissue	Enzyme treatment	Total pectic substances released (mg)	Extractable pectic substances in residue (mg)	
			0.5%[a] ammonium oxalate	0.05 N[b] NaOH
Potato	Control	1.26	1.22	4.47
	endo-PL	5.90	1.16	1.12
	endo-PG	4.30	0.60	3.24
Onion	Control	3.13	3.45	15.02
	endo-PL	16.21	3.10	2.11
	endo-PG	6.66	3.18	11.51
Radish	Control	3.91	16.50	8.26
	endo-PL	18.57	9.31	1.20
	endo-PG	19.78	4.36	4.57

[a] Each residue in Table 3 was suspended in 30 ml of 0.5% ammonium oxalate, stirred at room temperature for 1 hour, and filtered through Toyo No. 5C paper. The procedure was repeated by resuspension of the residue in 30 ml of ammonium oxalate.

[b] The NaOH-soluble pectic substances in the residue treated with ammonium oxalate was determined in the same manner except that 0.05 N-NaOH was used instead of 0.5% ammonium oxalate for extraction.

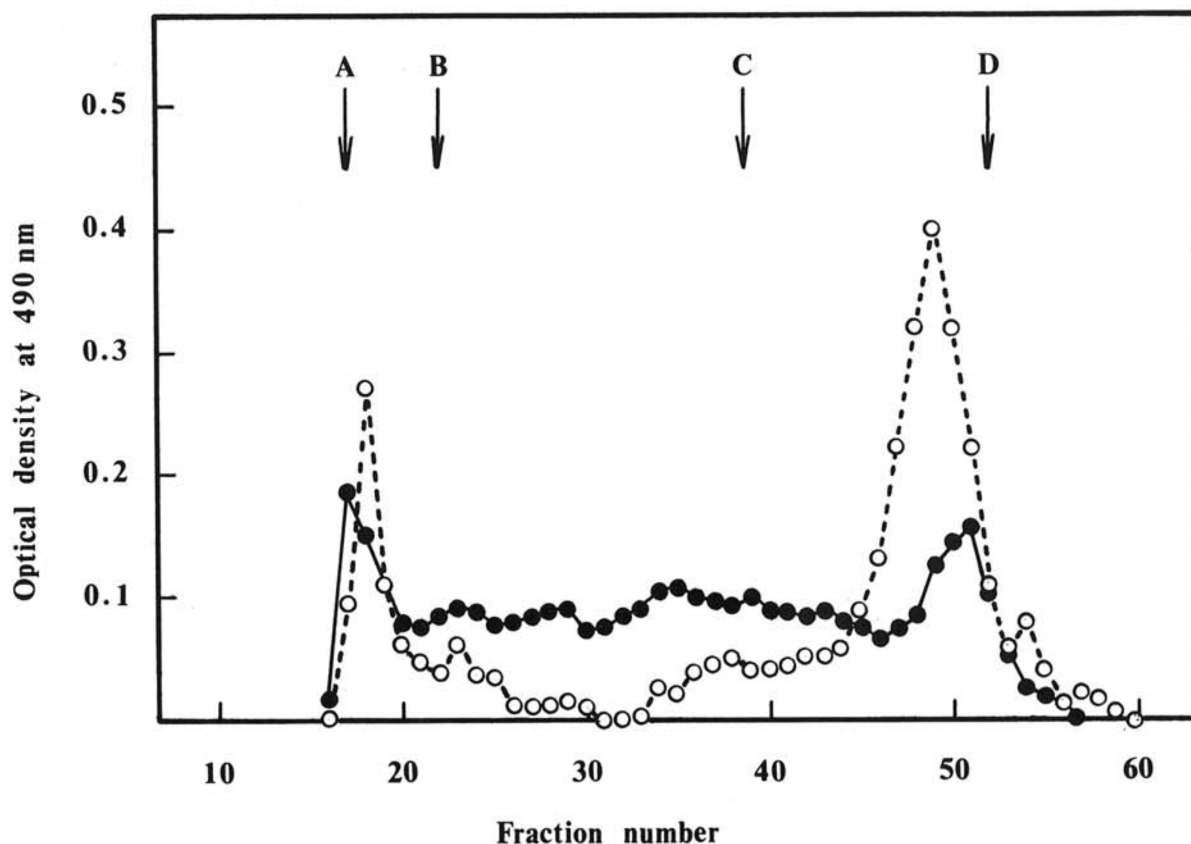

Fig. 5. Gel filtration patterns in Sephadex G-100 of soluble fractions from radish cell wall. The sample was applied in 1.0 ml volume to 1.9 × 98 cm of Sephadex G-100. The column was eluted with 0.2 M NaCl at a flow rate of 16 ml/hour and 5-ml fractions were collected. Sugar was determined by the phenol-sulfuric method. The markers A, B, C and D represent, respectively, the elution volumes of blue dextran (mol wt 2,000,000), dextran T-40 (mol wt 40,000), dextran T-10 (mol wt 10,000) and monogalacturonic acid. Soluble fractions of endo-PL (O) and endo-PG (•).

fractions of onion cell walls by the enzymes. However, high molecular weight polysaccharides derived from onion cell wall by endo-PL contained a very high percentage of netural sugar (galactose) with a neutral sugar to galacturonic acid ratio of 7.5.

4) *Fractional extraction of pectic substances in plant cell walls with or without enzyme treatment.*—To determine which part of the insoluble pectic substances were attacked by the enzymes, the cell walls with or without enzyme treatment were fractionated into two parts 0.5% ammonium oxalate-soluble pectic substances, and those soluble in 0.05 N NaOH. The ratio of pectic substances extractable with ammonium oxalate to those extractable with NaOH in the controls varied with plant species; potato and onion cell walls contained large amounts of pectic substances extractable with NaOH but radish cell wall contained greater amounts of pectic substances extractable with ammonium oxalate (Table 4). These results agree with those obtained by Kawabata, et al (18). The pectic substances extractable with NaOH were accessible to endo-PL, while those extractable with ammonium oxalate were accessible to endo-PG. This susceptibility of the substrate was consistent with the enzyme specificity; however, the pectic substances extractable with ammonium oxalate in onion cell wall were highly resistant to attack even by endo-PG.

The relationship between the susceptibility of plant tissue to enzymatic cell separation by endo-PL and endo-PG and the susceptibility of each pectic substance in the cell wall to attack by the enzymes may suggest the type of pectic substance that is important in cell wall cementing in the tissues. In onion tissue that was susceptible to cell separation by endo-PL, the pectic substances extractable with NaOH may be the main cementing materials. On the other hand, the pectic substances extractable with ammonium oxalate and accessible to endo-PG may play an important role in cell wall cementing in radish tissue, which was more susceptible to cell separation by endo-PG than by endo-PL.

DISCUSSION

In this study differences in enzymatic separation of cells and release of galacturonides were studied in terms of the effect of pH on enzymatic activity, susceptibility of plant species to the enzymatic attack, and the effect of Ca^{++} on the enzymatic activity. Macerating activity was determined by measuring release of pectic substances from plant cell walls, tissue softening, and cell separation. But on the basis of the results obtained it should be noted that enzymatic maceration can vary according to the assay method.

Comparative studies on enzymatic maceration by endo-PL and endo-PG from *A. japonicus* revealed that there are differences between the two enzymes. The enzymes apparently differed in ability to macerate tissues of different plant species. The susceptibility of plant tissues to cell separation by the enzymes largely depended on the enzyme specificity but not enzyme concentration. Onion tissue was quite susceptible to cell separation by endo-PL, while radish tissue was more accessible to endo-PG than to endo-PL.

Garibaldi and Bateman (9) reported that carrot tissue was relatively resistant to maceration by one of two endo-PAL's from *Erwinia chrysanthemi,* but not to the other, whereas potato and cucumber tissues were quite susceptible to maceration by both enzymes.

Endo-PL and endo-PG from *A. japonicus* also differed in their response to Ca^{++}. Endo-PG was not affected by Ca^{++}, but endo-PL was strongly inhibited. However, the activity of endo-PL was not affected when onion tissue was used as the substrate. Protopectinase activity of endo-PL, which can release pectic substances from plant cell walls, was not inhibited by Ca^{++} in all plant species.

Endo-PL and endo-PG attacked insoluble pectic substances in cell walls fractionated into ammonium oxalate-soluble and NaOH-soluble materials, but the enzymes apparently differed in ability to release galacturonide from each insoluble pectic substance. The former pectic substance was more susceptible to endo-PG than endo-PL and the latter was more susceptible to endo-PL than to endo-PG. It was also demonstrated, from the results of fractionation with ethanol and gel filtration of soluble fractions from plant cell wall, that endo-PL converts insoluble pectic substances into smaller products than does endo-PG.

Although there are several differences in enzymatic maceration by endo-PL and endo-PG when the two enzymes were combined, a stimulation did not occur in maceration of all plant tissues. This suggests that the mode of action of the two enzymes in cell separation may be similar. From the relationship between the susceptibility of plant tissue to enzymatic cell separation by endo-PL and endo-PG and the susceptibility of each insoluble pectic substance to attack by the enzymes, it may be concluded that in radish tissue the pectic substance extractable with ammonium oxalate and in onion tissue the pectic substance extractable with NaOH are the principal binding agents between plant cells.

From the results it seems reasonable to conclude that both enzymes attack the same pectic fraction in the cell wall when the enzymes give rise to cell separation.

Purified enzymes with definite specificity are useful to elucidate the chemical nature of cell wall structure (20, 29, 30, 35). The use of endo-PL and endo-PG in a given plant tissue can suggest which pectic substances in cell walls may play an important role in cell wall cementing.

Neutral sugars were released from plant cell walls together with pectic substances by the action of the enzymes. Arabinose and galactose were detected from potato and radish cell walls but only galactose was obtained from onion cell walls. These neutral sugars may be covalently linked to the pectin as arabinogalactan and galactan. Further studies are needed before the interconnection and the functional role of these neutral sugars in cell walls can be established.

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