Purification and Characterization of a Factor that Stimulates Tissue Maceration by Pectolytic Enzymes

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ABSTRACT

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Aspergillus japonicus produces a factor that stimulates tissue maceration by pectolytic enzymes but cannot macerate plant tissues by itself. The factor was purified from the culture medium of this fungus by ammonium sulfate fractionation, chromatography on columns of DEAE-Sephadex and SE-Sephadex, and by gel filtration. The purified factor was homogeneous by disc electrophoresis. The pH optimum of the factor for stimulation of tissue maceration in the presence of endo-pectin lyase or endo-polygalacturonase was between 4.5 and 5.0. The factor did not stimulate the activities of endo-pectin lyase and endo-polygalacturonase when pectin and sodium polypectate were used as the substrates. The factor alone could not release any sugars from potato tuber cell walls, but it released additional galacturonides, arabinose, and galactose when incubated simultaneously with pectolytic enzymes. Similar results were obtained when cell walls which had been pretreated with pectolytic enzymes were incubated with the factor. The factor affected endo-pectin lyase and endo-polygalacturonase similarly. The ability of the factor to release additional sugars from cell walls was considerably less than its ability to stimulate tissue maceration.

Additional key words: polysaccharide-degrading activity, glycosidase activity.

Plant cells are cemented together with intercellular materials which are composed mainly of pectic polysaccharides. Breakdown of these insoluble pectic polymers leads to the loss of tissue coherence and separation of individual cells, a phenomenon which is referred to as maceration.

Pectic polysaccharides contain neutral sugars such as arabinose, galactose, rhamnose, and xylose covalently linked to galacturonan chains. Talmadge et al. (25) and Keegstra et al. (14) have indicated that arabinogalactans of sycamore cell wall may serve as a cross-link between galacturonan and other cell wall components.

It has been demonstrated in many instances that the degradation of α-1,4 glycosidic linkages in galacturonan at random by enzymes is sufficient to cause tissue maceration (1, 2, 9, 22). The possibility that enzymes other than endo-pectolytic enzymes can contribute to maceration should not be eliminated, however. Arabanase (3) and galactanase (4, 16, 17) have been suspected of playing a role in the maceration process.

Aspergillus japonicus produces a factor that stimulates tissue maceration by pectolytic enzymes but cannot macerate plant tissues by itself (8). The factor showed no ability to degrade beet arabinan, lupin galactan, or soybean arabinogalactan. However, the factor was a heat-labile, high molecular weight substance which suggested that it may be an enzyme. The present paper describes the purification and characterization of the factor from *A. japonicus*.

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MATERIALS AND METHODS

Plant materials.—Fresh tissues of potato tuber (*Solanum tuberosum* L.) were cut into small pieces (about 5 × 5 × 5 mm), washed several times with water, and the excess water was removed with filter paper. Potato tuber cell walls were obtained by the following procedures. The fresh tissues (1 kg) were rapidly sliced and boiled in 4 liters of 96% (v/v) ethanol for 20 min. 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% ethanol, and blotted almost dry. The cell wall residues were suspended in 500 ml 0.05 M acetate buffer (pH 4.5), treated with 100 mg crystallized α-amylase from *Bacillus subtilis* (Seikagaku Kogyo Co., Ltd.) at 30 C for 2 hr, and then filtered on a Büchner funnel. The procedure was repeated twice by resuspending the residues in 500 ml 0.05 M acetate buffer (pH 4.5) containing 50 mg α-amylase. The cell wall residues were washed successively with water, 75% (v/v) ethanol, absolute ethanol and ether, and dried in vacuo at room temperature.

Enzyme substrates.—Carboxymethyl cellulose (DP 500, DS 0.42), mannan (yeast), and laminarin (from *Laminaria hyperborea*) were purchased from Tokyo

Kasei Kogyo Co., Ltd. Xylan (wood) was purchased from Nutritional Biochemicals Corporation, galactan (larch) from K & K Laboratories, Inc., and pectin N.F. and sodium polypectate from Sunkist Growers, Inc. Arabinan was prepared from sugar-beet by the method of Hirst and Jones (6), galactan from lupin seed by the method of Jones and Tanaka (13), and polymethyl polygalacturonate methyl glycoside by the method of Morell et al. (20, 21). The reagents *p*-nitrophenyl-α-D-glucopyranoside, *p*-nitrophenyl-β-D-glucopyranoside, *p*-nitrophenyl-β-D-galactopyranoside, *p*-nitrophenyl-α-D-xylopyranoside, *p*-nitrophenyl-β-D-xylopyranoside, *p*-nitrophenyl-α-L-fucopyranoside, and methyl-α-L-rhamnoside were purchased from Koch-Light Laboratories Ltd.; *p*-nitrophenyl-α-D-galactopyranoside and *p*-nitrophenyl-α-D-mannopyranoside were purchased from P-L Biochemicals, Inc.

Enzyme preparations.—A crude enzyme was prepared from the culture medium of *A. japonicus* isolate 1744 (ATCC 20236) by the method described previously (8). Purified endo-pectin lyase (endo-PL) and endo-polygalacturonase (endo-PG) were obtained by methods described previously (11, 12). One unit of endo-PL caused an increase in absorbance of 1.0/min at 235 nm and 40 C. The reaction mixture contained 1 ml 1% pectin N.F., 1 ml 0.1 M citrate-phosphate buffer (pH 6.0), and 0.5 ml enzyme. One unit of endo-PG caused a 50% loss in viscosity in 1 min at 40 C. The reaction mixture contained 3 ml 1% sodium polypectate, 3 ml 0.1 M citrate-phosphate buffer (pH 4.5), and 1 ml enzyme in an Ostwald viscometer.

Enzyme assays.—The activity of stimulating factor (ST) was determined by measuring the increase of single cells released from potato tuber tissues. The activity was routinely assayed in the presence of endo-PG because its action cannot be detected in the absence of pectolytic enzymes (Fig. 1). The method of Bateman (1) was modified as follows: 3 g of potato tuber pieces ($5 \times 5 \times 5$ mm) were placed in a 50-ml Erlenmeyer flask containing 5 ml 0.1 M citrate-phosphate buffer (pH 4.5), 2 mg bovine serum albumin, 100 units endo-PG, ST, and water to a final volume of 10 ml. The tissue from a single potato tuber served as the substrate for all treatments in a given experiment. The flasks were shaken on a rotary shaker at 160 rpm and 40 C for 3 hr. The reaction mixtures were filtered through a 0.862-mm (24-mesh) sieve, and 5 ml of filtrate was placed in a graduated test tube. The volume (milliliters) of single cells passed through the sieve was measured when the cells had sedimented to a constant level (about 2 hr at 25 C).

Cellulase activity was determined by measuring reducing sugars liberated from carboxymethyl cellulose (CMC). Reaction mixtures contained 0.5 ml 0.5% CMC in 0.1 M acetate buffer (pH 4.5) and 0.5 ml enzyme. They were incubated at 40 C for 10 min. Reactions were terminated by addition of 1 ml 0.1 N NaOH, and reducing sugars were determined by the method of Nelson (23) and Somogyi (24). One unit of cellulase was defined as the amount of enzyme that liberates 1 μg reducing sugar (as glucose) per minute.

Polysaccharide-degrading activity was determined by measuring the release of reducing sugars from 0.5% solution of the appropriate polysaccharide in 0.1 M acetate buffer (pH 4.5). Reaction mixtures containing 0.5 ml 0.5% polysaccharide and 0.5 ml enzyme were incubated at 40 C for 3 hr. Reactions were terminated by addition of 1 ml 0.1 N NaOH, and reducing sugars were determined as above.

Glycosidase activity was determined by measuring the increase in absorbance resulting from the release of *p*-nitrophenol from the respective *p*-nitrophenyl glycoside. One ml of a solution containing 1 mg the appropriate *p*-nitrophenyl glycoside in 0.1 M acetate buffer (pH 4.5) was mixed with 1 ml enzyme, and incubated at 40 C for 10 min. The reactions were terminated by addition of 2 ml 0.1 M Na_2CO_3. The increase in absorbance (400 nm) was determined with a Hitachi Model 101 spectrophotometer. In the case of α-rhamnosidase, the release of reducing sugar was determined by the method of Nelson (23) and Somogyi (24).

Cell wall-degrading activity was determined by measuring the release of galacturonides and neutral sugars from potato tuber cell walls. The cell walls were suspended in 0.05 M acetate buffer (pH 4.5) at a concentration of 2 mg/ml in Erlenmeyer flasks. The flasks were shaken at 115 rpm and 30 C on a rotary shaker after addition of enzymes. After 3 hr the reaction mixtures were filtered through Toyo No. 5C paper, and the galacturonide content in the filtrates was estimated by determining anhydrogalacturonic acid by the carbazole method (19). Polysaccharides in the filtrates were hydrolyzed in 1 N H_2SO_4 at 100 C for 6 hr, and the hydrolyzates were neutralized with $BaCO_3$ and then

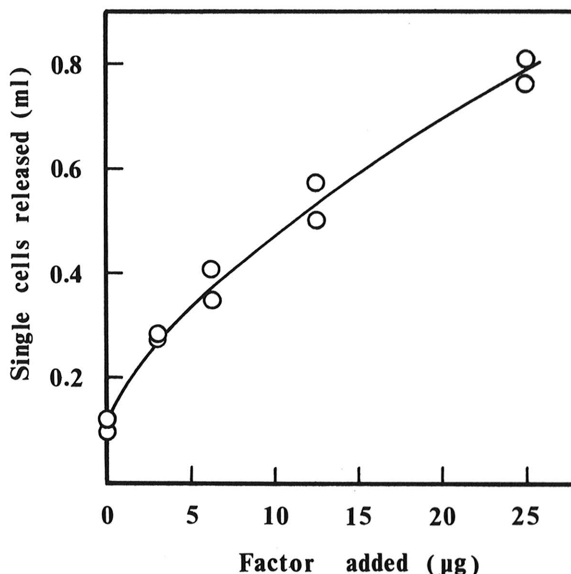

Fig. 1. Activity of a maceration-stimulating factor (ST) produced by *Aspergillus japonicus*; relation between ST concentration and the volume of single potato tuber cells released. Reaction mixtures contained 3 g potato tuber tissue ($5 \times 5 \times 5$ mm), 5 ml 0.1 M citrate-phosphate buffer (pH 4.5), 2 mg bovine serum albumin, 100 units endo-PG, purified ST and water to a final volume of 10 ml. They were shaken on a rotary shaker at 160 rpm and 40 C for 3 hr. The volume (ml) of single cells released from potato tissues was measured as described in the Materials and Methods section of this paper.

filtered. Analyses of neutral sugar compositions of the hydrolyzates were carried out by means of the automated liquid chromatography (JEOL, JLC-6AH Type). Sample solution (0.8 ml) was injected into the column (0.8 × 13 cm) packed with an anion exchange resin (JEOL Resin IC-R-3, borate form), and eluted successively with 0.13 M borate buffer (pH 7.5), 0.25 M borate buffer (pH 9.0), and 0.35 M borate buffer (pH 9.6) at a flow rate of 0.51 ml/min. An orcinol-sulfuric acid reagent (15) was used to detect sugars in the effluent and the absorbance at 440 nm was monitored and counted by the KD integrator. The amounts of each sugar were calculated by comparison with the absorbance of the standard.

Protein determination.—Protein concentrations were determined by the method of Lowry et al. (18) with bovine serum albumin as a standard.

Electrophoresis.—Disc electrophoresis was carried out in polyacrylamide gel (pH 9.5) by the method of Davis (5). About 70 μg ST was applied to the column, and electrophoresis was carried out at 4 C for 2 hr at 2.5 mA per gel. Protein bands were detected by staining the gel with a 1% solution of amido black dissolved in 7% acetic acid.

RESULTS

Purification of stimulating factor (ST).—Purification of ST was carried out at 4 C.

The crude enzyme (10 g) was dissolved in 250 ml of water, and the insoluble material was removed by centrifugation at 8,000 *g* for 10 min. To the supernatant liquid was added 70 g ammonium sulfate with continuous stirring. The precipitate formed was removed by centrifugation at 8,000 *g* for 10 min. To the supernatant fluid 75 g of ammonium sulfate was added. After the mixture was allowed to stand overnight, the precipitate was collected by centrifugation at 8,000 *g* for 10 min. The precipitate was dissolved in 20 ml 0.1 M acetate buffer (pH 5.0), and subjected to dialysis against the same buffer for 24 hr. The precipitate formed during dialysis was removed by centrifugation at 8,000 *g* for 10 min. One half (about 17 ml) of the dialyzate was applied on a DEAE-Sephadex A-50 column (2 × 33 cm) previously equilibrated with 0.1 M acetate buffer (pH 5.0). After the column was washed with 500 ml 0.1 M acetate buffer (pH 5.0), elution was carried out with a linear 0.0 to 0.5 M NaCl gradient prepared in the same buffer at a flow rate of 20 ml/hr. The eluate was collected in 10-ml fractions. Active fractions (100 ml) eluted between 0.20 and 0.22 M NaCl concentration, corresponding to fraction IV (8), were pooled and the eluates from two runs were combined. The combined eluate was dialyzed against 0.05 M ammonium acetate buffer (pH 5.0) for 8 hr and then lyophilized.

The powder was dissolved in 2 ml 0.1 M ammonium acetate buffer (pH 5.0), and the solution was applied to a Sephadex G-75 column (2.5 × 150 cm). Elution, at a flow rate of 15 ml/hr, was carried out with the same buffer and

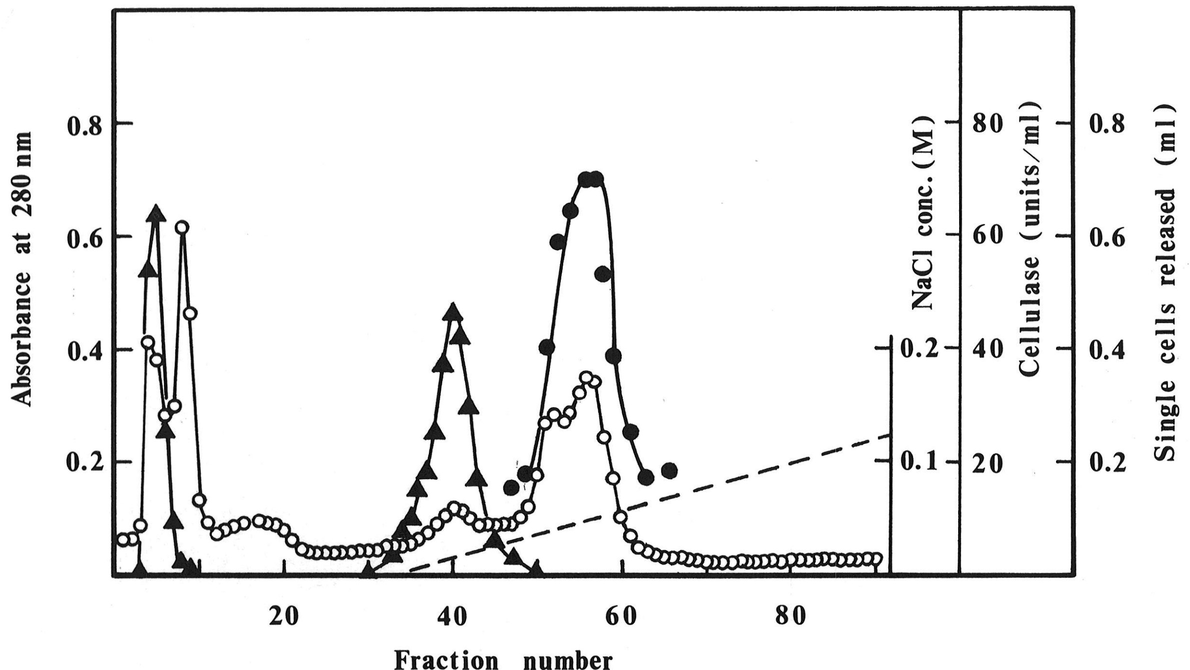

Fig. 2. Purification by SE-Sephadex column chromatography analysis of a maceration-stimulating factor produced by *Aspergillus japonicus*. The lyophilized active fraction from first gel filtration with Sephadex G-75 column was dissolved in 5 ml 0.1 M acetate buffer (pH 3.8) and dialyzed against the same buffer for 16 hr. The dialyzate was applied on a SE-Sephadex column (2 × 26 cm) previously equilibrated with 0.1 M acetate buffer (pH 3.8). The column was eluted with 200 ml 0.1 M acetate buffer (pH 3.8) and then with a linear 0.0 to 0.2 M NaCl gradient a flow rate of 20 ml/hr. The eluate was collected in 10-ml fractions and absorbance (A_{280nm}) O), maceration-stimulating (single-cell release from potato tuber tissue) activity (•), cellulase activity (Δ), and NaCl concentration (----) were measured.

10 ml-fractions were collected. The active fractions (40 ml) were collected, dialyzed against distilled water, and then lyophilized.

This powder was dissolved in 5 ml 0.1 M acetate buffer (pH 3.8) and dialyzed against the same buffer for 16 hr. The dialyzate was layered on a SE-Sephadex column (2 × 26 cm) previously equilibrated with 0.1 M acetate buffer (pH 3.8). After the column was washed with 200 ml of the same buffer, elution was carried out with a linear 0.0 to 0.2 M NaCl gradient at a flow rate of 20 ml/hr. The eluate was collected in 10-ml fractions and ST and cellulase activities were measured (Fig. 2). ST activity was detected only in fractions No. 50-60. A final preparation obtained in the previous study contained cellulase as a contaminant (8). However, SE-Sephadex column chromatography completely separated ST from cellulases. The active fractions (No. 55-57, 30 ml) were collected, dialyzed against 0.05 M ammonium acetate buffer (pH 5.0), and then lyophilized.

The powder was dissolved in 1 ml 0.1 M ammonium acetate buffer (pH 5.0), and the solution was subjected to a second gel filtration on a Sephadex G-75 column (2.5 × 150 cm). The elution, at a flow rate of 10 ml/hr, was carried out with 0.1 M ammonium acetate buffer (pH 5.0), and 5-ml fractions were collected. Two protein peaks were obtained (Fig. 3). The main protein peak, which had an estimated molecular weight of 21,000, had ST activity. It was demonstrated that this final preparation (No. 65-67), which contained 67 μg protein/ml, was homogeneous by disc electrophoresis.

Characterization of stimulating factor (ST).—The effect of pH on ST activity for tissue maceration was determined in the presence of endo-PG or endo-PL, because its action cannot be detected in the absence of pectolytic enzyme. The pH optimum for single-cell-releasing activity by endo-PG was about 4.0, but the highest activity was observed at pH 4.5 by adding ST (Fig. 4). In the case of endo-PL, the pH optimum for the activity was 5.0, either with or without ST. This result suggested that the pH optimum of ST for stimulation of tissue maceration lies between 4.5 and 5.0.

The factor could be stored for at least 1 mo at 4 C in 0.1 M acetate buffer (pH 5.0) without apparent loss in activity. It was remarkably stable between pH 4 and 6, but labile below pH 3 and above pH 8. It was stable at 40 C, but readily lost its activity above 50 C.

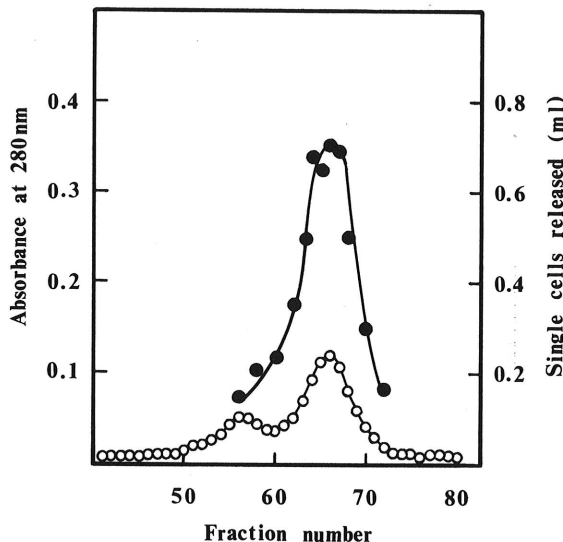

Fig. 3. Purification by Sephadex G-75 gel filtration of a maceration-stimulating factor produced by *Aspergillus japonicus*. The lyophilized active fraction from SE-Sephadex column was dissolved in 1 ml 0.1 M ammonium acetate buffer (pH 5.0), and the solution was applied on a Sephadex G-75 column (2.5 × 150 cm) equilibrated with 0.1 M ammonium acetate buffer (pH 5.0). The elution, at a flow rate of 10 ml/hr, was carried out with the same buffer and 5 ml were collected for each fraction. Absorbance at 280 nm (O) and stimulating activity (•) were measured.

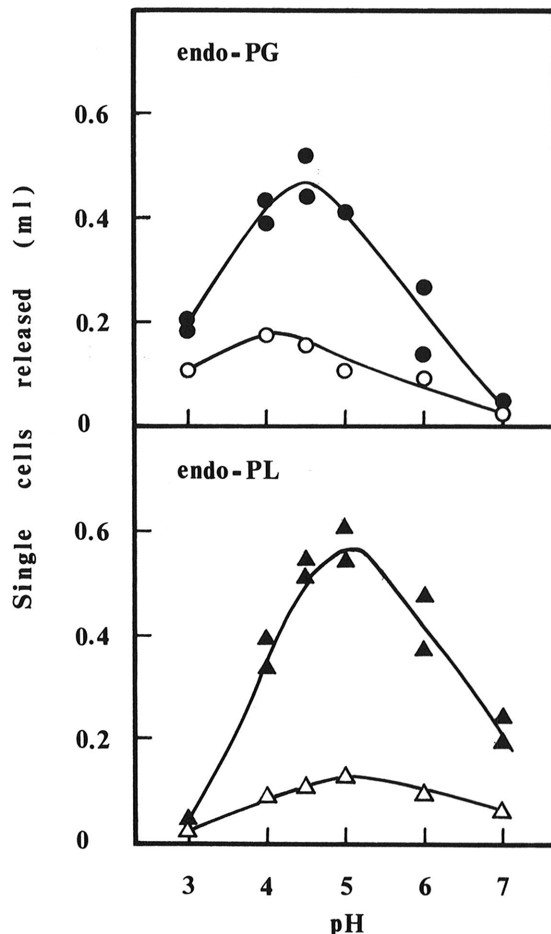

Fig. 4. Effect of pH on maceration-stimulating factor activity. Activity was measured in reaction mixtures containing 3 g potato tuber tissues, 5 ml 0.1 M citrate-phosphate buffer of various pH values, 2 mg bovine serum albumin, 100 units endo-PG (or 1.4 units endo-PL) with or without 6.7 μg purified stimulating factor, and water to a final volume of 10 ml. They were shaken on a rotary shaker at 160 rpm and 40 C for 3 hr. The volume (ml) of single cells released from potato tuber tissues by endo-PG (O), endo-PG and stimulating factor (•), endo-PL (Δ), and endo-PL and stimulating factor (▲) was measured as described in the Materials and Methods section of this paper.

The effect of ST on the activities of endo-PL and endo-PG was determined with pectin N.F. and sodium polypectate as the substrates. The effect on endo-PL activity also was determined by the viscosity-reducing method (10). Stimulating factor was added to each reaction mixture at a final concentration of 1 μg/ml. It was demonstrated that ST did not affect activities of endo-PL or of endo-PG on soluble pectic polysaccharides.

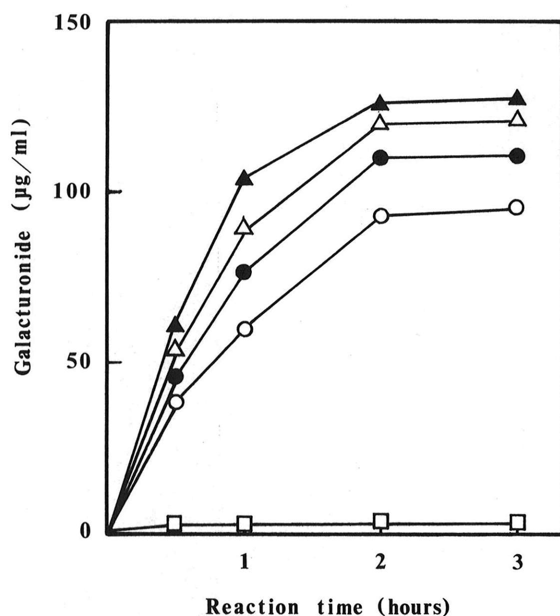

Fig. 5. Time-course of release of galacturonide from potato tuber cell walls. Reaction mixtures contained 50 mg cell walls and 25 ml 0.05 M acetate buffer (pH 4.5). They were shaken on a rotary shaker at 115 rpm and 30 C with the addition of 100 units endo-PG (0), 100 units endo-PG and 6.7 μg stimulating factor (●), 1.4 units endo-PL (Δ), 1.4 units endo-PL and 6.7 μg stimulating factor (▲), and 6.7 μg stimulating factor (□) and without enzyme (□). At intervals, reaction mixtures were withdrawn and filtered through Toyo No. 5C paper, and galacturonide released from cell walls was determined by the carbazole method.

The ST, by itself, did not catalyze the release of galacturonides from potato tuber cell walls (Fig. 5). The addition of ST, however, increased the rate and extent of galacturonide release from cell walls both by endo-PL and by endo-PG. But this ability was not correlated with the release of single cells from potato tuber tissues. The addition of ST increased only about 5 and 15% of galacturonides, respectively, from cell walls after 3 hr of incubation with endo-PL and with endo-PG.

Each 3-hr reaction mixture in Fig. 5 was filtered through Toyo No. 5C paper, and the filtrate was hydrolyzed by 1 N H_2SO_4. After neutralization with $BaCO_3$, neutral sugar compositions of the hydrolyzate were analyzed by liquid chromatography as described in the Materials and Methods section. Glucose and small amounts of xylose were detected in the control (without enzyme). Similar amounts of glucose were detected in all treatments (Table 1), and this may result from starch that was not completely eliminated from potato tuber cell wall preparations by the α-amylase treatment. Neutral sugars as well as galacturonides were not released from cell walls by action of ST alone. Both endo-PG and endo-PL could release arabinose, galactose, xylose, and rhamnose together with galacturonides from cell walls. This is in good agreement with the result of Talmadge et al. (25) who reported that these neutral sugars were released from sycamore cell walls by the endo-PG of *Colletotrichum lindemuthianum*. Further addition of ST did not release other neutral sugars from cell walls, but it affected the additional release of arabinose and galactose by both endo-PG and endo-PL, and failed to release additional xylose and rhamnose. Similar amounts of arabinose (2.7 ± 0.1 μg) and galactose (12.2 ± 0.2 μg) were released by

TABLE 1. Amounts of galacturonide and neutral sugars released from potato tuber cell walls by stimulating factor (ST), endo-polygalacturonase (endo-PG) and endo-pectin lyase (endo-PL) individually and in combination

Enzyme[a] treatment	Sugar released/ml of reaction mixture (μg)[b]					
		Neutral sugars[d]				
	Galacturonide[c]	Arabinose	Galactose	Xylose	Rhamnose	Glucose
Control	2.9	0	0	0.1	0	24.0
ST	2.4	0	0	0.1	0	23.2
endo-PG	95.2	5.3	23.4	1.8	2.0	21.7
endo-PG+ST	110.0	8.1	35.4	2.0	2.0	23.6
endo-PL	121.2	18.1	111.2	5.1	1.9	24.9
endo-PL+ST	127.0	20.7	123.6	4.9	2.1	22.0

[a]Reaction mixtures contained 50 mg cell walls and 25 ml 0.05 M acetate buffer (pH 4.5). They were shaken on a rotary shaker at 115 rpm and 30 C with the addition of 100 units endo-PG, 100 units endo-PG and 6.7 μg stimulating factor, 1.4 units endo-PL, 1.4 units endo-PL and 6.7 μg stimulating factor, and 6.7 μg stimulating factor and without enzyme.

[b]After a 3-hr incubation period reaction mixtures were filtered through Toyo No. 5C paper, and the filtrates were subjected to sugar analysis.

[c]Galacturonide content was estimated by determining anhydrogalacturonic acid by the carbazole method.

[d]After the sample was hydrolyzed in 1N H_2SO_4 at 100 C for 6 hr, neutral sugars were analyzed by automated liquid chromatography as described in the Materials and Methods section of this paper.

ST from cell walls treated with either endo-PG or endo-PL.

The release of protein from cell walls was not affected by ST.

We determined whether or not ST can attack cell walls pretreated with endo-PG or endo-PL. Potato tuber cell walls (100 mg) were incubated with 200 units endo-PG or 2.8 units endo-PL in 50 ml 0.05 M acetate buffer (pH 4.5) at 30 C for 3 hr. These treated cell walls, after being washed with the same buffer, were suspended in 75% ethanol (v/v) and allowed to stand at 30 C for 3 hr to inactivate endo-PG and endo-PL. Eighty-four mg of endo-PG-treated and 78 mg of endo-PL-treated cell walls were obtained. A sample (20 mg) from each pretreated cell wall was incubated in 10 ml 0.05 M acetate buffer (pH 4.5) with or without 10 μg ST at 30 C for 3 hr. The hydrolyzates of all soluble fractions contained large amounts of glucose which may arise from starch (Table 2). The presence of starch was confirmed by the iodine test. Considerable amounts of galacturonides, arabinose, galactose, and xylose were released from cell walls pretreated with endo-PL and endo-PG without ST. It is possible that the treatment of endo-PG and endo-PL may alter the cell walls to facilitate the release of these sugars by acetate buffer (pH 4.5) alone. The ST released additional galacturonides, arabinose, galactose, xylose, and rhamnose from both pretreated cell walls. The amounts of additional arabinose and galactose released from endo-PG-treated cell walls were similar to those released from endo-PL-treated cell walls.

The ST did not degrade beet arabinan, lupin galactan, larch galactan, xylan, yeast mannan, laminarin, polymethyl polygalacturonate methyl glycoside, and sodium polypectate. It cannot cleave the main linkages or the minor linkages in these polysaccharides, since after prolonged incubation no detectable reducing sugars were formed. Also, ST did not cleave glycosidic linkages such as α-glucoside, β-glucoside, α-galactoside, β-galactoside, α-mannoside, α-xyloside, β-xyloside, α-fucoside, and α-rhamnoside.

DISCUSSION

The factor (ST) that stimulates tissue maceration by pectolytic enzymes did not enhance the activities of endo-PL and endo-PG on pectin N.F. and sodium polypectate. The ST alone could not release any sugars but it did release additional galacturonides, arabinose, and galactose from cell walls incubated simultaneously with pectolytic enzymes. It was demonstrated that almost the same amounts of arabinose and galactose were released by ST from cell walls treated with either endo-PL or endo-PG. This may indicate that the action of ST is to release arabinose and galactose from cell walls modified by pectolytic enzymes.

There was little effect of ST on both tissue maceration and additional release of sugars when potato tuber tissues and the cell walls were treated initially with ST and then incubated with endo-PL or endo-PG (S. Ishii, *unpublished*). This suggested that pectic polysaccharides must be partially degraded before ST can attack plant tissues and cell walls.

However, the ability of ST to release additional sugars from cell walls was considerably lower than its ability to stimulate tissue maceration. For example, only 4.8% of galacturonides, 14.4% of arabinose, and 11.2% of galactose were increased by the action of ST.

The results of DEAE-Sephadex column chromatography suggested that components derived by ST and by pectolytic enzymes are covalently linked to galacturonides, most probably as arabinogalactans (S. Ishii, *unpublished*). It was demonstrated previously that endo-PL released larger amounts of pectic polysaccharides in the cell wall and converted them into smaller galacturonides than did endo-PG (7). Therefore, the cell walls modified by endo-PG may retain large

TABLE 2. Amounts of galacturonide and neutral sugars released from pretreated potato tuber cell walls with endo-polygalacturonase (endo-PG) or endo-pectin lyase (endo-PL) by the action of stimulating factor (ST)

		Sugar released/ml of reaction mixture (μg)[c]					
			Neutral sugars[e]				
Primary[a] treatment	Secondary[b] treatment	Galacturonide[d]	Arabinose	Galactose	Xylose	Rhamnose	Glucose
endo-PG	Buffer	7.8	1.4	4.5	0.6	0.1	18.9
endo-PG	ST	15.8	5.9	11.6	1.5	0.3	19.3
endo-PL	Buffer	6.5	2.4	7.2	1.4	0.1	21.2
endo-PL	ST	9.6	7.3	13.9	3.0	0.2	22.1

[a]Potato cell walls (100 mg) were incubated with 200 units endo-PG or 2.8 units endo-PL in 50 ml 0.05 M acetate buffer (pH 4.5) on a rotary shaker at 30 C. After 3 hr, reaction mixtures were filtered through Toyo No. 5C paper, and the residues were repeatedly washed with 0.05 M acetate buffer (pH 4.5). The residues were suspended in 75% ethanol and the suspension was allowed to stand at 30 C for 3 hr and then filtered through Toyo No. 5C paper. The residues were washed successively with 75% ethanol, absolute ethanol, and ether, and then dried in vacuo.

[b]The treated cell walls (20 mg) were placed in a 50-ml Erlenmeyer flask containing 10 μg of ST. The flasks were shaken on a rotary shaker at 115 rpm and 30 C.

[c]After a 3-hr incubation period, reaction mixtures were filtered through Toyo No. 5 C paper, and the filtrates were subjected to sugar analysis.

[d]Galacturonide content was estimated by determining anhydrogalacturonic acid by the carbazole method.

[e]After the sample was hydrolyzed in 1N H_2SO_4 at 100 C for 6 hr, neutral sugars were analyzed by automated liquid chromatography as described in the Materials and Methods section of this paper.

amounts of long-chain galacturonides. This is probably the reason why ST released larger amounts of galacturonides with the same amounts of arabinose and galactose from cell walls modified by endo-PG than by endo-PL.

An arabinogalactan, released by endo-PL or endo-PG alone, may not be attached to other cell wall components. On the other hand, it seems likely that an arabinogalactan, not released by pectolytic enzyme alone, but released by the further action of ST, may be attached to other cell wall components. Conceivably this compound is a cross-link between pectic polymers and other components in the middle lamella (14).

How ST-derived arabinogalactan is attached to other cell wall components cannot be determined from the data presented. A glycosidic attachment is not likely, since ST could not catalyze the cleavage of various kinds of polysaccharides and glycosides. However, it appears that ST may cleave the attachment point and that once the linkages are broken plant tissues become more susceptible to maceration by pectolytic enzymes.

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