

Evidence for a Factor that Stimulates Tissue Maceration by Pectolytic Enzymes

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ABSTRACT

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A factor that stimulates tissue maceration by pectolytic enzymes was isolated from the culture medium of *Aspergillus japonicus* by DEAE-Sephadex column chromatography and isoelectric focusing. The factor alone could not macerate plant tissues but it greatly stimulated the maceration of potato, onion, and radish tissues both by endo-pectin lyase and by endo-polygalacturonase. The stimulating factor was a

heat-labile, high-molecular-weight substance, which suggested that it may be an enzyme. It showed little or no ability to degrade pectin, pectic acid, arabinan, galactan, arabinogalactan, xylan, and protein. Only low cellulase activity was detected in the final preparation of the stimulating factor. However, there was a remarkable difference in heat stability between this factor and cellulase.

Additional key words: assay of macerating activity, endo-pectate lyase.

The maceration of plant tissue represents a phenomenon associated with pathogenesis by numerous phytopathogenic microorganisms (6, 27). Up to now highly purified and well-characterized macerating enzymes are endo-pectic hydrolases or lyases: endo-polygalacturonase (endo-PG, EC 3.2.1.15) (4, 5), endo-pectin lyase (endo-PL, EC 4.2.2.3) (7, 15), and endo-pectate lyase (endo-PAL, EC 4.2.2.1) (3, 23). Most phytopathogens produce cellulase and hemicellulases together with pectolytic enzymes (9, 10, 12). These enzymes, especially hemicellulases, are generally considered to play a role in the maceration process (8, 26, 27).

Aspergillus japonicus, a nonpathogenic fungus, produces two macerating enzymes that were identified as endo-PG and endo-PL (16, 17). We found, however, that the macerating activity of *A. japonicus* is not attributable only to the combined action of the two enzymes, suggesting the existence of a third factor which is responsible for maceration. The present paper describes the isolation and partial characterization of the factor from *A. japonicus* that stimulates the tissue maceration by endo-PL and endo-PG.

MATERIALS AND METHODS

Enzyme preparations.—*Aspergillus japonicus* isolate 1744 (ATCC 20236) was grown on moistened wheat bran in 500-ml Erlenmeyer flasks at 25 C for 65 hours. Twenty grams of wheat bran and 17 ml of water were mixed, autoclaved at 120 C for 30 minutes, and inoculated with six loopsful of conidia. The culture medium was incubated with five volumes of water, and the pH of the mixture was adjusted to pH 4.5 with 1 N HCl. The suspension was held for 2 hours at room temperature,

then squeezed through cotton cloth and filtered with Celite. The filtrate was concentrated to about one-tenth its original volume by ultrafiltration (Diaflo Model 402 ultrafiltration cell fitted with a UM-10 membrane) under nitrogen gas. Three volumes of cold (−4 C) ethanol were added to the concentrated extract with continuous stirring. After standing at 4 C for 4 hours, the resultant precipitate was collected by centrifugation, washed with 96% cold ethanol, and dried in vacuo. This was used as a crude enzyme which contained 1.4 units of endo-PL and 16.9 units of endo-PG per milligram. Purified endo-PL and endo-PG were obtained from the crude enzyme by methods described previously (16, 17). Both purified enzymes were homogeneous on ultracentrifugation and disc electrophoresis, and were free from other enzymes (16, 17).

Polysaccharide substrates.—Pectin N.F. and sodium polypectate were purchased from Sunkist Growers Inc. Xylan was purchased from Nutritional Biochemicals Corporation and carboxymethyl cellulose (CMC) from Tokyo Kasei Kogyo Co., Ltd. Arabinan was prepared from sugar beet by the method of Hirst and Jones (13), galactan from lupin seed by the method of Jones and Tanaka (18), and arabinogalactan from soybean seed by the method of Morita (22).

Enzyme assays.—Macerating activity was determined by measuring the volume of single cells released from plant tissues as described previously (14). Seven grams of tissue pieces (5×5×5 mm) were placed in a 150-ml Erlenmeyer flask containing 25 ml of 0.1 M citric-phosphate buffer (pH 4.5), 10 mg of bovine serum albumin, enzyme, and water to a final volume of 50 ml. The flasks were shaken on a rotary shaker at 160 rpm and 40 C. At intervals, 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 (ml) of single cells passed through the sieve was measured when

the cells had sedimented to an unchanged level (about 2 hours at room temperature). Endo-PL was determined by spectrophotometry (2). Reaction mixture contained 1 ml of 1% pectin N.F., 1 ml of 0.1 M citric-phosphate buffer (pH 6.0), and 0.5 ml of enzyme at 40 C. One unit of endo-PL was defined as an increase in absorbance of 1.0 per minute at 235 nm. Endo-PG was determined by the viscosity-reducing method of Roboz et al. (24). The reaction mixture contained 3 ml of 1% sodium polypectate, 3 ml of 0.1 M citric-phosphate buffer (pH 4.5), and 1 ml of enzyme. One unit of endo-PG was defined as a 50% loss in viscosity in 1 minute at 40 C. Cellulase and hemicellulases were determined by measuring reducing sugars liberated from each substrate. Reaction mixtures contained 0.5 ml of 0.5% polysaccharide in 0.1 M acetate buffer (pH 4.5) and 0.5 ml of enzyme. They were incubated at 40 C for 10 minutes. Reducing sugars were determined by the method of Somogyi (25). One unit of cellulase or hemicellulase was defined as the amount of enzyme that liberates 1 μ g of reducing sugar (as glucose) per minute at 40 C. *Aspergillus japonicus* produces acid protease with an optimum pH of 3.0 (S. Ishii and K. Kiho, unpublished). Protease activity was determined by the casein-275 nm method of Hagiwara et al. (11). The reaction mixture containing 0.5 ml of enzyme and 2.5 ml of 0.6% Hammarstein milk casein in 0.05 M acetate buffer (pH 3.0) was incubated at 40 C for 10 minutes. The reaction

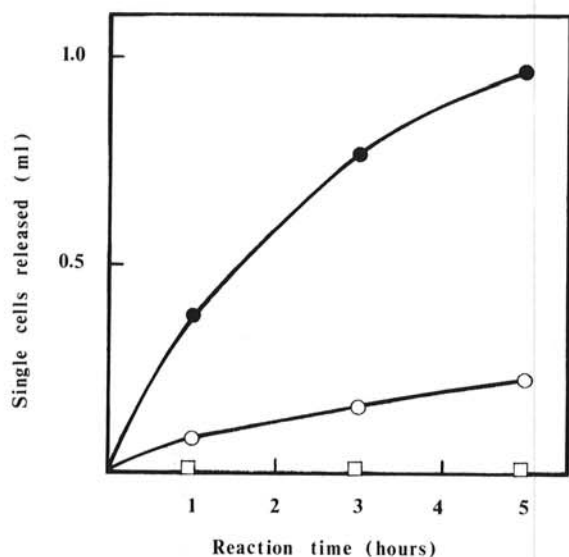


Fig. 1. Maceration of potato tissues by crude enzyme and mixture of endo-pectin lyase (endo-PL) and endo-polygalacturonase (endo-PG). Macerating activity was measured in reaction mixtures containing 7 g of potato tuber tissues (5×5×5 mm), 25 ml of 0.1 M citric-phosphate buffer (pH 4.5), 10 mg of bovine serum albumin, enzyme, and water to a final volume of 50 ml. Purified endo-PL and endo-PG were added to the reaction mixture, making the amount of their activities equal to the crude enzyme added. They were shaken on a rotary shaker at 160 rpm and 40 C. At intervals, 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 of single cells released from the tissue was measured. Macerations by 10 mg of crude enzyme (●), mixture of 14 units of endo-PL and 169 units of endo-PG (○), and autoclaved crude enzyme (□).

was stopped by adding 2.5 ml of 0.11 M trichloroacetic acid containing 0.22 M acetic acid and 0.33 M sodium acetate. The inactive reaction mixture was allowed to stand for 30 minutes at 40 C and then was filtered. The extinction values at 275 nm were read against a blank containing the reagents. One unit of protease activity was defined as an increase in absorbance of 1.0 under the above conditions.

DEAE-Sephadex column chromatography.—One gram of the crude enzyme was dissolved in 25 ml of 0.1 M acetate buffer (pH 5.0), and dialyzed against the same buffer for 16 hours at 4 C. The dialyze was applied to a DEAE-Sephadex A-50 column (2 × 33 cm) that had been equilibrated with 0.1 M acetate buffer (pH 5.0). After the column was washed with 500 ml of 0.1 M acetate buffer (pH 5.0), the elution was performed with a linear gradient of NaCl in the same buffer at a flow rate of 16 ml/hour.

Isoelectric focusing.—Isoelectric focusing was performed according to manufacturers' directions in a 110-ml Ampholine LKB 8100-1 electrofocusing apparatus (LKB-Produkter AB, Bromma, Sweden) containing pH 2.5-4 Ampholine carrier ampholytes (LKB). The density gradient was made up of ethylene glycol by the method of Ahlgren et al. (1). Fraction IV (fraction no. 104-106) from a DEAE-Sephadex column was pooled, dialyzed against distilled water for 24 hours, and lyophilized. This was electrofocusing in a pH 2.5-4 ampholyte gradient at 700 V for 45 hours. The column was eluted at a flow rate of 2 ml/minute and 3.9-ml fractions were collected. The pH and absorbance at 280 nm of each fraction were determined.

RESULTS

Isolation of a stimulating factor from crude enzymes of

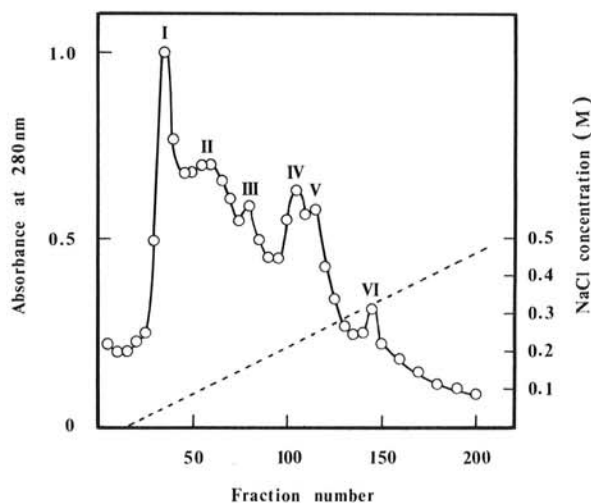


Fig. 2. Fractionation of crude enzyme of *Aspergillus japonicus* by DEAE-Sephadex column chromatography. Crude enzyme solution (1 g/25 ml of 0.1 M acetate buffer, pH 5.0) was applied on a DEAE-Sephadex A-50 column (2 × 33 cm) equilibrated with the same buffer. After the column was washed with the same buffer, elution was performed with a linear gradient of NaCl at a flow rate of 16 ml/hour. 10-ml fractions were collected. Absorbance at 280 nm (○) and NaCl concentration (---). Peak fractions were numbered I to VI.

Aspergillus japonicus.—Macerating activity of the crude enzyme could not be explained only by the combined action of endo-PL and endo-PG. The crude enzyme was more active than the mixture of the two enzymes at the same ratio as in the crude enzyme (Fig. 1). This suggested the existence of some factor other than endo-PL and endo-PG in the crude enzyme which is responsible for the tissue maceration. An autoclaved crude enzyme did not show macerating activity and had no stimulating effect on maceration by the mixture of endo-PL and endo-PG.

The crude enzyme solution, after passage through DEAE-Sephadex at pH 5.0, retained most of its endo-PL and endo-PG activity. This solution contained 12.7 and 146 units of activity. It showed similar macerating activity as a mixture of purified enzymes containing 12.7 units of endo-PL and 146 units of endo-PG. This indicated that the factor was adsorbed on DEAE-Sephadex. Then 1 g of the crude enzyme was dissolved in 25 ml of 0.1 M acetate buffer (pH 5.0), dialyzed against the same buffer, and applied on a DEAE-Sephadex A-50 column (2 × 33 cm). The column was washed with 500 ml of 0.1 M acetate buffer (pH 5.0) to remove endo-PL and endo-PG. Elution was performed with a linear (0.0 to 0.5 M) NaCl gradient prepared in the same buffer. The eluent was collected in 10-ml fractions. Six protein peaks were obtained and were numbered in the order they were eluted (Fig. 2).

Each peak fraction from the column was assayed for macerating activity in the presence of endo-PL and endo-PG. A remarkable stimulation was observed by the addition of fraction IV (Table I). The effect of fraction V could be due to the contamination with fraction IV. Fractions 104-106 were collected, dialyzed against distilled water, and lyophilized. This was electrofocused in a pH 2.5-4 gradient of Ampholine ampholytes at 4C for 45 hours. Fractions (3.9 ml) were collected and absorbance at 280 nm and pH values were measured. The main protein peak appeared around fraction number 23 with an isoelectric point of 4.1 (Fig. 3). This fraction showed an ability to stimulate the maceration of potato tissues by the mixture of endo-PL and endo-PG. The final preparation obtained as above was not homogeneous following disc electrophoresis. However, it was used as a stimulating factor in this study without further treatment.

TABLE I. Stimulating activity of fractions obtained by DEAE-Sephadex chromatography of crude enzyme preparations from *Aspergillus japonicus* on tissue maceration by endo-pectin lyase (PL) and endo-polygalacturonase^a (PG)

Enzyme	Single cells released (ml) after a reaction time (hours) of:		
	1	3	5
PL + PG ^b	0.08	0.21	0.31
PL + PG + Fraction ^c I	0.10	0.20	0.30
PL + PG + Fraction II	0.08	0.20	0.22
PL + PG + Fraction III	0.12	0.30	0.35
PL + PG + Fraction IV	0.33	1.00	1.30
PL + PG + Fraction V	0.17	0.42	0.70
PL + PG + Fraction VI	0.10	0.28	0.40

^aReaction mixtures and assay method were the same as in Fig. 1.

^bMixture of 14 units of endo-PL and 169 units of endo-PG.

^cEach fraction (2 ml) designated in Fig. 2 was added to the reaction mixture.

Characterization of the stimulating factor.—The stimulating factor was unable, by itself, to release single cells from potato tissues (Fig. 4). Maceration by both endo-PL and by endo-PG were greatly enhanced by the addition of the stimulating factor. In both cases about eight times the volume of single cells were released from potato tissues by the addition of the factor after 3 hours of incubation. Also, onion and radish tissues were greatly macerated by endo-PL and endo-PG in the presence of

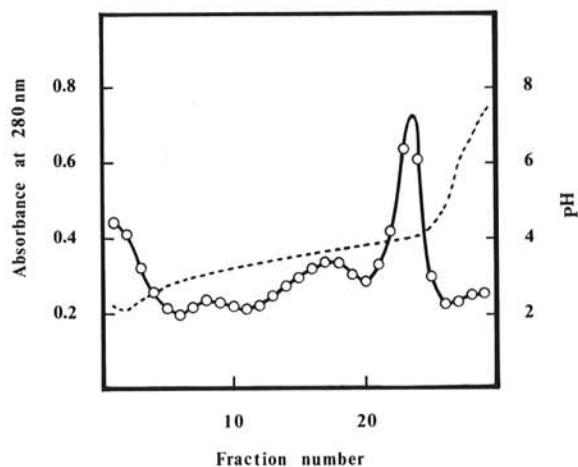


Fig. 3. Isoelectric focusing of fraction IV from a DEAE-Sephadex column using Ampholine carriers with a pH range of 2.5 to 4.0. Electrofocusing at 4 C for 45 hours. Fractions (3.9 ml) were collected and absorbance at 280 nm (O) and pH values (---) were measured.

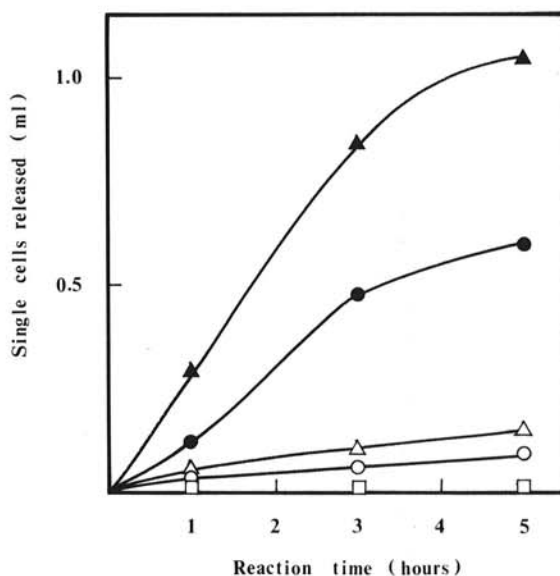


Fig. 4. Effect of the stimulating factor on maceration of potato tissues. Reaction mixtures were the same as in Fig. 1. Macerations by 0.3 ml of the stimulating factor (□), 7 units of endo-PL (Δ), 85 units of endo-PG (O), mixture of 7 units of endo-PL and 0.3 ml of the stimulating factor (▲), and mixture of 85 units of endo-PG and 0.3 ml of the stimulating factor (●).

the stimulating factor (Fig. 5). Onion tissue was resistant to maceration by endo-PG, but the factor facilitated the maceration of the tissue by the enzyme. Onion and radish tissues, as well as potato tissue, were not macerated by the stimulating factor alone.

Treatment of the factor at 60 C for 10 minutes brought about the complete loss of the stimulating activity. Enzyme activities present in the stimulating factor and crude enzyme were determined (Table 2). The fraction with pI 4.1 after isoelectric focusing was completely free of endo-PL and endo-PG. The crude enzyme could hydrolyze beet-arabinan, lupin-galactan, soybean-arabinogalactan, and xylan, but the purified stimulating factor did not contain enzymes which hydrolyze arabinan, galactan, and arabinogalactan, and had

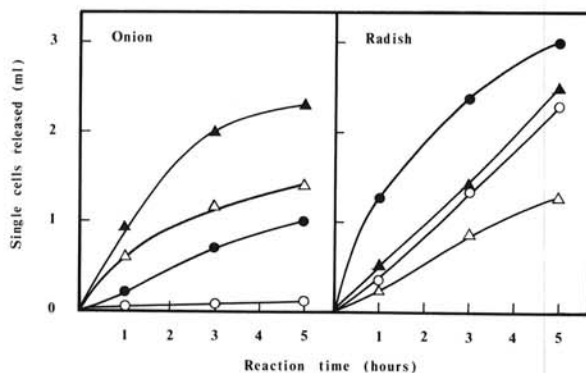


Fig. 5. Effect of the stimulating factor on maceration of onion and radish tissues. Reaction mixtures were the same as in Fig. 1 except that onion or radish tissues were used instead of potato tissues. Macerations by 7 units of endo-PL (Δ), 85 units of endo-PG (O), mixture of 7 units of endo-PL and 0.3 ml of the stimulating factor (\blacktriangle), and mixture of 85 units of endo-PG and 0.3 ml of the stimulating factor (\bullet).

TABLE 2. Determination of enzyme activities present in crude enzyme and purified stimulating factor from *Aspergillus japonicus*

Enzyme	Enzyme activity ^a	
	Crude enzyme ^b	Stimulating factor ^c
Pectinase		
endo-PL	14.0	0
endo-PG	169.0	0
Hemicellulase		
Arabanase	3.6	0
Galactanase	5.0	0
Arabinogalactanase	16.6	0
Xylanase	67.8	0.2
Cellulase		
CMCase	35.8	1.9
Protease	1.1	0

^aEnzyme activity was determined by the procedures described in "Materials and Methods".

^bActivity/ml of 1% crude enzyme.

^cActivity/ml of pI 4.1 fraction from isoelectric focusing.

negligible xylanase activity. Only a low activity of cellulase (carboxymethylcellulase) was detected in the stimulating factor.

DISCUSSION

A factor in the crude enzyme extract of *A. japonicus* alone was unable to macerate plant tissue, but it greatly stimulated tissue maceration by pectolytic enzymes. This is probably the reason why the macerating activity of the crude culture filtrate of *A. japonicus* is not attributable only to the combined action of endo-PL and endo-PG. The stimulating factor greatly facilitated the maceration of potato, onion, and radish tissues both by endo-PL and by endo-PG. It seems probable that the factor might be effective for the maceration of a wide variety of plant tissues by endo-pectolytic enzymes including endo-PAL. It is quite likely that such a factor may be secreted by phytopathogens, and may play an important role in pathogenesis.

The fact that the stimulating factor was heat-labile and non-dialyzable, with an approximate molecular weight of 21,000 (S. Ishii and K. Kiho, unpublished), suggests that it may be an enzyme.

Hemicellulases have been suspected of playing a role in maceration of plant tissues (12, 21, 26, 27). Cole and Bateman (8) reported that arabanase of *Sclerotium rolfsii* could not macerate plant tissues by itself, but could play a secondary role in maceration. Cole and Wood (9) and Knee and Friend (19, 20) indicated the possibility that galactanase may be associated with tissue maceration. However, the stimulating factor of *A. japonicus* was neither arabanase nor galactanase. The final preparation of the factor was shown to degrade cellulose. However, there was a remarkable difference between the stimulating factor and cellulase in heat stability. Treatment at 60 C for 10 minutes resulted in complete loss of stimulating activity, but about 60% of cellulase activity remained after similar treatment. Therefore, we do not think that the stimulating factor is a cellulase. It is likely that cellulase is a contaminant because the final preparation obtained in this study was not homogeneous.

An investigation on the purification, nature, and biological significance (of the stimulating factor) is being continued.

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