Purification and Crystallization of a Protopectin-solubilizing Enzyme from *Trichosporon penicillatum*†

Takuo SAKAI* and Minoru OKUSHIMA

Department of Agricultural Chemistry, College of Agriculture, University of Osaka Prefecture, Sakai, Osaka 591, Japan

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A protopectin-solubilizing enzyme (an endo-polygalacturonase) was purified and crystallized, with an overall yield of 75%, from the culture filtrate of *Trichosporon penicillatum* SNO-3 by a procedure involving ammonium sulfate fractionation and chromatographies on CM-Sephadex C-50 and Sephadex G-75 columns. The purified enzyme was homogeneous on polyacrylamide gel electrophoresis and ultracentrifugation. The sedimentation coefficient ($s_{20,w}$) was determined to be 3.66 S, and the molecular weight was determined to be 30,000 by gel filtration and ultracentrifugation. The enzyme was a glycoprotein containing 1.7% sugar, and had an isoelectric point of around pH 7.8. The enzyme catalyzed the liberation of a pectin substance from protopectin of various plant tissues. The enzyme was different immunologically from endo-polygalacturonases produced by *Saccharomyces fragilis* and *Aspergillus niger*.

The decomposition of protopectin, and insoluble pectin substance, was originally attributed to a specific enzyme protopectinase,[1] but further research on pectolytic enzymes has revealed that the decomposition of protopectin is due to the action of a system of enzymes, including pectinesterase, endo-polygalacturonase, endo-pectate lyase and pectin lyase. In most of these studies, the protopectin-decomposing enzymes were regarded only as enzymes macerating plant tissues.

Previously, the authors found yeasts producing protopectin-solubilizing enzymes which liberated water-soluble pectin substances from protopectin,[2～4] and demonstrated that these enzymes could be applied for the production of pectin.

This paper deals with the purification and some properties of a protopectin-solubilizing enzyme from *Trichosporon penicillatum* SNO-3.

† Studies on Enzyme Produced by Yeasts. Part III. For Part II, see ref. 3.

* To whom correspondence should be addressed.

MATERIALS AND METHODS

Chemicals. All chemicals, unless otherwise specified, were from Wako Pure Chemical Industries (Osaka) and of certified reagent grade. CM-Sephadex C-50 and Sephadex G-75 were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Ampholite was obtained from LKB Co. (Bromma, Sweden). Standard proteins for determination of the molecular weight and isoelectric point of the enzyme were from Sigma Chemicals (St. Louis, U.S.A.) and Oriental Yeast Co. (Tokyo), respectively.

Microorganism. A strain of *Trichosporon penicillatum* (Strain SNO-3) isolated as a protopectinase-producing strain[2] was used in this study.

Cultivation of microorganism. The organism was maintained on agar slants containing 1% glucose, 0.2% peptone, 0.2% pectin and 0.1% yeast extract, pH 5.0. For the production of enzyme, the microorganism was aerobically grown in a medium containing 2% glucose, 0.4% peptone, 0.2% yeast extract and 0.08% Silicone KM-70 (Shin-Etsu Chemical Co., Ltd., Tokyo), pH 5.0, for 25 hr.

Enzyme assay. Protopectinase activity was determined by measuring the amount of pectin substance liberated from protopectin by the carbazole-sulfuric acid method, as follows. The reaction mixture was composed of 10 mg protopectin, 4 mmol acetate buffer, pH 5.0, and 0.5 ml of enzyme solution in a total volume of 2.5 ml, and the reaction was carried out at 37°C for 30 min. One unit of enzyme activity was defined as the activity which liberated

pectin substance corresponding to 1 μmol of D-galacturonic acid per ml reaction mixture at 37°C in 30 min. Protopectin was prepared by the following procedure: The albedo layer of *Citrus unshiu* peel was scooped out, pooled, washed with distilled water until the water-soluble substance which reacted with carbazole-sulfuric acid was washed off, and then lyophilized. The dried protopectin preparation was powdered to a 100~200 mesh powder.

Pectic acid-liquefying activity (viscosity-reducing activity) was determined as follows: To 6 ml of 0.5% pectic acid solution in MacIlvaine buffer (which was preincubated at 37°C for 3 min), pH 5.0, was added 1 ml of enzyme solution, and this mixture was incubated at 37°C for 5 min. The rate of viscosity reduction (A) was calculated with the equation $A=(Ta-T)/(Ta-To)\times 100$, where, Ta is the flow time (in seconds) of pectic acid solution added to the heat-inactivated enzyme, T is the flow time (sec) of the reaction mixture, and To is the flow time (sec) of water added to the heat-inactivated enzyme. One unit of enzyme activity was defined as the activity reducing the viscosity by 50%.

Pectic acid-saccharifying activity was determined by measuring the release of reducing groups according to the method of Jansen and MacDonnell.[5] One unit of enzyme activity was defined as the activity producing reducing groups corresponding to 1 μmol of D-galacturonic acid per ml reaction mixture at 37°C in 10 min.

Electrophoresis. Polyacrylamide gel disc electrophoresis was carried out with 7% polyacrylamide gel according to the method described by Davis[6] (at pH 9.4) and by Reisfeld *et al.*[7] (at pH 4.0). Polyacrylamide gel electrophoresis in the presence of 1% sodium dodecyl sulfate was performed as described by Weber and Osborn.[8]

Isoelectric focusing. For determination of the p*I* value of the enzyme, gel disc isoelectric focusing[9] was performed in 7.5% polyacrylamide gel containing 2% Ampholine (pH 3.5 to 10.0). Electrofocusing was carried out at a constant voltage (200 V) for 4 hr at 5°C. As anode and cathode solutions, 0.02 M H_3PO_4 and 1 M NaOH were used, respectively. After electrofocusing, the gels were stained with Coomassie Brilliant Blue G-250-trichloroacetic acid solution. The isoelectric point of the enzyme was determined from a graph of the relation between the mobility of marker proteins and their isoelectric points.

Determination of molecular weight.

i) Gel filtration of Sephadex G-75. Gel filtration was performed at 5°C on a Sephadex G-75 column (2.0 × 100 cm) preequilibrated with 0.05 M acetate buffer, pH 5.0. Proteins were eluted with the same buffer at a flow rate of 10 ml/hr.

ii) Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis was performed according to Weber and Osborn,[8] using a 10% gel in 0.1% SDS-0.1 M phosphate buffer, pH 7.2, at 7 mA per gel for 5 hr. Prior to electrophoresis, the protein samples were boiled for 5 min in 0.01 M phosphate buffer, pH 7.2, containing 1% SDS, 25% glycerol, and 1% mercaptoethanol. The gels were stained with 0.1% Coomassie Brilliant Blue G-250, and destained in 7% acetic acid. The molecular weight of the enzyme was determined from a graph of the relation between the mobility of marker proteins and their molecular weights.

iii) *Sedimentation equilibrium.* The molecular weight of the enzyme was also determined by sedimentation equilibrium analysis in a Hitachi model 252 analytical ultracentrifuge, at 14,000 rpm (at 20°C) in a 0.02 M acetate buffer, pH 5.0, containing 0.1 M NaCl, and the molecular weight of the enzyme was determined according to the method of Yphantis.[10]

Immunochemical methods.

i) *Preparation of antiserum.* The antiserum was prepared by injection of rabbits with crystallized enzyme: One milliliter of crystalline enzyme solution which contained 2 mg of enzyme protein was emulsified with an equal volume of Freund's complete adjuvant (Difco). The emulsion was injected subcutaneously into a Japanese white rabbit. A titer of 1 : 32 was obtained after 7 injections given at intervals of 7 days. Fifty-seven days after the first injection, blood was collected and the serum was isolated, heated at 56°C for 30 min, and stored at 0°C.

ii) *Gel diffusion.* Immunological tests were performed by Ouchterlony double immunodiffusion at 37°C. Gel diffusion plates were prepared with 1% agar in 1.2% NaCl solution containing 0.05% NaN_3.

iii) *Inhibition test for enzyme activity.* One milliliter of the enzyme solution (containing 50 units of enzyme) was mixed with 0.2 to 1 ml of antiserum in 0.02 M acetate buffer, pH 5.0, and kept for 18 hr at 37°C, and the remaining enzyme activity was then determined.

Determination of protein. Protein was determined by the method of Lowry *et al.*[11] using bovine serum albumin as a standard.

RESULTS

Cultural conditions for enzyme production

From the results of experiments on enzyme production, the medium described in the text was determined to be the most favorable. Figure 1 shows the time course of enzyme production under these conditions. The enzyme began to be produced after 5 hr of cultivation and reached a maximum at 25 hr of cultivation, after which the activity disappeared rapidly.

The production of enzymes possibly catalyz-

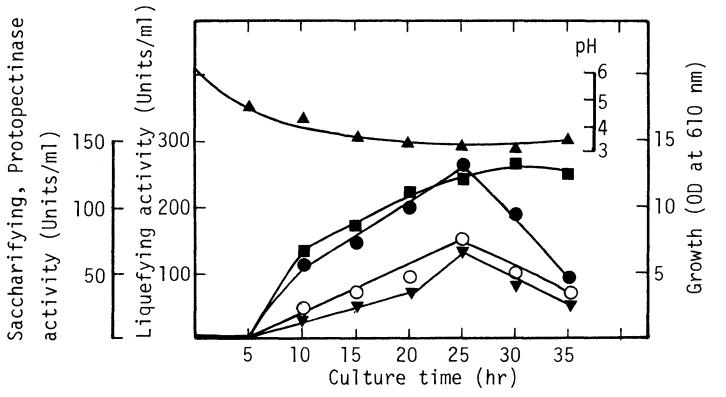

FIG. 1. Time Course of Enzyme Formation.

Details of cultivation of the organism are given in the text. —▲—, pH; —■—, growth; —●—, liquefying activity; —○—, protopectinase activity; —▼—, saccharifying activity.

TABLE I. ENZYME ACTIVITY FOUND IN THE CULTURE FILTRATE OF *Trichosporon penicillatum* SNO-3

Enzyme activity tested	Substrate used	Activity
Cellulase[a]	Carboxymethyl-cellulose	−
	Filter paper	−
Hemicellulase[a]	Wheat bran	−
Xylanase[a]	Xylan	−
Mannase[a]	Yeast mannan	−
Protopectinase	Protopectin	+
Pectin lyase[b]	Pectin	−
Pectate lyase[b]	Pectic acid	−
Polygalacturonase		
Liquefying activity	Pectin acid	+
	Pectin	+
Saccharifying activity	Pectic acid	+
	Pectin	+
Pectin esterase[c]	Pectin	−

The reaction mixture contained 1% substrate, 6 ml MacIlvain buffer, pH 5.0, and 1 ml culture filtrate, and the reaction was performed at 37°C for 30 min. The enzyme activities were determined by the following methods:

[a] Determined by measuring the amount of reducing groups formed.

[b] Determined by the method described by Weissbach and Hurwitz.[12]

[c] Determined by measuring the amount of carboxyl groups formed by titration of the reaction mixture with 0.01 N NaOH.

ing the degradation of protopectin in the culture filtrate was investigated. As shown in Table I, pectin- and pectic acid-degrading activities were detected besides protopectinase activity.

Purification of enzyme

Forty liters of culture filtrate (cultured for 24 hr) was concentrated by evaporation *in vacuo*, at 30°C, to 1/10 of the initial volume, and used as the starting material for enzyme purification. Solid ammonium sulfate was gradually added to the concentrated culture filtrate, with mechanical stirring, to 70% saturation, and the solution was stood overnight at 5°C. The precipitate was collected by centrifugation and dissolved in 100 ml of 0.02 M acetate buffer, pH 5.0. The enzyme solution obtained was dialyzed thoroughly against 0.02 M acetate buffer, pH 5.0, and then applied to a CM-Sephadex C-50 column (3 × 70 cm) equilibrated with the dialysis buffer. The column was washed with 100 ml of dialysis buffer, and then the enzyme was eluted with a linear NaCl concentration gradient, at a flow-rate of 20 ml/hr. The linear gradient was made up of two column volumes, one of 0.02 M acetate buffer, pH 5.0, and the other of 0.4 M NaCl in 0.02 M acetate buffer, pH 5.0. The active fractions were pooled and concentrated to 5 ml by evaporation *in vacuo*, at 30°C, and applied to a column of Sephadex G-75 (2 × 70 cm) equilibrated with 0.02 M acetate buffer, pH 5.0, and chromatography was performed at a flow-rate of 7 ml/hr. The chromatography was performed two times, and the active fractions ob-

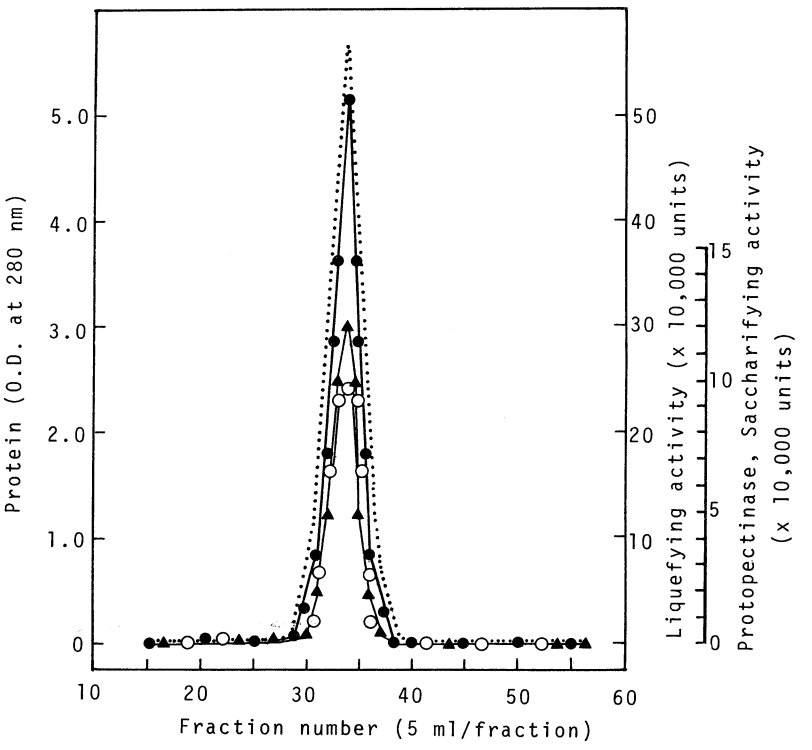

FIG. 2. Second Sephadex G-75 Column Chromatography of Protopectin-solubilizing Enzyme.
Chromatography was performed under the conditions described in the text. ·····, protein; —●—, liquefying activity; —▲—, saccharifying activity; —○—, protopectinase activity.

TABLE II. PURIFICATION OF PROTOPECTIN-SOLUBILIZING ENZYME FROM CULTURE FILTRATE OF *Trichosporon penicillatum* SNO-3

Step	Total protein (mg)	Total activity ($\times 10^3$ units)	Specific activity (units/mg protein)	Relative purification	Yield (%)
Culture filtrate	—	600.0	—	1	100
$(NH_4)_2SO_4$ Fractionation	4,614	595.4	129	1	100
CM-Sephadex chromatography	80	386.6	4,830	37.4	64
1st Sephadex G-75 chromatography	78	448.7	5,750	44.6	75
2nd Sephadex G-75 chromatography	75	432.5	5,770	44.7	72

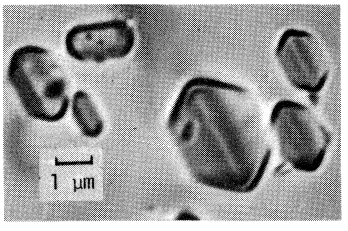

FIG. 3. Microphotograph of Crystals of Protopectin-solubilizing Enzyme.

tained were pooled and concentrated *in vacuo*, at 30°, to 20 ml. As shown in Fig. 2, the second Sephadex G-75 chromatography yielded a single protein peak, and the enzyme activities were entirely associated with the peak. To this fraction was added solid ammonium sulfate until faint turbidity was observed. After standing overnight in a refrigerator, the enzyme was crystallized. The crystallization was repeated

TABLE III. COMPARISON OF ENZYME ACTIVITIES IN THE PURIFICATION STEPS

Step	Pectic acid-degrading activity						PA/P[a]
	Protopectinase		Saccharifying		Liquefying		
	Specific activity	Relative purification	Specific activity	Relative purification	Specific activity	Relative purification	
$(NH_4)_2SO_4$ Fractionation	129	1	130	1	559	1	20.3
CM-Sephadex chromatography	4,830	37.4	4,940	38.0	21,242	38.0	20.8
1st Sephadex G-75 chromatography	5,750	44.6	5,800	44.6	24,940	44.6	20.0
2nd Sephadex G-75 chromatography	5,770	44.7	5,810	44.7	24,983	44.7	20.4
3rd Crystallization	5,775	44.8	5,820	44.8	24,900	44.5	20.1

[a] Pectic acid-degrading activity/pectin-degrading activity (determined from liquefying activity).

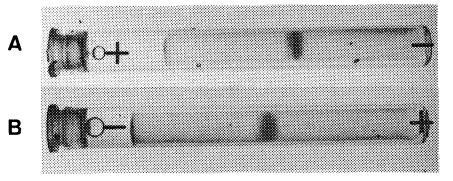

FIG. 4. Disc Gel Electrophoresis of Protopectin-solubilizing Enzyme.

(A) Polyacrylamide gel electrophoresis. A sample of crystalline enzyme (40 μg) was applied to a 7.5% gel column and run at pH 4.5 for 2.5 hr at 5 mA per column. The direction of electrophoresis was from left (anode) to right (cathode).

(B) SDS-Polyacrylamide gel electrophoresis. Crystalline enzyme (40 μg) treated with SDS at 100°C for 5 min was applied on a 10% gel containing 0.1% SDS, and run at 7 mA per column for 5 hr. The direction of electrophoresis was from left (cathode) to right (anode).

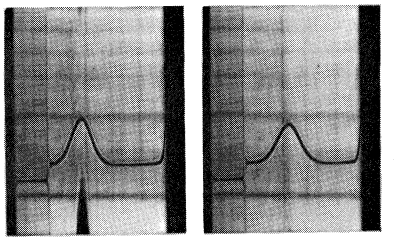

FIG. 5. Ultracentrifugal Analysis of Protopectin-solubilizing Enzyme.

The crystalline enzyme (10 mg) was centrifuged at 52,000 rpm at 20°C.

three times. Table II is a summary of the enzyme purification. From 40 liters of culture filtrate, about 75 mg of crystalline enzyme was obtained, with a recovery of 72%. The preparation from the third crystallization procedure is shown in Fig. 3. As shown in Table III, the ratio of three enzyme activities — protopectinase, pectic acid-liquefying and -saccharifying activity — was around 1 : 1 : 4.3 at each step of the purification procedure, and the ratio of pectic acid- and pectin-degrading activity (determined from liquefying activity) was 20 : 1 at each step of the purification procedure.

Homogeneity

The enzyme preparation thus obtained was completely homogeneous as judged by polyacrylamide gel electrophoresis, both in the presence and absence of sodium dodecyl sulfate (SDS) (Fig. 4), and by sedimentation experiments (Fig. 5).

Molecular properties

The molecular weight was determined by gel filtration on Sephadex G-75, sedimentation equilibrium and SDS-polyacrylamide gel electrophoresis. From the results of gel filtration with marker proteins, the molecular weight of the enzyme was determined to be 30,000 (Fig. 6). The results of sedimentation equilibrium studies were plotted as the logarithm of the equilibrium protein concentration ($\log A_{280\,nm}$) *vs.* the square of the radial distance from the center of the rotor (r^2), as shown in Fig. 7. Taking a partial specific volume of 0.730 cm^3/g together with the slope from Fig. 7, a mo-

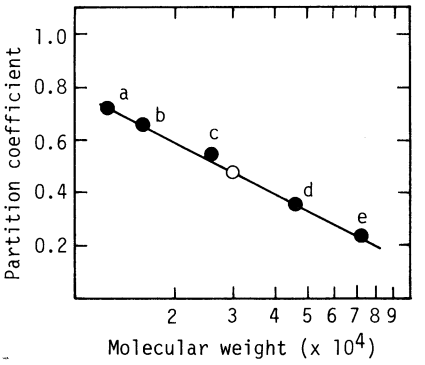

FIG. 6. Determination of Molecular Weight of Protopectin-solubilizing Enzyme by Sephadex G-75 Gel Filtration.

Standard marker proteins: a, cytochrome *c* (M_w 12,500); b, lysozyme (M_w 14,300); c, α-chymotrypsinogen *A* (M_w 25,000); d, ovalbumin (M_w 45,000); e, bovine serum albumin (M_w 68,000). The open circle indicates the elution position of the enzyme.

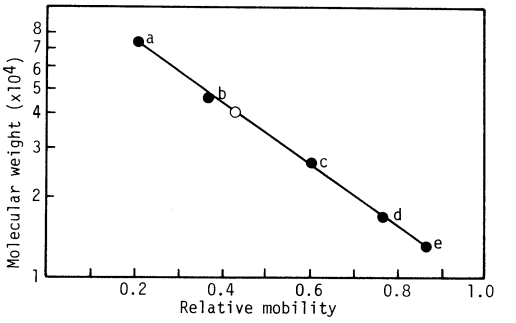

FIG. 8. Determination of Molecular Weight of Protopectin-solubilizing Enzyme by SDS-Polyacrylamide Gel Electrophoresis.

Conditions of electrophoresis are described in the text. Standard marker proteins: a, bovine serum albumin (M_w 68,000); b, ovalbumin (M_w 45,000); c, α-chymotrypsinogen *A* (M_w 25,000); d, myoglobin (M_w 17,300); e, cytochrome *c* (M_w 12,500). The open circle indicates the migration position of the enzyme.

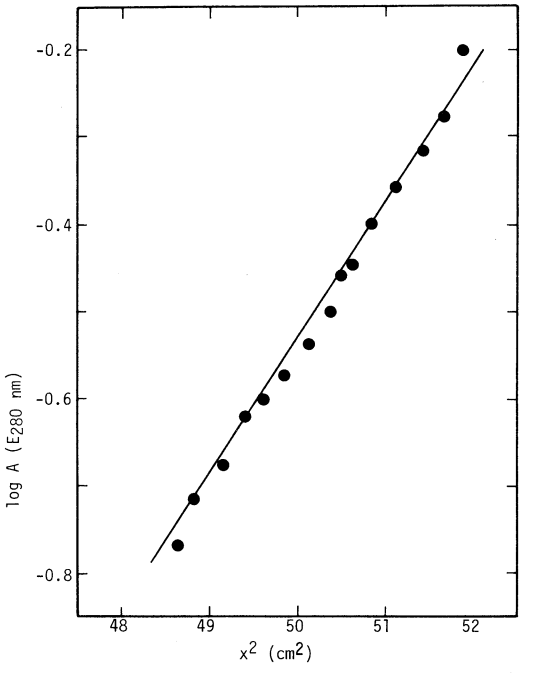

FIG. 7. Plot of Equilibrium Protein Concentration of Protopectin-solubilizing Enzyme *vs.* the Radial Distance in Ultracentrifugation.

Details of the sedimentation experiment are given in the text.

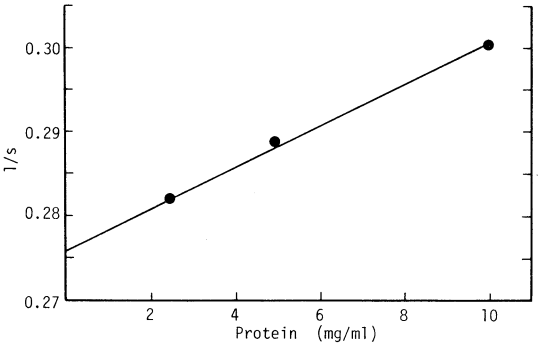

FIG. 9. Sedimentation Coefficient ($s_{20,w}$) of Protopectin-solubilizing Enzyme as a Function of Protein Concentration.

The crystalline enzyme was used at the concentrations of 2.5, 5.0, and 10 mg of protein per ml of 0.01 M acetate buffer, pH 5.0, containing 0.1 M NaCl.

lecular weight of 29,300 was obtained for the enzyme. On the other hand, the molecular weight of the enzyme was determined to be 40,000 by SDS-polyacrylamide gel electrophoresis (Fig. 8).

The sedimentation velocity of the enzyme was measured by analytical centrifugation. The purified enzyme was centrifuged under the conditions described in the legend to Fig. 5. By extrapolation of the linear plot of 1/s *vs.* protein concentration to the intercept, the corrected sedimentation coefficient, $s_{20,w}$, was calculated to be 3.66S, using a value of 0.730 cm^3/g for the partial specific volume of the protein (Fig. 9).

Ultraviolet absorption spectrum

The enzyme showed λ_{max} at 283 nm and λ_{min} at 249 nm with a shoulder around 290 nm, and $E^{1\%}_{280\ nm}$ was determined to be 9.20.

Amino acid composition

The amino acid composition of the enzyme was determined with the hydrolyzates of the enzyme obtained after 12-, 24-, and 36-hr hydrolysis at 105°C, and the average values for these samples are shown in Table IV. The chromatogram from the amino acid analyzer showed no unusual peak, the enzyme contains glycine, glutamic acid, serine, and aspartic acid at relatively high levels. From the results of separate studies, the enzyme was observed to contain 1.7% sugar.

Isoelectric point

The isoelectric point of the enzyme was determined by isoelectric electrophoresis on Ampholine-polyacrylamide gel. The mobility on the gel was plotted with marker proteins, as shown in Fig. 10, and the isoelectric point was determined to be 7.8.

Immunological analysis

The enzyme was a variety of endo-polygalacturonase and its molecular weight was found to be similar to that of endo-polygalacturonases produced by *Aspergillus niger* and *Saccharomyces fragilis*. To confirm the difference of this enzyme from these two endo-polygalacturonases, immunological analyses were performed as shown in Fig. 11, rabbit antiserum against the enzyme (*S*-antibody) did not cross-react with endo-polygalacturonases from *Aspergillus niger* and *Saccharomyces fragilis* (crystalline preparation obtained by Sakai and Yoshitake).[15] The effects of *S*-antibody on the inactivation of these enzymes were investigated. As shown in Fig. 12, the enzyme from *Saccharomyces fragilis* was inactivated by *S*-antibody, though the inactivation ratio was much less than that for

TABLE IV. AMINO ACID COMPOSITION OF PROTOPECTIN-SOLUBILIZING ENZYME

Amino acid	Number of residues[a] per mol of enzyme
Lysine	9.6
Histidine	3.6
Arginine	2.7
Tryptophane[b]	3.9
Aspartic acid (Asparagine)	27.9
Threonine[c]	22.8
Serine[c]	28.8
Glutamic acid (Glutamine)	15.3
Proline	4.2
Glycine	25.7
Alanine	12.0
Cysteine (half cystine)[d]	4.5
Valine	11.7
Methionine	0.9
Isoleucine	15.6
Leucine	8.1
Tyrosine	3.0
Phenylalanine	7.2

[a] Calculations were based on a molecular weight of 30,000.
[b] Cysteine and cystine were determined by the method of Crestfield *et al.*[13]
[c] Determined by extrapolation to zero time of hydrolysis.
[d] Determined spectrophotometrically by the method of Edelhoch.[14]

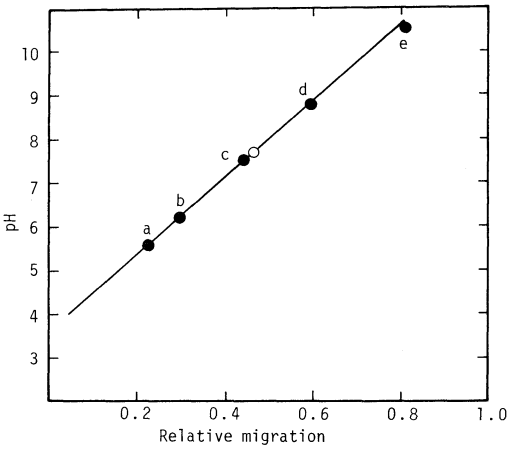

FIG. 10. Determination of Isoelectric Point of Protopectin-solubilizing Enzyme by Isoelectric Focusing.
Conditions of electrofocusing are described in the text. p*I* marker proteins: a, cytochrome *c'* from *Rhodospirillum rubrum* (p*I* 5.6); b, cytochrome c_2 from *R. rubrum* (p*I* 6.2); c, myoglobin from horse (p*I* 7.6); d, myoglobin from whale (p*I* 8.7); e, cytochrome *c* from *R. rubrum* (p*I* 10.6). The open circle indicates the migration position of the enzyme.

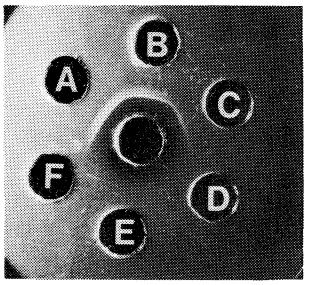

FIG. 11. Immunodiffusion of Some Enzymes with Rabbit Antiserum against Protopectin-solubilizing Enzyme from *Trichosporon penicillatum* SNO-3.

Each well was filled with about 60 μl of sample. Center well, rabbit antiserum against protopectin-solubilizing enzyme from *Tr. penicillatum* SNO-3 (Enzyme S); Well A, crystalline Enzyme S (1 mg/ml); Well B, crystalline Enzyme S (10 μg/ml); Well C, culture broth of *Tr. penicillatum* SNO-3; Well D, purified endo-polygalacturonase from *Aspergillus niger* (1 mg/ml); Well E, crystalline endo-polygalacturonase from *Saccharomyces fragilis* (1 mg/ml); Well F, Macerozyme® (a macerating enzyme mixture from *Rhizopus* sp.). The plate was incubated at 37°C in a moist chamber for 48 hr.

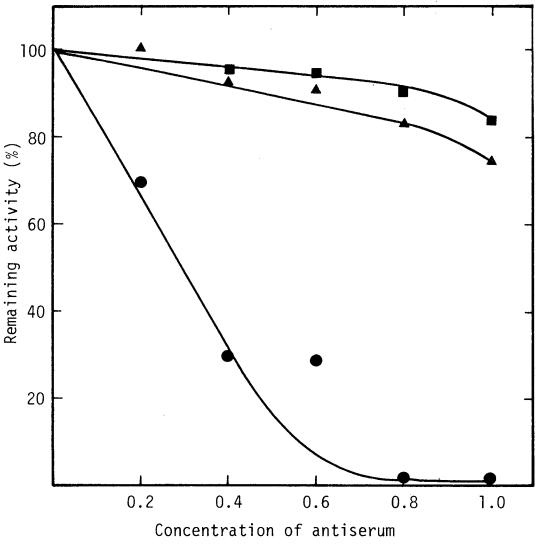

FIG. 12. Effect of Rabbit Antiserum against Protopectin-solubilizing Enzyme from *Trichosporon penicillatum* SNO-3 Inactivation of Some Enzymes.

Details of the experiment are given in the text. —■—, endo-polygalacturonase from *Aspergillus niger*; —▲—, endo-polygalacturonase from *Saccharomyces fragilis*; —●—, protopectin-solubilizing enzyme from *Tr. penicillatum*.

TABLE V. LIBERATION OF PECTIN FROM VARIOUS PROTOPECTINS BY PROTOPECTIN-SOLUBILIZING ENZYME

Origin of protopectin	Pectin liberated (g/100 g sample)	Yield[a] (%)
Citrus unshiu peel	7.8	78
Burdock	11.6	77
Radish	11.8	81
Watermelon peel	17.8	81
Carrot	16.3	82

[a] Ratio of pectin liberated to whole pectin substance in sample. Whole pectic substance was determined by the method described by Stoddart *et al.*[16]

TABLE VI. SOME PROPERTIES OF PECTIN FROM *Citrus unshiu* PEEL LIBERATED BY PROTOPECTIN-SOLUBILIZING ENZYME

Relative viscosity of 0.1% solution	2.12
Methoxyl group (%)	8.46
Esterified carboxyl group (%)	55.4
Galacturonic acid (%)	92.1
Neutral sugar (%)	7.3
pH of 0.5% solution	5.8
Molecular weight[a]	170,000

[a] Determined by the method of Smit and Bryant.[17]

the enzyme from Strain SNO-3.

Protopectin-solubilizing activity

The protopectin-solubilizing activity of the enzyme on protopectin from various plant tissues was determined. The enzyme showed protopectin-solubilizing activity on various protopectins as shown in Table V. The pectin liberated from protopectin (from *Citrus unshiu* peel) was observed to have a high molecular weight and the enzyme was confirmed to exhibit protopectinase activity (Table VI).

DISCUSSION

Degradation of protopectin was originally attributed to a specific enzyme, "protopectin." However, the results of studies on pectolytic enzymes have shown that enzymic decomposition of protopectin is due to the action of a system of pectolytic enzymes such as pectinesterase, endo-polygalacturonase, endo-pectin

lyase and pectate lyase. In these studies, much attention was paid to the degradation of protopectin (maceration) but little to the liberation of water-soluble polymers (pectin substances) from protopectin.

There are some observations for enzymes which liberate a pectin substance from protopectin. Kaji[18,19] demonstrated a microbial enzyme from the culture filtrate of *Clostridium felsineum*, catalyzing the breakdown of middle lamella pectin from the bark of Gampi (Wikstremia sikokiana Fr *et.* Sav.) to a soluble pectin substance. The existence of an enzyme having activities which liberate pectin from plant tissues was also demonstrated in the culture filtrate of *Aspergillus japonicus* by Ishii[20]: He separated the enzymes, endo-polygalacturonase and endo-pectin lyase. Karr and Albersheim,[21] studying Pectenol R-10®, a mixture of enzymes produced by *Aspergillus niger*, isolated an enzyme which liberated a pectin substance from protopectin but only degraded pectic acid to a limited extent. However, details of the enzyme were not clarified.

Thus, there have been many studies on protopectinases, and the mechanism of protopectin-solubilizing activity, especially the polymer-pectin substance-liberating activity, is not clear.

In the preceding work, the authors found that *Trichosporon penicillatum* SNO-3 produced a protopectin-solubilizing enzyme.[2] In the investigations performed in the present work, our enzyme was found to liberate a polymer pectin substance from various protopectins and was reconfirmed to be a protopectinase. The enzyme catalyzed the hydrolysis of pectic acid and decreased the viscosity of the pectic acid-containing reaction medium. However, the enzyme increased the reducing value very slightly; a 50% decrease of viscosity of the substrate solution corresponded to the splitting of 2% of the glycosidic bonds, as mentioned in the preceding paper.[2] From the results, the enzyme was confirmed to be a endo-polygalacturonase.

Endo-polygalacturonases are known to be produced by such saprophytic fungi as *Aspergillus, penicillium, Monilia, Rhizopus, Sclerotinia* and *Conothyrium*, and some bacteria and a few yeasts.

However, detailed studies on the properties of endo-polygalacturonases are not as numerous as those on such other polymer-degrading enzyme as amylase and protease.

Purification of an endo-polygalacturonase from a surface culture of *Aspergillus niger*[22] was one of the first attempts at obtaining a homogeneous pectolytic enzyme. After that, some endo-polygalacturonases were isolated from the culture filtrates of fungi, such as *Aspergillus japonicus*,[23] *Aspergillus saitoi*,[24] *Verticillium albo-atrum*,[25] and a strain of *Acrocylindrium*.[26] These fungi each produced several pectolytic enzymes, and complicated procedure was used for their purification.

In the present study, the production of various enzymes, which possibly catalyze the degradation of protopectin, in the culture filtrate of Strain SNO-3 was studied, and polygalacturonase and protopectinase activity was found. The culture filtrate catalyzed the degradation of pectin, pectic acid and protopectin. The ratio of these three activities was found to be the same in each step of the purification procedure. From these results it appears that degradation of protopectin, pectic acid and pectin is catalyzed by the same enzyme in the culture filtrate. Thus, Strain SNO-3 seemed to produce this enzyme as the sole pectolytic enzyme, and it was purified by a simple procedure with a relatively high yield.

The molecular weight of the enzyme was determined to be 30,000 by sedimentation and gel filtration, and to be 40,000 by SDS-polyacrylamide gel electrophoresis. The enzyme is a glycoprotein, and the molecular weight determined by SDS-polyacrylamide gel electrophoresis seems to be erroneous.[27] Thus, the molecular weight of the enzyme was determined to be 30,000. Endo-polygalacturonases having molecular weights around 30,000 were isolated from *Aspergillus niger*[28] and *Saccharomyces fragilis*.[29] Our enzyme is very similar in molecular weight to the enzymes

from *Aspergillus niger* and *Saccharomyces fragilis*. Martini *et al.*[30] found that a polysaccharide degrading activity of *Fusarium oxysporum*, a phytopathogenic fungi, is determined by extrachromosomal DNA which is maintained in *Escherichia coli* K-12 as a plasmid, and suggested that exocellular cell wall degrading enzyme (protopectin-solubilizing enzyme) determinants are plasmid borne. The authors assumed that some endo-polygalacturonases (protopectin-solubilizing enzyme) are determined by the same type of extrachromosomal DNA, from the facts that enzymes having similar properties are produced by organisms belonging to different genera.

In order to clarify the degree of similarity of our enzyme with the above enzymes, immunological experiments were performed. However, the antibody against our enzyme did not cross-react with the endo-polygalacturonases from *Aspergillus niger* and *Saccharomyces fragilis*, and our enzyme seems to be different from the above endo-polygalacturonases from the criteria of its characteristics as a protein.

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