Purification, Crystallization, and Characterization of a Novel Protopectinase from *Bacillus subtilis*†

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We found a novel protopectinase that did not catalyze the degradation of polygalacturonic acid in the culture filtrate of *Bacillus subtilis* IFO 12113. The enzyme was purified by CM-Sephadex C-50 column chromatography, heat treatment at 60°C, and column chromatography on DEAE-Toyopearl 650M and Toyopearl HW-55S. The enzyme was isolated as a homogeneous protein, and was finally isolated as crystals. The molecular weight of the enzyme was estimated to be 30,000 by gel permeation chromatography and by SDS-polyacrylamide gel electrophoresis, and 29,800 by sedimentation analysis. Its isoelectric point was around pH 9.4. The enzyme was thermostable; its activity was not lessened by being heated at 60°C for 10 min. The enzyme catalyzed the degradation of arabinogalactan from soybean seeds. Some strains belonging to the genus *Bacillus*, such as *B. amyloliquefaciens*, *B. cereus*, *B. circulans*, *B. coagulans*, *B. firmus*, *B. licheniformis*, *B. macerans*, and *B. pumilus*, produced the same kind of protopectinase.

Protopectin is a water-insoluble parent pectic substance found in plant tissue. The features of the insolubility of pectin in protopectin are complicated, and include (1) bonding of pectin molecules to other cell wall constituents, such as hemicellulose or cellulose, (2) binding of pectin molecules with polyvalent ions, such as Ca^{2+}, $Fe^{2+,3+}$, and Mg^{2+} leading to insolubility, (3) secondary valency bonding between pectin molecules or with other cell wall constituents such as cellulose or hemicellulose, (4) salt bridging between the carboxyl groups of pectin molecules and the basic groups of protein and, (5) mechanical entwining of the pectin molecules with each other and with other cell wall constituents.[1] Protopectin is solubilized by its restricted hydrolysis, resulting in the liberation of water-soluble pectin. An enzyme catalyzing the solubilization of protopectin to form a highly polymerized pectic substance soluble in water has tentatively been named protopectinase.[2] Sakai and Okushima found protopectinase in a yeast strain in 1978. Since then, some other protopectinases have been isolated.[3~6] Those enzymes have the same mechanism to solubilize protopectin; they cause restricted hydrolysis of the polygalacturonic acid domain in the pectin molecule in protopectin.

The features of the insolubility of pectin in protopectin suggest that various enzymes that have protopectinase activity with different mechanisms may exist. We screened for a protopectinase with a different mechanism from the enzymes found previously, and found a protopectinase that does not catalyze the degradation of polygalacturonic acid.[7]

This paper deals with the purification and characterization of the novel protopectinase.

Materials and Methods

Chemicals. CM-Sephadex C-50 was obtained from Pharmacia K.K. (Tokyo). DEAE-Toyopearl 650M and Toyopearl were obtained from the Tosoh Corp. (Tokyo).

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Standard proteins for the estimation of the molecular weight of the enzyme were purchased from the Sigma Chemical Co. (St. Louis). Soybean flour was obtained from the Fuji Seiyu Co. (Osaka), and soybean flour extract (SBFE) was prepared by the method of Morita.[8] Unless otherwise specified, other chemicals were from Wako Pure Chemicals (Osaka) and of reagent grade.

Microorganisms. The organisms used were supplied by the Institute of Fermentation, Osaka (Osaka). The organisms were maintained on agar slants containing 1% glucose, 0.5% peptone, 0.5% meat extract, and 0.5% NaCl, at pH 7.0.

Cultivation of microorganisms. For the study of enzyme production, medium containing 0.5% soluble starch, 0.05% yeast extract, 1% $(NH_4)_2PO_4$, 0.05% $CaCl_2 \cdot 2H_2O$, 0.02% $MgSO_4 \cdot 7H_2O$, and 0.02% KCl, pH 6.8 (B-medium) was used as the basal medium. The organisms were cultured in a 500-ml shaking flask containing 100 ml of the medium on a shaker (120 rpm) at 37°C.

Enzyme assay. Protopectinase activity was assayed by the measurement of the amount of pectin liberated from protopectin by the carbazole–sulfuric acid method.[9] The reaction was done in a mixture containing 10 mg of protopectin (prepared from lemon peel), 2 mmol of acetate buffer, pH 5.0, and 50 μl of enzyme solution in a total volume of 1 ml at 37°C for 60 min. One unit of enzyme activity is defined as the activity liberating pectin corresponding to 1 μmol of D-galacturonic acid per ml of reaction mixture at 37°C in 60 min. Protopectins were prepared by a method described previously.[4] Polygalacturonase activity was assayed by the measurement of the release of reducing groups by the method of Somogyi,[10] and one unit of activity was defined as the activity liberating reducing groups corresponding to 1 μmol of D-galacturonic acid in 60 min.

Electrophoresis. Polyacrylamide disc gel electrophoresis (PAGE) was done at pH 4.5 in a 7.5% gel by the method of Reisfeld *et al.*[11] Electrophoresis in the presence of sodium dodecyl sulfate (SDS) was done in a 10% gel by the method of Weber and Osborn.[12] The protein was stained with Coomassie brilliant blue R-250.

Isoelectric focusing. To find the *p*I value of the enzyme, gel disc isoelectric focusing[13] was done in 7.5% polyacrylamide gel containing 2% Ampholine (pH 3.5 to 10.0). Electrofocusing was done at a constant voltage (200 V) for 4 hr at 5°C. As the anode and cathode solutions, 20 mM H_3PO_4 and 1 M NaOH were used, respectively. After electrofocusing, the gels were stained with Coomassie brilliant blue G-250 in a trichloroacetic acid solution. The isoelectric point of the enzyme was estimated from a graph of the relationship between the mobility of marker proteins and their isoelectric points.

Estimation of molecular weight. The molecular weight of the enzyme was estimated by the following three methods.

(i) *Permeation chromatography on Toyopearl HW-55S.* Permeation chromatography was done at 5°C on a Toyopeal HW-55S column (2.0 × 100 cm) equilibrated with 50 mM acetate buffer containing 200 mM KCl, pH 5.0. Proteins were eluted with the same buffer at a flow rate of 10 ml/hr.

(ii) *SDS-PAGE.* SDS-PAGE was done on a 10% gel in 0.1% SDS/100 mM phosphate buffer, pH 7.2, at 7 mA per gel for 5 hr.

(iii) *Sedimentation equilibrium.* Sedimentation equilibrium was done in a Hitachi model 252 analytical ultracentrifuge at 16,000 rpm and 20°C, in a 20 mM acetate buffer, pH 5.0, containing 100 mM NaCl. The molecular weight of the enzyme was estimated by the method of Yphantis.[14]

Identification of amino acid composition. The amino acid composition of the enzyme was identified using a hydrolysate of the enzyme on a Hitachi KLA-3B amino acid analyzer. To analyze the amino acid composition of the enzyme, 1.5 mg of enzyme was hydrolyzed for 24, 48, or 72 hr at 105°C with 6 N HCl in a sealed tube filled with nitrogen gas. Half-cystine was assayed by the method of Crestfield *et al.*[15] Tryptophan was measured spectrophotometrically.[16]

Protein concentration. The protein concentration was found spectrophotometrically at 280 nm, with the assumption that the absorbance at a protein concentration of 1 mg/ml was about 1.5. The protein concentration was also measured by the method of Lowry *et al.*[17] with bovine serum albumin as a standard.

Results

Selection of microorganisms

To select suitable microorganisms for production of a novel protopectinase that did not catalyze the degradation of polygalacturonic acid, a wide variety of stains of *Bacillus* were screened. Table I compares the protopectinase productivities of selected strains. Protopectinase that did not catalyze the degradation of polygalacturonic acid was found in the culture filtrate of some *Bacillus* strains, including *B. amyloliquefaciens*, *B. cereus*, *B. circulans*, *B. coagulans*, *B. firmus*, *B. licheniformis*, *B. macerans*, *B. pumilus*, and *B. subtilis*. *B. subtilis* IFO 12113 produced this protopectinase at a higher level than the other strains. The strain also produced polygalacturonase, at a lower level. The activities of the two enzymes

Table I. PRODUCTION OF PROTOPECTINASE AND POLYGALACTURONASE BY *Bacillus* STRAINS

Organisms were cultured in a medium containing 5% soybean flour, 0.5% soluble starch, 1% $(NH_4)_2HPO_4$, 0.05% $CaCl_2 \cdot 2H_2O$, 0.02% $MgSO_4 \cdot 7H_2O$, 0.02% KCl, and 0.05% yeast extract, pH 6.8, for 45 hr on a shaker.

Starin	Protopectinase activity (Unit/ml)		Polygalacturonase activity (Unit/ml)	
Culture temperature (°C)	30	37	30	37
Bacillus subtilis IFO 12113	2.85	12.1	<0.01	<0.01
Bacillus subtilis IFO 13719	<0.01	9.8	<0.01	<0.01
Bacillus subtilis IFO 13721	<0.01	4.2	<0.01	<0.01
Bacillus amyloliquefaciens IFO 14141	<0.01	0.8	<0.01	<0.01
Bacillus cereus IFO 3132	<0.01	2.5	<0.01	<0.01
Bacillus circulans IFO 13632	<0.01	0.2	<0.01	<0.01
Bacillus coagulans IFO 12583	0.8	2.6	<0.01	<0.01
Bacillus firmus IFO 3330	1.7	1.2	<0.01	<0.01
Bacillus licheniformis IFO 14206	1.4	2.5	<0.01	<0.01
Bacillus macerans IFO 3490	0.1	0.1	<0.01	<0.01
Bacillus pumilus IFO 12087	<0.01	4.1	<0.01	<0.01

Table II. EFFECTS OF HEAT TREATMENTS ON ENZYME ACTIVITIES IN CULTURE FILTRATE OF *Bacillus subtilis* IFO 12113

Concentrated culture filtrate was dialyzed against 20 mM acetate buffer, pH 5.0, treated under the conditions shown here, and cooled rapidly.

Treatment		Enzyme activity			
EDTA (50 mM)	Heat	PGase[a] (U/ml)	Yield (%)	PPase[b] (U/ml)	Yield (%)
−	—	16.8	100	170	100
+	—	3.8	23	170	100
+	55°C, 3 min	4.8	29	170	100
+	55°C, 6 min	5.9	29	181	106
+	60°C, 3 min	8.2	49	193	114
+	60°C, 6 min	2.7	17	172	101
+	60°C, 8 min	0	0	169	99
+	60°C, 12 min	0	0	160	94

[a] Polygalacturonase activity.
[b] Protopectinase activity.

had different patterns of stability; the polygalacturonase actvity was more sensitive to treatment with a chelating reagent, ethylenediaminetetraacetic acid-2Na (EDTA) and to heat treatment. In Table II, the effects of heat treatment on the protopectinase and polygalacturonase are summarized. Polygalacturonase was unstable when heated at 60°C in the presence of 50 mM EDTA, but protopectinase was stable in this condition.

Culture conditions

To establish suitable conditions for enzyme formation, the following experiments were done.

(i) *Effects of carbon sources.* The use of various carbon sources, such as glucose, maltose, sucrose, soluble starch, glycerol, citrate, tartrate, and succinate, was examined. Among the substances tested, soluble starch was the most favorable for the enzyme formation and glucose repressed enzyme formation. Soluble starch was selected as the carbon source, and its optimum concentration was 0.5 ~ 1.0%.

(ii) *Effects of nitrogen sources.* Bacterial growth and enzyme formation was affected by the nitrogen source used (Table III). $NH_4H_2PO_4$ was effective on both growth and enzyme formation, and its optimum concentration was around 1% (Fig. 1a). Soybean flour (defatted) was also a good nitrogen source. However, as it contains much hemicellulose, a possibility existed that it might have

Table III. EFFECTS OF NITROGEN SOURCES ON PROTORECTINASE PRODUCTION

Culture was done on a shaker for 20 hr at 37 °C in 100 ml of the B-medium in which $(NH_4)_2HPO_4$ was replaced with the indicated substance in the equivalent amount on the basis on nitrogen.

Nitrogen source	Concentration (%)	pH	Growth (OD_{660})	PGase (U/ml)	PPase (U/ml)	PPase /PGase
$(NH_4)_2CO_3$	0.85	7.7	1.5	4.0	8.2	2.0
$(NH_4)_2SO_4$	1.0	4.8	1.2	4.6	8.4	1.8
NH_4Cl	0.81	4.3	1.7	3.5	4.3	1.2
$(NH_4)_2HPO_4$	1.0	6.6	5.5	4.8	13	2.7
$NH_4H_2PO_4$	1.7	6.7	4.3	6.9	24	3.5
NH_4NO_3	1.2	4.6	1.4	2.0	4.4	2.2
$NaNO_3$	1.3	4.5	1.3	4.4	2.9	0.66
Urea	0.45	6.7	1.6	2.7	1.7	0.63
Peptone	1.5	6.2	5.0	6.0	7.7	1.3
Yeast extract	0.72	7.0	5.6	6.2	7.8	1.3

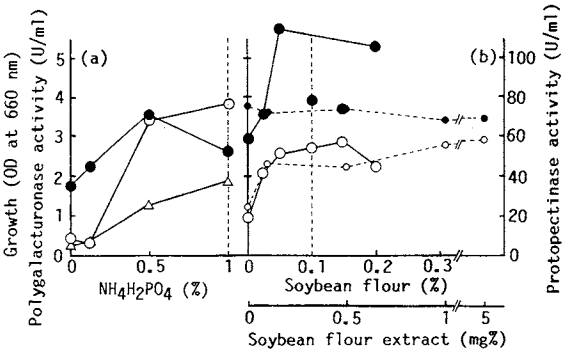

Fig. 1. Effects of $NH_4H_2PO_4$ (a) and Soybean Extract (b) on Growth and Enzyme Production.

Cultivation was done in B-medium without $NH_4H_2PO_4$ (a) or in B-medium (b) supplemented as indicated at 37°C for 24 hr on a shaker.

—●—, growth in medium with added $NH_4H_2PO_4$ and in medium with soybean flour; —○—, protopectinase activity in medium with added $NH_4H_2PO_4$ or soybean flour; —△—, polygalacturonase activity in medium with added $NH_4H_2PO_4$; ---●---, growth in medium with added soybean flour extract; ---○---, protopectinase activity in medium with added soybean flour extract.

acted as an inducer of enzyme formation, rather than as a nitrogen or carbon source. Therefore, the effects of SBFE (the main component of which is arabinogalactan) was examined. SBFE promoted enzyme formation (Fig. 1b). At the optimum concentration (around 1 mg%), SBFE promoted the enzyme formation more than 8-fold of the activity produced in its absence. As 1 mg of SBFE was prepared from about 300 mg of soybean flour, the optimum SBFE concentration corresponded to 0.3% soybean flour.

(iii) *Course of enzyme formation.* From the above experiments, a favorable medium for the enzyme formation was devised: 0.5% soluble starch, 1% $NH_4H_2PO_4$, 0.05% yeast extract, 0.05% $CaCl_2 \cdot 2H_2O$, 0.02% KCl, 0.02% $MgSO_4 \cdot 7H_2O$, and 0.3% soybean flour, pH 6.8. When the microorganism was inoculated into this medium, the enzyme formation increased during the first 24 hr, reached a maximum at 24 hr of cultivation, and gradually declined thereafter (Fig. 2). Polygalacturonase was also produced, as a low level. However, production of this enzyme was inconsistent, and the ratio of the enzymes changed with

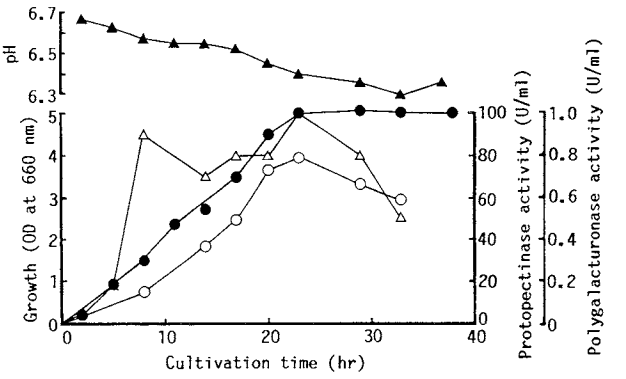

Fig. 2. Course of Growth and Enzyme Formation.

Cultivation was done in medium as described in the text.

●, growth; ▲, pH; ○, protopectinase activity; △, polygalacturonase activity.

Table IV. PURIFICATION OF PROTOPECTINASE FROM *Bacillus subtilis* IFO 12113

Step	Protein (mg)	PPase (U/ml)	Specific activity (U/mg)	Purification (-fold)	Yield (%)
Culture filtrate[a]	3500	170,000	50	1	100
CM-Sephadex C-50	250	57,000	230	4.6	33
Heat treatment[b]	180	56,000	320	6.4	33
DEAE-Toyopearl 650 M	65	51,000	780	16	30
Toyopearl HW-55S	42	35,000	840	17	21

[a] From 20 l of culture.
[b] At 60°C for 7 min.

each cultivation.

Purification of enzyme

The enzyme was purified from the culture filtrate of *B. subtilis* IFO 12113 as follows. To the culture filtrate (20 l) was added NaCl (final concentration, 500 mM); and this was concentrated to 2 l by ultramembrane filtration with a Labomodule SEP 1013 (Asahi Chemical Industry Co., Osaka). The concentrated filtrate was dialyzed against 20 mM acetate buffer, pH 5.0 (AcB), and then used as the starting material for enzyme purification. The enzyme solution (about 2 l) was put on a CM-Sephadex C-50 column (5×46 cm) equilibrated with AcB. After the column was washed thoroughly with AcB, the adsorbed enzyme was eluted by a linear gradient of NaCl (0 to 800 mM NaCl) with 1 l each of AcB and 800 mM NaCl (Fig. 3). The fractions containing enzyme activity were pooled and EDTA was added to make 50 mM. The mixture (about 255 ml) was heated at 60°C for 7 min, cooled

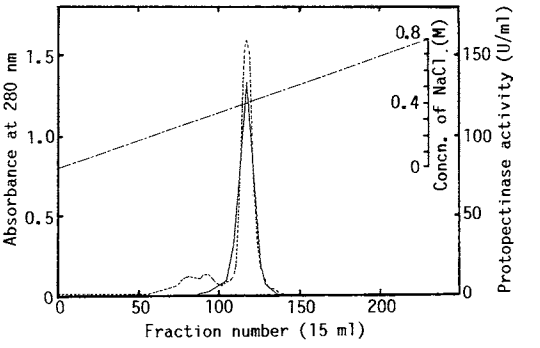

Fig. 3. Chromatography of Protopectinase on CM-Sephadex C-50.

Chromatography was done as described in the text.

------, protein; ——, protopectinase activity; – – –, NaCl concentration.

rapidly, dialyzed against AcB, and passed through a column (2.1 × 15 cm) of DEAE-Toyopearl 650M equilibrated with AcB. The effluents were collected and concentrated to 4 ml; finally, the enzyme was purified by chromatography on a column (2.5 × 75 cm) of Toyopearl HW-55S equilibrated with AcB containing 200 mM KCl. By this procedure, 42 mg of purified enzyme was obtained with the overall yield of about 20% of the starting material. The purification steps are summarized in Table IV. This process purified the enzyme about 17-fold, and 42 mg of enzyme protein was obtained from 20 l of culture filtrate.

The purified enzyme solution was concentrated under reduced pressure. Solid ammonium sulfate was gradually added to the

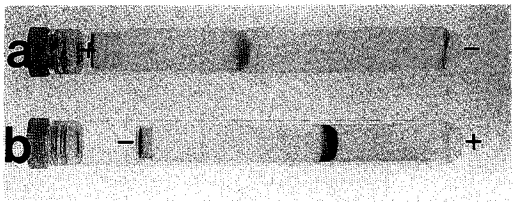

Fig. 5. Polyacrylamide Gel Electrophoresis of Protopectinase in the Absence (a) and Presence (b) of SDS.
A sample of crystalline enzyme (40 μg) was put on the gel. The direction of electrophoresis was from left to right.

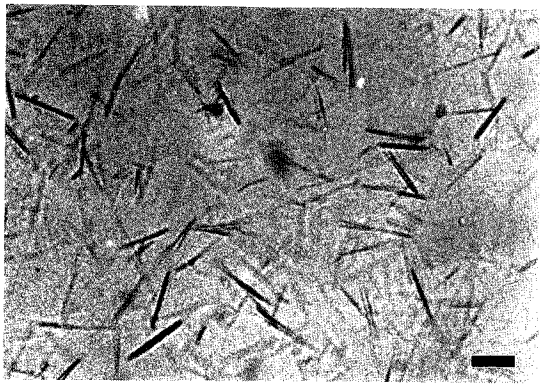

Fig. 4. Photomicrograph of Crystals of Protopectinase.
The bar represents 20 μm.

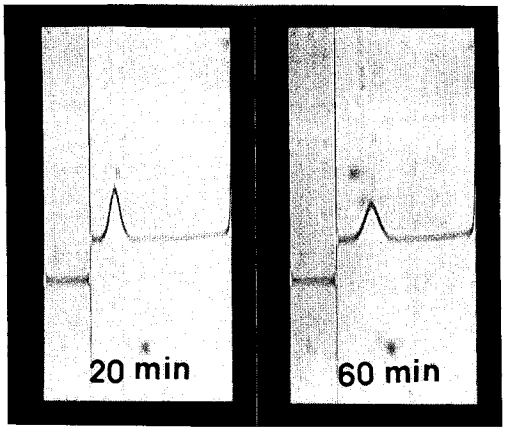

Fig. 6. Ultracentrifugal Analysis of Protopectinase.
A sample of crystalline enzyme (5 mg/ml) was centrifuged at 52,000 rpm at 20°C.

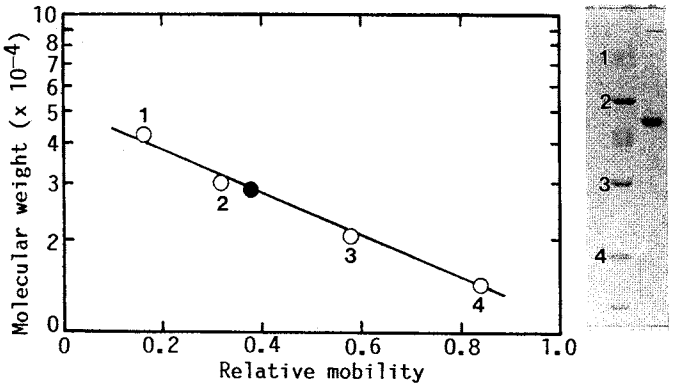

Fig. 7. Estimation of Molecular Weight of Protopectinase by SDS-Polyacrylamide Gel Electrophoresis.
Conditions of electrophoresis were as described in the text. Standard marker proteins: 1, ovalbumin (M_w 43,000); 2, carbonic anhydrase (M_w 30,000); 3, soybean trypsin inhibitor (M_w 20,100); 4, α-lactoalbumin (M_w 14,400). ●, protopectinase.
Photographs show gels with applied marker proteins (left) and protopectinase (right).

concentrated enzyme solution until the solution became slightly turbid. When left for a few days in the refrigerator, needle-like crystals formed without an increase in specific activity (Fig. 4).

Homogeneity

The enzyme preparation obtained was homogeneous as judged by PAGE, both in the presence and absence of SDS (Fig. 5), and by sedimentation experiments. The enzyme sedimented as a single symmetrical peak during sedimentation runs (Fig. 6). The sedimentation constant ($s_{20,w}$) was 2.74 S at an enzyme concentration of 5.0 mg/ml.

Molecular properties

The molecular weight was estimated by permeation chromatography on Toyopearl HW-55S, SDS-PAGE, and sedimentation equilibrium. From the results of permeation chromatography and SDS-PAGE, the molecular weight of the enzyme was 30,000 (Fig. 7). By the sedimentation equilibrium method, when

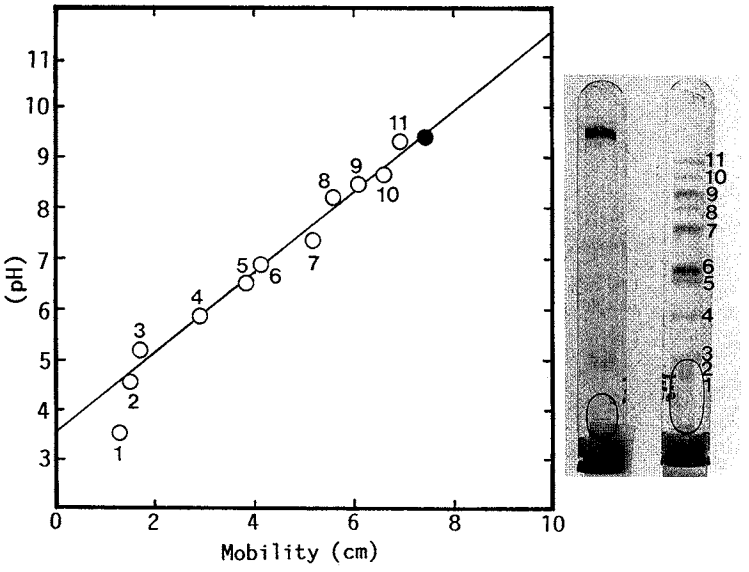

Fig. 8. Identification of the Isoelectric Point of Protopectinase by Isoelectric Focusing.

Conditions of electrofocusing were as described in the text. *p*I maker protains: 1, amyloglucosidase (*p*I 3.50); 2, soybean trypsin inhibitor (*p*I 4.55); 3, β-lactoglobulin A (*p*I 5.20); 4, bovine carbonic anhydrase B (*p*I 5.85); 5, human carbonic anhydrase B (*p*I 6.55); 6, horse myoglobin (*p*I 6.85); 7, horse myoglobin (*p*I 7.35); 8, lentil lectin (*p*I 8.15); 9, lentil lectin (*p*I 8.45); 10, lentil lectin (*p*I 8.65); 11, trypsinogen (*p*I 9.30). ●, protopectinase. Photographs show gels with applied protopectinase (left) and marker proteins (right).

Table V. Amino Acid Analysis of Protopectinase from *Bacillus subtilis* IFO 12113

Amino acid	mol[a]	Amino acid	mol[a]	Amino acid	mol[a]
Glycine	22	Phenylalanine	5	Aspartic acid	
Alanine	14	Tyrosine	14	+Asparagine	32～44
Valine	10	Tryptophan	12	Glutamic acid	
Leucine	10	Methionine	1	+Glutamine	14
Isoleucine	13	Proline	3～6	Cysteine	
Threonine	14	Lysine	13	+Cystine	—
Serine	23	Arginine	6	Histidine	6

[a] Calculated based on a molecular weight of 30,000.

the log of the fringe displacement was plotted against the square of the distance from the center of rotation, straight lines were obtained. The slope of the line and an assumed partial specific volume of 0.74 gave a molecular weight of 29,800.

Isoelectric point

The isoelectric point of the enzyme was found, using Ampholine-PAGE, to be pH 9.3～9.4 (Fig. 8).

Ultraviolet absorption spectrum

The enzyme had an E_{max} at 275 nm and E_{min} at 248 nm with a shoulder at around 282 nm, and $E^{1\%}_{280\,nm}$ was 15.00 at pH 7.0.

Amino acid composition

The amino acid composition of the enzyme is shown in Table V. The enzyme contained aspartic acid, serine, and glycine at relatively high levels.

Stability of the enzyme

The enzyme was somewhat thermostable. When it was incubated at pH 5.0 at various temperatures, the enzyme was stable up to 60°C, and was not denatured for 10 min under these conditions. The enzyme was stable at pH 5.0 to 9.0 at 50°C for 30 min (Fig. 9a). When the enzyme was incubated at 60°C (at pH 5.0)

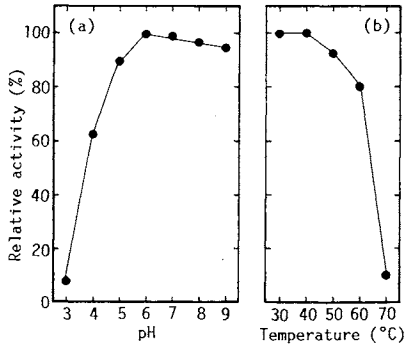

Fig. 9. Effects of pH (a) and Temperature (b) on Enzyme Stability.

(a): The enzyme (30 U/ml) in various buffers (20 mM acetate buffer, from pH 3 to 5, and 20 mM phosphate buffer, from pH 6 to 9) containing 50 μg/ml bovine serum albumin was incubated at 50°C for 30 min, and the remaining activity was assayed.

(b): The enzyme (30 U/ml) in 20 mM acetate buffer, pH 5.0, containing 50 μg/ml bovine serum albumin was incubated at various temperatures, and the remaining activity was assayed.

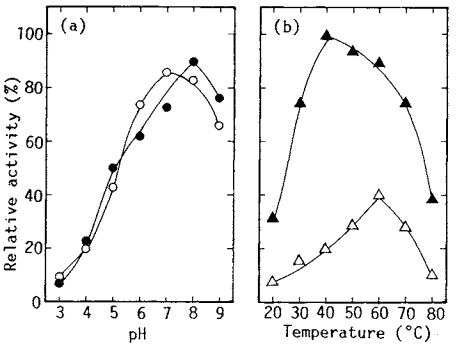

Fig. 10. Effects of pH (a) and Temperature (b) on Enzyme Activity.

(a): The enzyme reaction was done at various pH (20 mM acetate buffer, from pH 3 to 5, and 20 mM phosphate buffer, from pH 6 to 9) at 37°C (○) and at 60°C (●) for 60 min.

(b): The enzyme reaction was done at pH 5.0 (△, 20 mM acetate buffer) and pH 8.0 (▲, 20 mM phosphate buffer) at various temperatures.

Table VI. EFFECTS OF METAL SALTS ON PROTOPECTONASE ACTIVITY

Salt	(mM)	Relative activity[a] (%)
$AgNO_3$	1	90
$BaCl_2$	1	60
$CaCl_2$	1	70
$Cd(CH_3COO)_2$	1	70
$CoCl_2$	1	80
$CuSO_4$	1	90
$FeCl_2$	0.1	70
$FeCl_3$	0.01	80
$Hg(CH_3COO)$	0.1	90
$HgCl_2$	1	80
KCl	1	90
$MgCl_2$	1	80
$MnCl_2$	1	80
NaCl	1	110
$NiCl_2$	1	60
$PbCl_2$	1	100
$SnCl_2$	0.1	100

[a] Relative to control (without any metal salt added to the standard assay mixture).

Table VII. Effects of Common Inhibitors on Protopectinase Activity

Inhibitor	(mM)	Relative activity[a] (%)
NaF	1	80
NaN_3	1	110
Na_2HAsO_4	1	140
$NaNO_2$	1	80
α,α′-Dipyridyl	1	80
o-Phenanthroline	1	80
2-Mercaptoethanol	1	80
N-Methylmaleimide	1	70
p-Chloromercuribenzoate	1	70
p-Chloromercuriphenylsulfonate	0.1	90
Mersalyl acid	1	130
Iodoacetate	1	110

[a] Relative to control (without inhibitor).

for 30 min, the remaining activity was about 80% of the original value (Fig. 9b).

Effects of pH and temperature on enzyme activity

The pH dependence of the enzyme activity was measured. The enzyme was active in alkaline pH with a maximum at pH 8.0 at 37°C. However, when the enzyme activity was measured at 60°C, the maximum was at pH 7.0 (Fig. 10a). When the reaction was done at pH 8.0, the highest activity was at around 40°C. On the other hand, at pH 5.0, the highest activity was at around 60°C (Fig. 10b).

Effects of metal ions and enzyme inhibitors on enzyme activity

The effects of metal ions and enzyme inhibitors are summarized in Tables VI and VII. Most of the metal ions examined was not effective on the enzyme reaction, except that Ba^{2+} and Ni^{2+} inhibited it to some extent. Some mercury compounds also inhibited the enzyme reaction. However, mersalyl acid, a mercury compound, acted rather as a promoter of the enzyme reaction.

Enzyme activity

The enzyme acted on protopectin of various origins (Table VIII), although the activity changed depending on the substrate used. The enzyme preparation showed the activities of none of the following: polygalacturonase, amylase, xylanase, cellulase, or protease. However, it degraded arabinogalactan, which might connect the pectin molecule with cell wall constituents.

Table VIII. Activity of Protopectinase on Protopectin from Various Sources

The reaction was in a reaction mixture with 100 mg of protopectin and 8 units of enzyme in 20 mM acetate buffer, pH 5.0, in a total volume of 10 ml, with incubation for 60 min at 37°C.

Whole pectin was assayed by the method of Stoddart *et al.*[18]

Origin of protopectin	Pectin in Protopectin (mg/g)	Pectin released (mg/g of protopectin)	Relative activity (%)
Lemon peel (*Citrus limon*)	375	19.5	100
Buntan peel (*C. grandis*)	431	76.8	394
Ponkan peel (*C. reticulata*)	551	38.8	199
Yuzu peel (*C. Junos*)	448	30.4	156
Karatachi peel (*C. trifoliata*)	435	15.6	81
Lime peel (*C. aurantifolia*)	561	29.3	150
Valencia orange peel (*C. sinensis*)	428	36.7	188
Busshukan peel (*C. medica*)	512	54.8	281
Nagamikinkan peel (*Fortunella margrigata*)	293	39.2	201
Toukinkan peel (*C. microcarpa*)	365	39.8	204
Satsuma mandarin orange peel (*C. unshiu*)	576	23.6	121
Carrot	394	13.8	71
Burdock (*gobou*)	285	31.4	161
Radish	321	13.8	71
Sugar beet	327	2.7	14

Discussion

The degradation of protopectin liberating highly polymerized pectin was originally attrib-

uted to a specific enzyme, "protopectinase."[2] However, for a long time, little was found about this enzyme. In 1978, the first study of protopectinase was done.[3] Since then, some enzymes that catalyze the solubilization of protopectin have been isolated.[4~6] These enzymes have polygalacturonase activity as well as protopectinase activity and react with protopectin, hydrolyzing the polygalacturonic acid domain on pectin.

We assumed that a novel protopectinase that does not degrade polygalacturonic acid could exist. We searched for such enzyme in strains belonging to *Bacillus*, because this genus includes strains (such as *B. subtilis*) that grow on hay, which contains much protopectin, and partly degrade the hay. We found that many strains belonging to *Bacillus* produced protopectinases. They also produced polygalacturonase. However, this was denatured in the presence of EDTA, although protopectinase was not. Therefore, we assumed that such strains produced a novel protopectinase. To confirm our assumption, we isolated and characterized a protopectinase from *B. subtilis* IFO 12113 here.

The protopectinase described here is a novel one in respect to its reaction mechanism of the solubilization of protopectin, since it does not catalyze the degradation of polygalacturonic acid. The pectin liberated by the action of this protopectinase has a higher molecular weight than that obtained by the conventional method.[7] Because of these properties, we came to a conclusion that the enzyme was a new kind of protopectinase, and named it "protopectinase-B." Although the details of the reaction mechanism of the enzyme are still unclear, it influenced the hydrolysis of arabinogalactan from soybean. Albersheim has found that the pectin molecule is connected to cell wall constituents—cellulose or hemicellulose—with galactan or arabinan.[19] From this finding, protopectinase-B seems to split the glycosidic bond in polysaccharides that connects pectin molecules to cell wall constituents in protopectin, resulting in the solubilization of pectin.

The enzyme reacts with protopectin from various sources. However, the activity changed depending on the substrate used. For example, the activity on sugar beet protopectin is one-seventh and that on *Citrus grandis* (Buntan) protopectin is 3.9 times that of *C. limon* (lemon). These results suggest that there is a difference in their cell wall structure. Thus, the enzyme should be useful for studies of the structure of plant cell walls.

The enzyme was relatively thermostable.

Protopectinase-B should be useful for pectin production.[20] In pectin production, when protopectinase with polygalacturonase activity is used, pectin released from protopectin is degraded by the protopectinase. Therefore, control of the reaction is important. On the other hand, as this protopectinase does not degrade soluble pectin, when it is used for pectin production, control of the reaction is not difficult. Thus, this characteristic is advantageous for pectin production, as is the thermostable characteristics.

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References

1) W. M. Fogarty and O. P. Ward, *Prog. Indust. Microbiol.*, **13**, 59 (1974).
2) C. S. Brinton, W. H. Dore, H. J. Wichmann, J. J. Willaman and C. P. Wilson, *J. Am. Chem. Soc.*, **49**, 38 (1927).
3) T. Sakai and M. Okushima, *Agric. Biol. Chem.*, **42**, 2427 (1978).
4) T. Sakai and M. Okushima, *Agric. Biol. Chem.*, **46**, 667 (1982).
5) T. Sakai and S. Yoshitake, *Agric. Biol. Chem.*, **48**, 1941 (1984).
6) T. Sakai, M. Okushima and S. Yoshitake, *Agric. Biol. Chem.*, **48**, 1951 (1984).
7) T. Sakai and Y. Ozaki, *Agric. Biol. Chem.*, **52**, 1091 (1988).
8) M. Morita, *Agric. Biol. Chem.*, **29**, 564 (1965).
9) S. Furutani and Y. Osajima, *Science Bulletin of the Faculty of Agriculture, Kyushu University*, **22**, 35 (1965).
10) M. Somogyi, *J. Biol. Chem.*, **195**, 19 (1952).
11) R. A. Reisfeld, U. J. Lewis and D. E. Williams, *Nature*, **195**, 281 (1962).

12) K. Weber and M. Osborn, *J. Biol. Chem.*, **244**, 4406 (1969).
13) K. Miyazaki, "Seikagaku Jikken Koza," Vol. 1, ed. by Japanese Biochemical Society, Tokyo Kagaku Dozin, Tokyo, 1970, p. 262.
14) D. A. Yphantis, *Biochemistry*, **3**, 297 (1964).
15) A. M. Crestfield, S. Moore and W. H. Stein, *J. Biol. Chem.*, **238**, 622 (1963).
16) H. Edelhoch, *Biochemistry*, **7**, 1948 (1967).
17) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
18) R. W. Stoddart, A. J. Barrett and D. H. Northcote, *Biochem. J.*, **102**, 194 (1967).
19) P. Albersheim, *Scientific American*, **6**, 81 (1975).
20) T. Sakai and M. Okushima, *Appl. Environ. Microbiol.*, **39**, 908 (1980).