Purification, Crystallization and Some Properties of Endo-polygalacturonase from *Kluyveromyces fragilis*†

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The production of pectolytic enzyme in the genus *Kluyveromyces* was investigated. The production of the enzyme was dependent on the strain, and some strains belonging to *K. fragilis*, *K. marxianus*, and *K. wickerhamii* produced this enzyme among 11 species (29 strains) of the genus *Kluyveromyces*. *K. fragilis* IFO 0288 produced at least four endo-polygalacturonases which have different molecular weights. The dominant endo-polygalacturonase in the culture filtrate of the strain was purified and isolated as crystals. The purified enzyme was homogeneous based on analysis by polyacrylamide gel electrophoresis and ultracentrifugation. The enzyme was a glycoprotein having an isoelectric point around pH 5.6. The sedimentation coefficient ($s_{20,w}$) was 3.77 S, and the molecular weight was around 33,000. The enzyme contained aspartic acid (asparagine), serine,threonine, and glycine at relatively high levels. The enzyme showed the highest activity around pH 5.0 and was stable at pH 5.0 up to 30°C. With the enzyme, and activity which releases highly polymerized pectin from various protopectins (protopectinase activity) was found.

Endo-polygalacturonase is an enzyme catalyzing the depolymerization of pectic substances and has been used as a processing aid or clarification agent in the production of fruit and vegetable juices.[1] The endo-polygalacturonase from a yeast, *Saccharomyces fragilis* (*Kluyveromyces fragilis*), is also known to clarify citrus pectin medium.[2] The enzyme was purified and its properties were investigated by Phaff and Demain[3] and Lim *et al.*[4]

Recently, we found enzymes having protopectinase activity in culture filtrates of various yeasts and demonstrated that these enzymes are available for pectin production from citrus peel.[5～10] We isolated two protopectin-solubilizing enzymes and found that they have endo-polygalacturonase activity besides protopectinase activity.

Although to date there have been many reports on microbial endo-polygalacturonases, few have dealt with their protopectinase activity. To find whether the above protopectinase activities are unique among microbial endo-polygalacturonases, comparative studies of microbial endo-polygalacturonases are being performed.

The present paper deals with the purification, crystallization, and characterization of an endo-polygalacturonase from the culture filtrate of *Kluyveromyces fragilis* IFO 0288.

MATERIALS AND METHODS

Chemicals. All chemicals were Wako Pure Chemicals Industries (Osaka) certified reagent grade.

Protopectin was prepared by the following procedure: The ground plant tissues were washed with distilled water until the water-soluble substance which reacted with carbazole–sulfuric acid was washed off, and then they were lyophilized.

Microorgnisms and culture. All microorganisms used were supplied from the stock culture of the Institute for Fermentation, Osaka. Microorganisms were cultured in 500-ml flasks containing 100 ml of a medium containing

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3% glucose, 0.6% peptone, 0.2% yeast extract, and 0.08% Silicone KM-70 (Shin-Etsu Chemical Co., Ltd., Tokyo), pH 5.0, at 30°C for 36 hr on a shaker. The microorganisms were maintained on agar slants of a medium containing 2% glucose, 0.6% peptone, and 0.5% yeast extract, pH 5.0.

Enzyme assays. Endo-polygalacturonase activity (pectic acid-liquefying activity) was determined as follows: To 6 ml of 0.5% pectic acid solution in McIlvaine 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 (sec) 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 of 50% under the above conditions.

Pectic acid-saccharifying activity was determined by measuring the release of reducing groups according to the method of Jansen and MacDonnell.[11]

Electrophoresis. Polyacrylamide gel electrophoresis was performed with 7% polyacrylamide gel according to the method described by Reisfeld *et al.*[12] SDS-polyacrylamide gel electrophoresis was performed as described by Weber and Osborn.[13]

Isoelectric focusing. For determination of the pI value of the enzyme, gel disc isoelectric focusing[14] was performed in 7.5% polyacrylamide gel containing 2% Ampholine (pH 3.5 to 10.0). Electrofocusing was performed at 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, respectively, were used. After electrofocusing, the proteins in the gels were stained with Coomassie Brilliant Blue G-250-trichloroacetic acid solution. The isoelectric point of the enzyme was determined on a graph of the relation between the mobility of marker proteins and their isoelectric points.

Determination of molecular weight. (i) Gel filtration on Sephadex G-75: Gel filtration was performed at 5°C on a Sephadex G-75 column (2.0 × 100 cm) equilibrated 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) Sedimentation equilibrium: The molecular weight of the enzyme was also determined by sedimentation equilibrium in a Hitachi model 252 analytical ultracentrifuge at 16,000 rpm at 20°C, in 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.[15]

Determination of amino acid composition. The amino acid composition of the enzyme was determined with a hydrolyzate of the enzyme on a Hitachi KLA-3B Amino Acid Analyzer. For the determination of the amino acid composition of the enzyme, 2.54 mg was hydrolyzed for 24, 48, or 72 hr at 105°C with 6 N HCl in a sealed tube filled with nitrogen gas. Half-cystines were determined by the method of Crestfield *et al.*[16] Tryptophan was determined spectrophotometrically.[17]

Determination of carbohydrate in enzyme protein. Total carbohydrate was determined with a sample from which free carbohydrate had been removed by Bio-Gel P-100 column chromatography, by the phenol–sulfuric acid procedure.[18] For analysis of sugar in the enzyme, mannose was chosen as a standard.

Determination of protein. Protein was determined by the method of Lowry *et al.*[19] using bovine serum albumin as a standard.

RESULTS

Production of polygalacturonase- and protopectinase-activity by the genus Kluyveromyces

In Table I are listed the production of polygalacturonase- and protopectinase-activity by 29 strains 11 species belongong to the genus *Kluyveromyces*. Both enzyme activities were produced by 11 strains belonging to *K. marxianus*, *K. fragilis*, and *K. wickerhamii*. In *K. marxianus* and *K. fragilis*, enzyme prductions varied depending on strain. *K. fragilis* IFO 0288, IFO 1777, *K. marxianus* IFO 0428, and *K. wickerhamii* IFO 1675 produced enzyme at relatively high levels.

From these strains *K. fragilis* IFO 0288 was chosen as the most favorable endo-polygalacturonase-producing strain and its enzyme was purified in the following study.

Culture conditions for enzyme production

Figure 1 shows the course of enzyme production under the conditions described in MATERIALS AND METHODS. The enzyme began to be produced after 5 hr of cultivation and reached a maximum at 15 hr of cultivation.

Purification of enzyme

Thirty-seven liters of culture filtrate (cultured for 15 hr) was concentrated by evap-

TABLE I. PRODUCTION OF PECTOLYTIC ACTIVITY BY *Kluyveromyces* spp.

Strain		Enzyme activity (units/ml)		PPase activity / PG activity
		PG[a]	PPase[b]	
K. fragilis	IFO 0288	14.2	59.7	4.2
K. fragilis	IFO 0541	<0.1	<0.05	—
K. fragilis	IFO 1735	2.2	8.2	3.7
K. fragilis	IFO 1777	10.0	51.0	5.1
K. marxianus	IFO 0260	1.8	7.2	4.0
K. marxianus	IFO 0272	1.7	8.2	4.8
K. marxianus	IFO 0273	1.0	4.9	4.9
K. marxianus	IFO 0277	1.4	6.4	4.6
K. marxianus	IFO 0482	5.9	17.0	2.9
K. marxianus	IFO 0690	<0.1	<0.05	—
K. wickerhamii	IFO 1675	8.1	12.2	1.5

The following strains used did not produce pectolytic enzyme under the conditions used in this experiment: *K. africanus* IFO 1671, *K. drosophilarum* IFO 1012, *K. lactis* IFO 0433, IFO 0648, IFO 1090, IFO 1267, *K. marxianus* IFO 0219, IFO 0483, *K. phaffii* IFO 1672, *K. polysporus* IFO 0966, *K. thermotolerans* IFO 0662, IFO 1050, IFO 1674, IFO 1778, IFO 1779, IFO 1780, *K. vanudenii* IFO 1673 and *K. waltii* IFO 1666. All strains used produced neither pectinesterase nor pectin (pectate) lyase under the conditions used in this experiment.

[a] Endo-polygalacturonase.

[b] Protopectin-solubilizing enzyme.

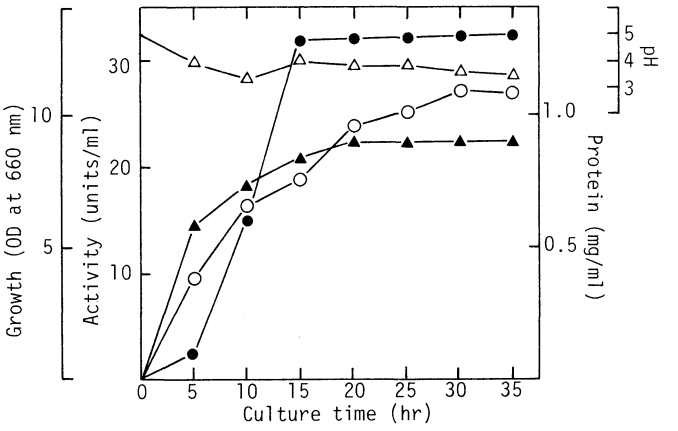

FIG. 1. Course of Formation of Endo-polygalacturonase by *Kluyveromyces fragilis* IFO 0288.

Details of cultivation of the microorganism are described in the text. —△—, pH; —○—, growth; —▲—, protein; —●—, enzyme activity.

oration *in vacuo* at 30°C to 1/10 of the initial volume, and used as the starting material for enzyme isolation. The concentrated cultured filtrate was dialyzed thoroughly against 0.02 M acetate buffer, pH 5.0, and then applied to a CM-Sephadex C-50 column (3 × 50 cm) equilibrated with 0.02 M acetate buffer, pH 5.0. The column was washed with 300 ml of acetate buffer, pH 5.0, and then the enzyme was eluted with 350 ml of a linear gradient of 0 ~ 0.4 M NaCl in the same buffer, at a flow rate of 20 ml/hr. The fractions containing enzyme activity were pooled and concentrated to about 5 ml by evaporation *in vacuo*, at 30°C, and applied to Sephadex G-75 column chromatograpy. The column chromatography was performed using a column (2.2 × 80 cm) equilibrated with

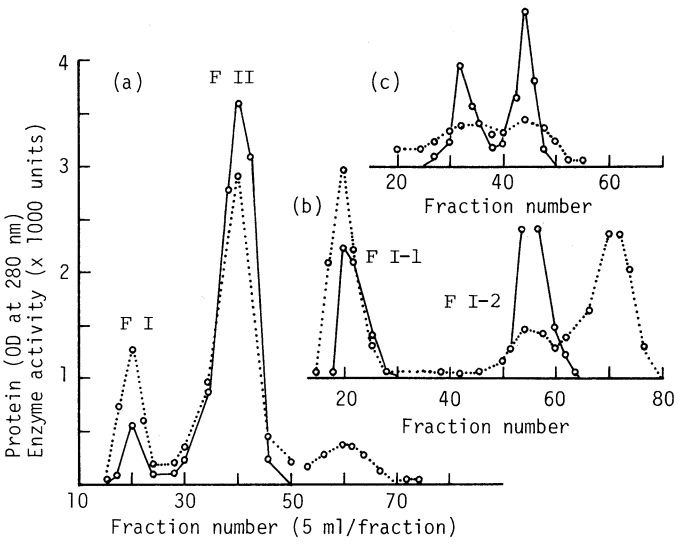

FIG. 2. Chromatograms of Endo-polygalacturonase from *Kluyveromyces fragilis* IFO 0288 on Sephadex Columns.

Chromatographies were performed on Sephadex (a) G-75, (b) G-100 and (c) G-200 under the conditions described in the text. —○—, enzyme activity; ---○---, protein.

0.02 M acetate buffer, pH 5.0, and elution was performed at a flow rate of 6.7 ml/hr. As shown in Fig. 2(a), the activity was recovered in two peaks (F I, II). Most of the activity was recovered in F II, which was concentrated to 2 ml at 30°C *in vacuo* and subjected to rechromatography on the same column and in the same manner. On the second Sephadex G-75 column chromatography, F II was recovered in a single protein peak, and the enzyme activities were entirely associated with the peak. The active fractions were pooled and concentrated *in vacuo*, at 30°C, to 10 ml. To the fraction was added solid ammonium sulfate until faint turbidity was observed. After standing a week in a refrigerator, the enzyme formed needle-like crystals. The crystallization was repeated three times (Fig. 3). Table II is a summary of the enzyme purification. From 37 liters of culture filtrate, about 50 mg of crystalline enzyme was obtained, with a recovery of about 40%.

On the other hand, F I, which was eluted around the void volume on Sephadex G-75 column chromatography, was applied to chromatography on a Sephadex G-100 column

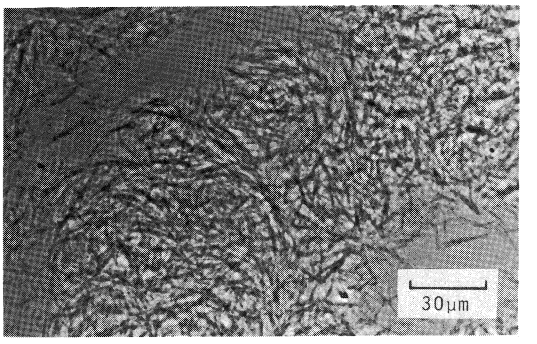

FIG. 3. Photomicrograph of Crystals of Endo-polygalacturonase from *Kluyveromyces fragilis* IFO 0288.

(2.5 × 60 cm) and chromatography was performed with 0.02 M acetate buffer, pH 5.0, at a flow rate of 2.7 ml/hr. The activities were recovered in two peaks, F I-1 seemed to have too high a molecular weight to be applied to chromatography on Sephadex G-100, so it was applied on a Sephadex G-200 column (1.6 × 80 cm) equilibrated with 0.02 M acetate buffer, pH 5.0. By the chromatography (eluted with 0.02 M acetate buffer, pH 5.0, at a flow rate of 0.7 ml/hr), activities were recovered in two peaks as shown in Fig. 2(c). Thus, the strain appeared to produce at least four endo-

TABLE II. PURIFICATION OF ENDO-POLYGALACTURONASE FROM CULTURE FILTRATE OF *Kluyveromyces fragilis* IFO 0288

	Total protein (mg)	Total activity ($\times 10^4$ units)	Specific activity (units/mg)	Relative purification	Recovery (%)
Concentrated culture filtrate	7,844	75	96	1	—
CM-Sephadex column chromatography	204	68.4	3,350	35	91
1st Sephadex G-75 column chromatography	98	48.5	4,950	52	65
2nd Sephadex G-75 column chromatography	65	38.5	5,920	62	51
Crystallization	50	29.5	5,900	61	39

polygalacturonases having different molecular weights.

Homogeneity of F II

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

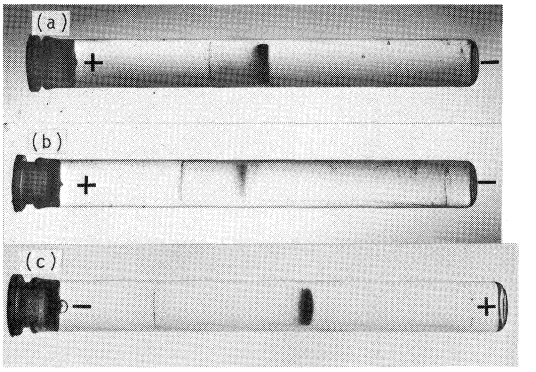

FIG. 4. Polyacrylamide Gel Electrophoresis of Endo-polygalacturonase from *Kluyveromyces fragilis* IFO 0288.
(a) and (b) Polyacrylamide gel electrophoresis. A sample of crystalline enzyme (40 μg) was applied to a 7% gel column and run at pH 4.5 for 2.5 hr at 5 mA per column. The direction of electrophoresis was from left (anode) to right (cathode), and (a) was stained with Amido Black 10 B for protein and (b) was stained with basic fuchsin for sugar in protein. (c) 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).

Molecular properties of F II

The molecular weight was determined by gel filtration on Sephadex G-75 and sedimentation equilibrium. By the gel filtration method, the molecular weight of the enzyme was determined to be 33,000 (Fig. 6). In the sedimentation equilibrium experiment, the plots of logarithum of the equilibrium protein concentration log A ($E_{280\,nm}$) *vs.* the square of the radial distance from the center of the rotor (x^2) were obtained (Fig. 7). Taking a partial specific volume of 0.370 cm^3/g together with the slope from Fig. 7, a molcular weight of 32,800 was obtained for the enzyme. The apparent sedimentation velocities measured at various protein concentrations by analytical centrifugation were plotted against the reciprocals of protein concentration ($1/s$) and extrapolated to the intercept. The corrected sedimentation coefficient, $s_{20,w}$, was calculated to be 3.77 S, using the values obtained in Fig. 5(b) and (c) and 0.370 cm^3/g for the partial specific volume for the protein.

Ultraviolet absorption spectrum of F II

The enzyme showed λ_{max} at 283 nm and λ_{min} at 250 nm with a shoulder around 293 nm, and $E^{1\%}_{280\,nm}$ was determined to be 9.98.

Amino acid composition of F II

The amino acid composition of the enzyme

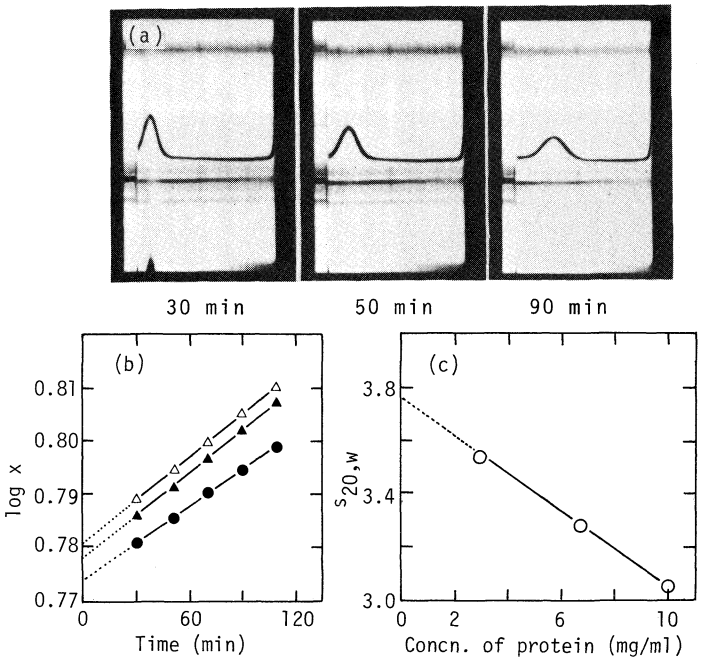

FIG. 5. Ultracentrifugal Analysis of Endo-polygalacturonase from *Kluyveromyces fragilis* IFO 0288.

(a) Sedimentation pattern. The crystalline enzyme (10 mg/ml) was centrifuged at 52,000 rpm at 20°C.

(b) Plot of log x (distance from the center of rotor to sedimentation peak of enzyme protein) *vs.* sedimentation time. The enzyme was used at a concentration of 10 mg —△—, 5 mg —▲— and 2.5 mg —●—, per ml of 0.02 M acetate buffer, pH 5.0, containing 0.2 M NaCl.

(c) Plot of $s_{20,w}$ *vs.* protein concentration for determination of sedimentation coefficient. The enzyme was used at a concentration of 3.3, 6.3, and 10 mg per ml of 0.02 M acetate buffer, pH 5.0, containing 0.2 M NaCl.

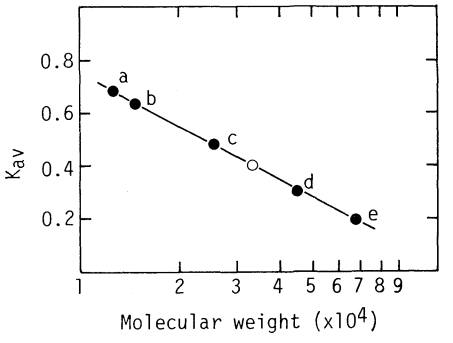

FIG. 6. Determination of Molecular Weight of Endo-polygalacturonase from *Kluyveromyces fragilis* IFO 0288 by Sephadex Gel Filtration.

Details of procedure are described in the text. 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.

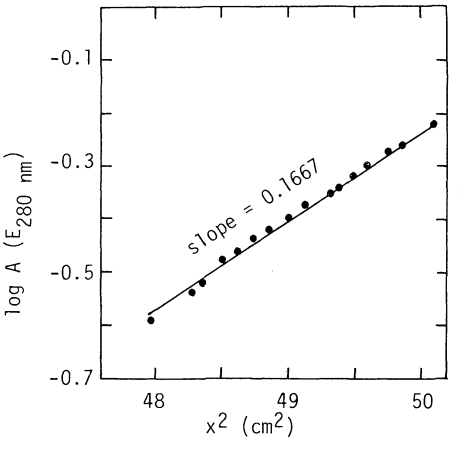

FIG. 7. Plot of Equilibrium Protein Concentration of the Enzyme *vs.* Radial Distance in Ultracentrifugation.

Details of the sedimentation experiment are described in the text.

TABLE III. AMINO ACID COMPOSITION OF ENDO-POLYGALACTURONASE FROM *Kluyveromyces fragilis* IFO 0288

Amino acid	μg/sample[a]	μmol/mg	Number of residues[b]	
			Calculated	Nearest integral
Lysine	171.0	0.46	15.2	15
Histidine	45.1	0.11	3.7	4
Arginine	45.0	0.12	4.0	4
Tryptophan[c]	70.3	0.14	4.5	5
Aspartic acid (Asparagine)	407.4	1.20	39.7	40
Serine	262.0	0.98	32.3	32
Threonine	285.0	0.94	31.1	31
Glutamic acid (Glutamine)	115.6	0.31	10.1	10
Proline	41.2	0.14	4.6	5
Glycine	172.4	0.90	29.8	30
Alanine	66.0	0.29	9.6	10
Half-cystine[d]	36.5	0.12	3.9	4
Valine	117.1	0.39	13.0	13
Methionine	11.0	0.03	1.0	1
Isoleucine	130.4	0.39	12.9	13
Leucine	112.1	0.34	11.1	11
Tyrosine	56.2	0.12	4.1	4
Phenylalanine	87.7	0.21	6.8	7
Ammonia	117.0			
Sugar (as mannose)	83.8	0.18	5.9	6
Recovery: 95.8%				

[a] Crystalline enzyme (2.54 mg) was used in the analysis.
[b] Calculated based on a molecular weight of 33,000.
[c] Determined spectrophotometrically by the method of Edelhoch.[17]
[d] Determined by the method of Crestfield *et al.*[16]

was determined with the hydrolyzates of the enzyme obtained by 24-, 48-, and 72-hr hydrolysis at 105°C, and the amino acid content was estimated by extrapolation of the amino acid content determined to zero time hydrolysis. The results are presented in Table III. The chromatogram from the amino acid analyzer showed no unusual peaks, and the enzyme contains glycine, aspartic acid, serine, and threonine at relatively high levels. In separate experiments, the enzyme was observed to contain 3.2% sugar as mannose.

Isoelectric point of F II

The isoelectric point of the enzyme was determined by isoelectric electrophoresis in Ampholine–polyacrylamide gel. The mobility on the gel was plotted with marker proteins, as shown in Fig. 8, and the isoelectric point was determined to be pH 5.6.

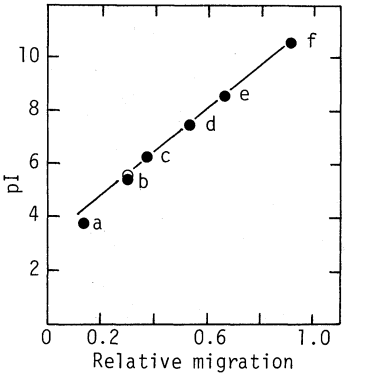

FIG. 8. Determination of Isoelectric Point of Endo-polygalacturonase from *Kluyveromyces fragilis* IFO 0288 by Isoelectric Focusing.

Conditions of electrofocusing are described in the text. pI marker proteins: a, horse cytochrome *c* (pI 3.9); b, cytochrome *c'* from *Rhodospirillum rubrum* (pI 5.6); c, cytochrome c_2 from *Rhodospirillum rubrum* (pI 6.2); d, horse myoglobin (pI 7.6); e, lentil lectin (pI 8.65); f, cytochrome *c* from *Rhodospirillum rubrum* (pI 10.6). Open circle indicates migration position of the enzyme.

Enzyme properties of F II

Pectic acid-degrading activity. The present enzyme had hydrolyzed 3% of the glycosidic bonds of pectic acid when the viscosity of the reaction mixture was decreased by 50%, as shown in Fig. 9.

Protopectinase activity. The present enzyme catalyzed the degradation of protopectin from various plant tissues and released pectic substances (Table IV).

In Table V are listed some properties of pectin released from *Citrus unshiu* peel.

Effects of pH on activity and stability of the enzyme. The pH optimum for the enzyme activity was observed to be around pH 5.0 (Fig. 10(a)), and it was stable in the pH range from 4 to 7 (Fig. 10(b)).

Effects of temperature on activity and stability of the enzyme. The enzyme was stable up to 30°C, but lost 50% its activity at 55°C and lost its activity completely at 70°C, with 30 min treatment (Fig. 11(b)). The temperature optimum was observed to be around 55°C (Fig. 11(a)).

Effects of various inhibitors on enzyme activity. The effects of some enzyme inhibitors on

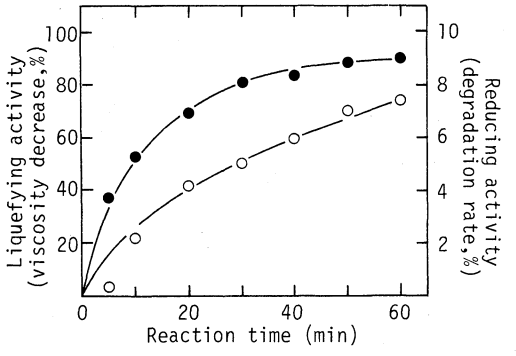

FIG. 9. Action of Endo-polygalacturonase from *Kluyveromyces fragilis* IFO 0288 on Pectic Acid.

Reactions were performed under the standard assay conditions. —●—, liquefying activity; —○—, reducing activity.

TABLE IV. LIBERATION OF PECTIN FROM VARIOUS PROTOPECTINS BY ENDO-POLYGALACTURONASE FROM *Kluyveromyces fragilis* IFO 0288

Origin of protopectin	Pectin liberated (mg/g protopectin)	Yield[a] (%)
Citrus unshiu peel	63	63
Burdock	136	91
Radish	143	99
Watermelon peel	216	98
Carrot	146	73

[a] Ratio of pectin liberated to whole pectin substance in protopectin. Whole pectin substance was determined by the method described by Stoddart *et al.*[31]

The reaction was performed in a reaction mixture containing 1 g protopectin and 120 units enzyme in 0.02 M acetate buffer, pH 5.0, in a total volume of 250 ml, with incubation for 30 min at 37°C.

TABLE V. SOME PROPERTIES OF PECTIN FROM *Citrus unshiu* PEEL LIBERATED BY ENDO-POLYGALACTURONASE FROM *Kluyveromyces fragilis* IFO 0288

Relative viscosity of 0.1% solution	2.28
Methoxyl groups (%)	12.1
Esterified carboxyl groups (%)	72.3
Galacturonic acids (%)	92.9
Neutral sugars (%)	71.
pH of 0.5% solution	4.5
Molecular weight[a]	188,000

[a] Determined by the method described by Smit and Bryant.[32]

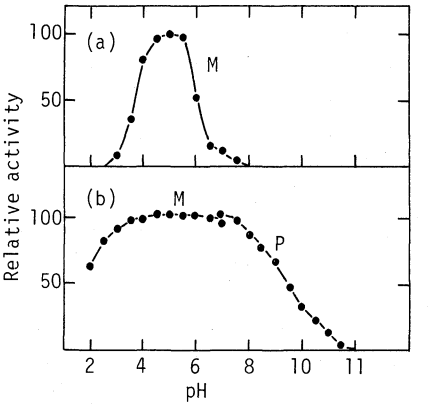

FIG. 10. Effect of pH on Activity and Stability of Endo-polygalacturonase from *Kluyveromyces fragilis* IFO 0288.

(a) Optimum pH. Enzyme activity was assayed in 0.02 M McIlvaine buffer. (b) pH-stability. The enzyme (50 units/ml) in 0.02 M McIlvaine buffer (M, pH 3.0~7.0) or 0.02 M KH_2PO_4–Na_2HPO_4 buffer (P, pH 7.0~11) containing 50 μg/ml bovine serum albumin was incubated at 37°C for 30 min, and remaining activities were determined under the standard assay conditions.

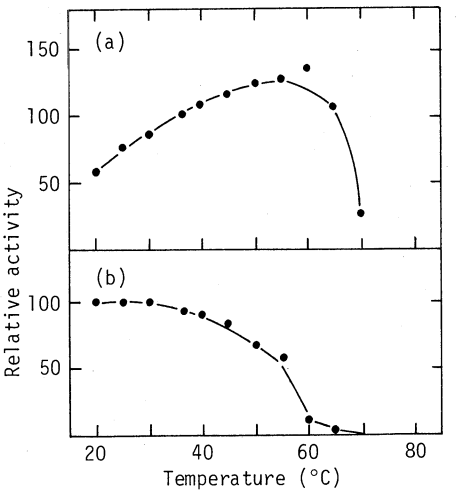

FIG. 11. Effects of Temperature on Enzyme Activity and Stability.

(a) Optimum temperature. The enzyme activities were assayed at various temperatures (20 to 70°C) under the standard assay conditions. (b) Thermo-stability. The enzyme (20 units/ml) in 0.02 M McIlvaine buffer, pH 5.0, containing 50 μg/ml bovine serum albumin was kept for 30 min at various temperatures (20 to 70°C), and the remaining activities were assayed under the standard assay conditions.

TABLE VI. EFFECTS OF INHIBITORS ON ENDO-POLYGALACTURONASE FROM *Kluyveromyces fragilis* IFO 0288

Purified enzyme solution was preincubated for 5 min in 2 mM acetate buffer, pH 5.0, containing the indicated inhibitor, and then the substrate was added followed by measurement of enzyme activity.

Compound	Concentration (mM)	Inhibition (%)
None	—	0
$HgCl_2$	0.1	0
$HgCl_2$	1	78
$Hg_2(CH_3COO)_2$	0.01	44
$Hg_2(CH_3COO)_2$	0.1	99
HgO	0.01	4
HgO	0.1	97
p-CMB[a]	0.05	9
p-CMB	0.1	56
p-Chloromercuriphenyl sulfonic acid	0.1	4
Mersalyl acid	0.1	−9
$AgNO_3$	1	49
$BaCl_2$	1	49
$CaCl_2$	1	41
$PbCl_2$	0.1	−23
$PbCl_2$	0.5	58
$PbCl_2$	1	99

[a] *p*-Chloromercuribenzoic acid.

the enzyme activity were determined at pH 5.0. As shown in Table VI, the enzyme activity was inhibited by such metal ions as Hg^+, Hg^{2+}, Ag^{2+}, Ba^{2+}, Ca^{2+}, and Pb^{2+}. The enzyme activity was not affected by such chelating agents as EDTA, α,α'-dipyridyl, or *o*-phenanthroline, nor by thiol reagents such as *N*-ethylmaleimide and monoiodoacetic acid.

DISCUSSION

There are several reports concerning endo-polygalacturonases from *Kluyveromyces fragilis* (*Saccharomyces fragilis*).[3,4,20~26] In the earlier works on an endo-polygalacturonase of *Kluyveromyces fragilis*, Phaff and Demain[3] isolated the enzyme in a single form, and Lim *et al.*[4] obtained three enzymes from the culture filtrate of a strain of *K. fragilis*, but their detailed properties have not been reported.

In the present study, we found that *K. fragilis* IFO 0288 produces at least four endo-polygalacturonases, having different molecular weights, in its culture broth. The smallest was isolated in crystalline form. *K. fragilis*' three endo-polygalacturonases (enzymes I, II, and III) obtained by Lim *et al.* have different pIs and molecular weights (enzyme I: pI 6.1, M_w 46,000; enzyme II: pI 6.1, M_w 50,000; enzyme III: pI 5.8, 30,000). Our enzyme has a pI around 5.6 and molecular weight around 33,000, and those values are close to enzyme III. However, we determined the molecular weight by a different method from Lim *et al.*: Our enzyme is a glycoprotein, and as SDS-polyacrylamide gelelectrophoresis seems not to be a suitable method for the determination of the molecular weights of glycoproteins,[27] we determined it by Sephadex G-75 column chromatography and a sedimentation equilibrium method, whereas Lim *et al.* used SDS-polyacrylamide gel electrophoresis. The molecular weight of the present enzyme determined by the same method as that of

IM *et al.* is 40,000, which is different from enzyme III. Thus, our enzyme is different from those isolated by Lim *et al.* in molecular weight.

Concerning the hydrolysis of pectic acid by endo-polygalacturonases, it is known that the degree of hydrolysis of pectic acid for a 50% viscosity decrease of the reaction mixture varies according to the enzyme source.[28,29] Three endo-polygalacturonases from *K. fragilis* obtained by Lim *et al.* were reported to hydrolyze 6% of the glycosidic bonds of polygalacturonic acid when the viscosity of the reaction mixture was decreased by 50%, whereas the present enzyme hydrolyzes around 3% of the glycosidic bonds when the viscosity of the reaction mixture is decreased by 50%.

We found that the productivity of pectolytic enzymes in the genus *Kluyveromyces* varies depend on the strain (Table I). From these results, it is supposed that in this genus not only the productivity of pectolytic enzymes but also the properties of the pectolytic enzymes vary depending on the strain.

Previously, we found protopectin-solubilizing enzymes (tentatively called protopectinases) which have endo-polygalacturonase activity, and isolated them from culture filtrates of *Trichosporon penicillatum* and *Galactomyces reessii* (called S-ezyme and L-enzyme, respectively).[5,8,10] The present enzyme also catalyzes the release of pectin from various protopectins, and is confirmed to be a protopetinase.

Protopectinases are enzymes attacking plant tissues and injuring plants. Garibaldi and Bateman isolated three forms of pectate transeliminase from *Erwinia chrysanthemi* and found that two of them, which were basic proteins, macerated plant tissues, whereas the other, which was an acidic protein, did not.[30] The facts suggest that the damage of plant cell walls caused by pectolytic enzymes may be influenced by their basic properties. The endo-polygalacturonases isolated as protopectinases (S- and L-enzyme) are basic proteins,[5,10] whereas the present enzyme is an acidic protein, and they seem to be favorable materials to investigate the mechanisms of protopectinase activity of endo-polygalacturonases. The studies on comparison of the S- and L-enzymes and the present enzyme are proceeding and the details will be published elsewhere.

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