Purification and Some Properties of Three Components of Endo–Polygalacturonase from Irpex lacteus (Polyporus tulipiferae)

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Three components of endo–polygalacturonase (endo–PG) were purified by gel filtration and ion exchange chromatography from Driselase, a commercial enzyme preparation from *Irpex lacteus* (*polyporus tulipiferae*). The purified enzymes were almost homogeneous as judged through polyacrylamide gel disc electrophoresis. The three pure enzymes were designated as endo–PG I, II, and III.

Endo–PG I, II, and III were purified approximately 13–, 93–, and 118–fold from the starting solution, and their molecular weights were estimated to be 16,000, 32,000, and 70,000 by gel filtration on Bio-gel P–100, respectively. The three enzymes show similar pH and thermal optima for pectic acid hydrolysis, while they are different from one another in pH and thermal stability. The activity of endo–PG I is slightly stimulated by Fe^{++}, Mg^{++}, or Mn^{++}, whereas those of the other are somewhat inhibited by the same ions. The three enzymes are completely inhibited by Hg^{++}. All the three enzymes show macerating activity toward potato tissue. The enzymes rapidly decrease the viscosity of solutions of pectic acid and pectin, but give little release of reducing groups. The enzymes each also attack galacturonic acid oligomers at random, except di–galacturonic acid. These results indicate that each of the three endo–PGs is a typical endo–polygalacturonase.

Introduction

It has been reported that a commercial cellulase preparation, Driselase, obtained from *Irpex lacteus* (*Polyporus tulipiferae*) can easily macerate plant tissues such as patato tuber and carrot root,[1] and that pectic enzymes in this fungus, probably endo–PG (endo–polygalacturonase), is mainly responsible for the macerating activity.[2]

However, no studies have so far been reported on enzymatic properties of

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endo–PG (EC. 3. 2. 1. 15) from *Irpex lacteus* in its purified state, although the cellulases from this fungus have been investigated in detail.[3-6]

In dealing with fractionation of the polygalacturonase from this fungus using Driselase, we noticed that the fungus produced at least several endo–PG components differing from one another in chromatographic behavior.

This paper describes the purification of three major endo–PG components found in preparation of *Irpex lacteus* and some enzymatic properties of these purified endo–PGs.

Materials and Methods

Enzyme Source. Driselase, a commercial enzyme preparation from *Irpex lacteus*, supplied by Kyowa Hakko Co. , Ltd. , was used as the starting material for purification of endo–polygalacturonases.

Chemicals and Reagents. Pectic acid and Pectin were purchased from Nakarai Chemicals Ltd. They were washed three times with 70% ethanol to remove soluble sugar, and then treated with absolute ethanol and ether. The degree of esterification of pectin was 72–75%. For most experiments, pectic acid was dissolved by neutralization with NaOH and used without additional treatment. Sodium carboxymethyl cellulose (CMC, DS = 0.63) was obtained from Daiichi Industrial Pharmaceutical Co. , and *p*–Nitrophenyl β–D–glucoside (PNPG) from Nakarai Chemical Co. DEAE–Sephadex A–50 and CM–Sephadex C–50 were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden), and Bio–gel P–60 and P–100 from Bio–Rad Laboratories (Calif. , U. S. A.). Di–, tri–, and tetra–galacturonic acids were isolated by paper chromatography from the hydrolyzate of pectic acid. The other chemicals used were of reagent grade, commercially available.

Enzyme Assays. *a) Pectic Acid–Saccharifying Activity.* The reaction mixture (2 ml), consisting of 0.25% pectic acid, 0.05 M sodium acetate buffer (pH 5.0), and enzyme, was incubated at 30°C for a given period (10 min in most cases). The reducing power produced in the reaction mixture was determined by the method of Somogyi[7] and Nelson.[8] One unit of endo–polygalacturonase (endo–PG) is defined as the amount of enzyme which produces a reducing power from the substrate equivalent to 1 μmol of galacturonic acid per min.

b) Pectin–Saccharifying Activity. The hypoiodate method of Hatanaka[9] was employed for determination of the reducing power of pectin solution, under the same conditions as used for the pectic acid–saccharifying activity.

c) CMC–Saccharifying Activity. The assay was carried out under the same conditions as used for the pectic acid–saccharifying activity, except for the use of 1% CMC in place of 1% pectic acid.

d) β–Glucosidase Activity. The reaction mixture consisted of 0.1 ml of 0.048 M

PNPG, 0.2 ml of 0.1 M sodium acetate buffer (pH 5.0), and 0.1 ml of enzyme solution. After incubation at 30°C for 10 min, 5 ml of 1% Na_2CO_3 was added to the mixture, and the *p*–nitrophenol liberated was estimated from the absorbance at 420 nm.

e) Pectin–and Pectic Acid–Liquefying Activities. The reaction mixture consisting of 2 ml of 1% substrate solution, 3 ml of 0.1 M sodium acetate buffer (pH 5.0), and 1 ml of enzyme solution, was incubated in an Ostwald viscometer at 30°C, and the flow time (seconds) was measured to estimate the relative viscosity (η_{rel}) of mixture; the activity is expressed as decrease (%) in η_{rel}.

f) Macerating Activity. Maceration of plant tissues was determined according to the method of Suzuki *et al.*[10] Two potato discs (1.3×0.5 cm) were placed in an L–shaped tube containing 2 ml of 0.1 M sodium acetate buffer (pH 5.0), 1 ml of water, 1 ml (105 μg) of enzyme solution, and 0.2 ml of toluene. The macerating activity of potato disc is defined by maceration value expressed in %; maceration value $=(Wc - Wt)/Wo$, where Wo is the dry weight of untreated disc and Wt and Wc are the dry weights of discs remaining after incubation at 30°C for 5 hr in the presence and absence of enzyme solution, respectively.

Determination of Protein. The protein in a reaction mixture was determined by the method of Lowry *et al.*[11] or from the absorbance at 280 nm, using crystalline bovine serum albumin (from Miles Laboratories, Inc.) as a standard.

Disc Electrophoresis on Polyacrylamide Gel. Polyacrylamide gel electrophoresis was carried out in glycine-Tris buffer at pH 8.9 as determined according to the procedure described by Ornstein[12] and Davis.[13] A sample containing about 10 μg of protein was applied on the top of the gel and then subjected to electrophoresis at 2.5 mA/gel. Gels were stained for enzyme protein with 0.05% coomassie brilliant blue R250.

Paper Chromatography. Hydrolysis products produced by the enzymes were detected by paper chromatography. After an appropriate period of incubation, aliquots of enzymatic hydrolyzate of substrates and solution of authentic sugars used as standard were spotted individually on a Whatman No.1 filter paper and chromatograms were obtained after development by the descending technique with *n*–butanol: acetic acid: water (4:3:3, v/v) at room temperature for an appropriate period. Spots of the products were detected by the dipping procedure using silver nitrate reagent.[14]

Results

Purification of Endo–polygalacturonase Components.

Step 1. First DEAE–Sephadex A–50 Column Chromatography. A crude enzyme solution obtained from Driselase powder (50 g) was applied to a DEAE–Sephadex
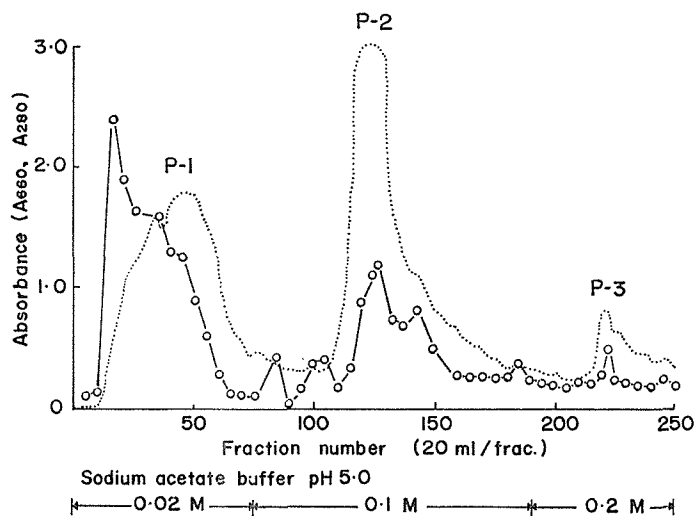
Fig. 1 Elution pattern of the crude enzyme preparation from a
DEAE–Sephadex A–50 column.

—○—, pectic acid–saccharifying activity (10 min incuba-
tion, A_{660}); ••••, protein (A_{280}).
Column, 5.0×40 cm; flow rate, 24 ml/h; fraction size, 20 ml.

column (acetate form) equilibrated previousely with 0.02 M sodium acetate buffer
(pH 5.0). Elution was carried out stepwise with 0.02, 0.1, and 0.2 M sodium acetate
buffers at the same pH. The results are shown in Fig. 1. Three fractions of
pectic acid–saccharifying activity from P–1 to P–3 were obtained, which overlapped
the main peaks of protein. P–1 and P–2, which are more active toward pectic
acid and contain the greater amount of protein of the three, were subjected to
further purification. These two fractions were each brought to 80% saturation by
addition of solid ammonium sulfate, and allowed to stand overnight. The resulting
precipitates of P–1 and P–2 were each collected by centrifugation and then dissolved
in a small volume of water, dialyzed, and lyophilized.

Purification of P–1 Component.

Step 2. CM–Sephadex C–50 Column Chromatography. An aqueous solution of
P–1 was applied to a CM–Sephadex C–50 column, previously equilibrated with
0.02 M sodium acetate buffer (pH 5.0). The column was eluted stepwise with 0.02
and 0.1 M sodium acetate buffers, and then with 0.1 M sodium acetate buffer
containing 0.1 M NaCl at the same pH. As shown in Fig. 2, P–1 gives three
protein fractions (P–1–1 to –3) and the activity is recovered in the two main
fractions, P–1–2 and P–1–3, which possess almost none of CMCase or β–glucosidase
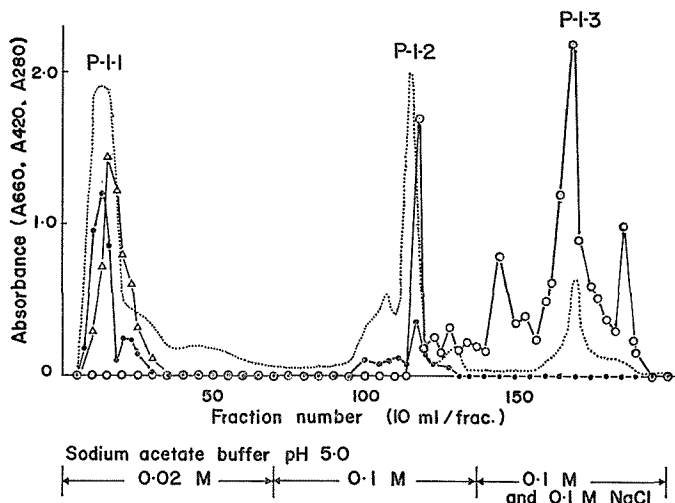
Fig. 2 Elution pattern of P–1 on a CM–Sephadex C–50 column.
—○—, pectic acid–saccharifying activity (15 min incubation,
A_{660}) of eluates diluted 5–fold; —●—, CMC–saccharifying
activity (15 min incubation, A_{660}) of eluates diluted 5–fold;
—△—, β–glucosidase activity (15 min incubation, A_{420});
····, protein (A_{280}). Column, 2.0×45 cm; flow rate, 17 ml/h;
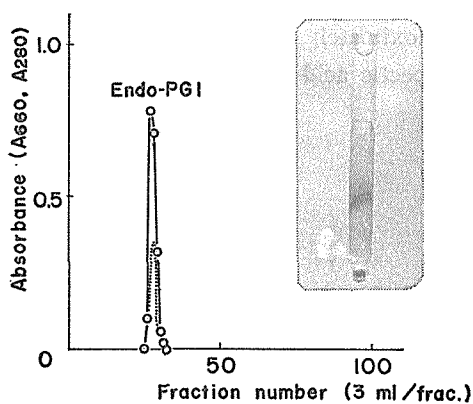fraction size, 10 ml.

Fig. 3 Elution pattern of P–1–2–2 on a Bio-gel P–100 column
and disc electrophoresis of endo–PG I on polyacrylamide
gel.
—○—, pectic acid–saccharifying activity (20 min
incubation, A_{660}) of eluates diluted 2–fold; ····,
protein (A_{280}). Column, 1.0×150 cm; flow rate,
2 ml/h; fraction size, 3 ml. Disc electrophoresis of
the purified enzyme (endo–PG I) was carried out by
the method of Ornstein[12] and Davis,[13] as described
in the "Materials and Methods" section.

activity. These two fractions were each concentrated in a collodion bag and then lyophilized.

Purification of P-1-2 Component.

Step 3a. Bio-gel P-60 Gel Filtration. The P-1-2 fraction was applied to a Bio-gel P-60 column (2 × 110 cm) equilibrated with 0.1 M sodium acetate buffer (pH 5.0) and eluted with the same buffer. The pectic acid–saccharifying activity was separated into three fractions (P-1-2-1 to -3), of which P-1-2-2 showed a high pectic acid–saccharifying activity and was almost free from CMCase. This fraction was concentrated in the same way as described above.

Step 4a. Bio-gel P-100 Gel Filtration. The P-1-2-2 fraction gave no other peaks on this gel filtration, which was carried out in the same way as described above, except for the use of Bio-gel P-100 in the place of Bio-gel P-60 and for the column size. As shown in Fig. 3, a single peak fraction of pectic acid–saccharifying activity coincided entirely with the protein peak. This fraction was then lyophilized after concentration and named endo-PG I.

Purification of P-1-3 Component.

Step 3b. Bio-gel P-100 Gel Filtration. The P-1-3 fraction obtained in Step 2 was subjected to gel filtration on a Bio-gel P-100 column in the same manner as described in Step 4a, except for the column size. As shown in Fig. 4, the eluates were separated into approximately three fractions (P-1-3-1 to -3), of which P-1-3-2 showed the highest pectic acid–saccharifying activity and was present in the
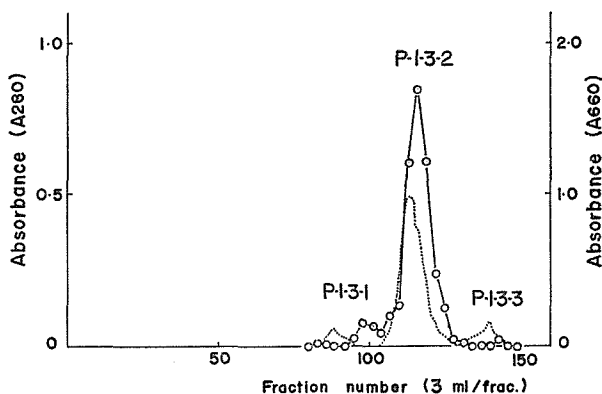

Fig. 4 Elution pattern of P-1-3 on a Bio-gel P-100 column.

—○—, pectic acid–saccharifying activity (6 min incubation, A_{660}); ••••, protein (A_{280}). Column, 3.0 × 150 cm; flow rate, 12 ml/h; fraction size, 3 ml.
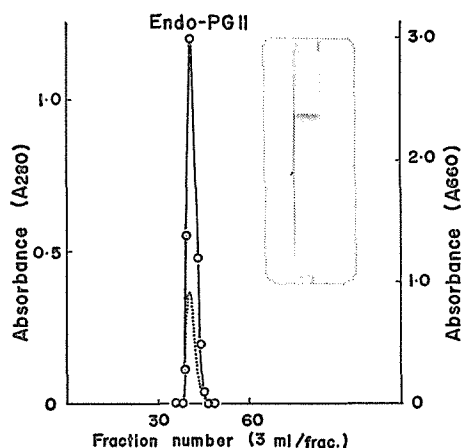
Fig. 5 Elution pattern of P–1–3–2–2 on a Bio-gel P–60 column and disc electrophoresis of endo–PG II on polyacrylamide gel.

—○—, pectic acid–saccharifying activity (10 min incubation, A_{660}) of eluates diluted 5–fold; ••••, protein (A_{280}). Column, 2.0 × 105 cm; flow rate, 3 ml/h; fraction size, 3 ml. Disc electrophoresis of the purified enzyme (end–PG II) was carried out under the same conditions as in Fig. 3.

largest amount. The fraction was lyophilized after concentration in the same way as described before.

Step 4b. Second DEAE–Sephadex A–50 Column Chromatography. The P–1–3–2 fraction was again subjected to DEAE–Sephadex A–50 column chromatography under the same conditions with the first DEAE Sephadex A–50 chromatography, except for the column size (2 × 45 cm). Three fractions (P–1–3–2–1 to –3) were obtained, of which P–1–3–2–2 showed the highest activity. This fraction was concentrated in the same way as described before.

Step 5b. Bio–gel P–60 Gel Filtration. The P–1–3–2–2 fraction was subjected to gel filtration on a Bio–gel P–60 column in the same way as described in Step 3a, except for the column size. As shown in Fig. 5, a single peak fraction of pectic acid–saccharifying activity entirely coinciding with the protein was obtained and named endo–PG II after concentration.

Purification of P–2 Component

Step 2c. CM–Sephadex C–50 Column Chromatography. An aqueous solution of P–2 obtained in Step 1 was applied to a CM–Sephadex C–50 column in the same manner as described in Step 2. As shown in Fig. 6, three fractions (P–2–1 to –3) were obtained. The P–2–2 fraction which showed the highest activity of the three fractions was concentrated and lyophilized.
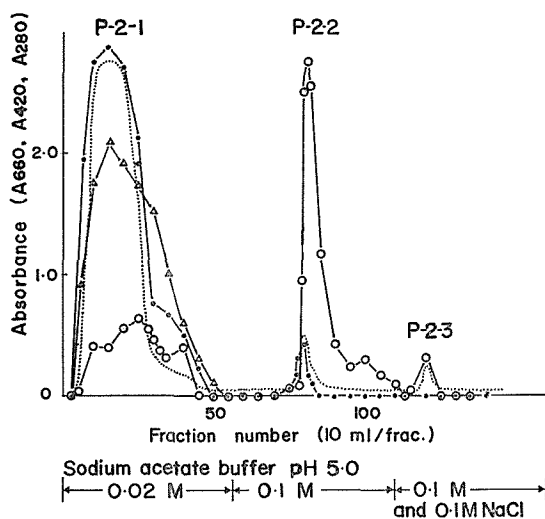
Fig. 6   Elution pattern of P–2 on a CM–Sephadex C–50 column.

   —◯—, pectic acid–saccharifying activity (10 min incubation, A_{660}); —●—, CMC–saccharifying activity (10 min incubation, A_{660}); —△—, β–glucosidase activity (15 min incubation, A_{420}); ····, protein (A_{280}). Column, 2.0×20 cm; flow rate, 20 ml/h; fraction size, 10 ml.
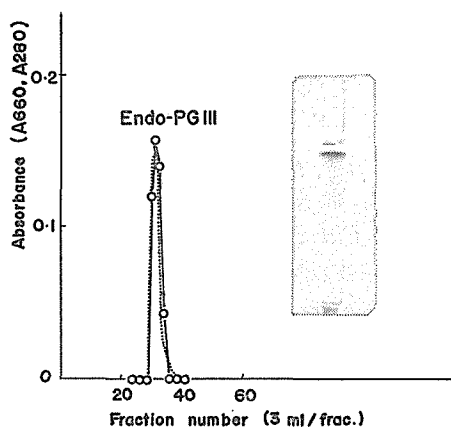

Fig. 7   Elution pattern of P–2–2–1 on a Bio–gel P–60 column and disc electrophoresis of endo–PG III on polyacrylamide gel.

   —◯—, pectic acid–saccharifying activity (20 min incubation, A_{660}) of eluates diluted 5–fold; ····, protein (A_{280}). Column, 2.0×107 cm; flow rate, 2 ml/h; fraction size, 3 ml. Disc electrophoresis of the purified enzyme (end–PG III) was carried out under the same conditions as in Fig. 3.

Step 3c. Second CM–Sephadex C–50 Column Chromatography. In an attempt to remove the accompanying CMCase, the P–2–2 fraction was again subjected to CM–Sephadex C–50 column chromatography in the same way as described in Step 2, except for the column size (2×26 cm). A single fraction of pectic acid–saccharifying activity, almost free from CMCase, was obtained.

Step 4c. Bio–gel P–60 Gel Filtration. The concentrated fraction (P–2–2–1) obtained above was further subjected to gel filtration on a Bio–gel P–60 column, in the same way as described in Step 5b. As shown in Fig. 7, the pectic acid–saccharifying activity coincided fully with the protein peak, suggesting its homogeneity. After concentration, it was named endo–PG III.

The final preparations of endo–PG I, II, and III each gave a single protein band on disc gel electrophoresis at pH 8.9 (Figs. 3, 5, and 7). Tables 1, 2, and 3 summarize the specific activities and recoveries during the purification procedure for the three endo–PGs from the starting solutions. The specific activities of endo–PG I, II, and III toward pectic acid were increased 13–, 93–, and 118–fold

Table 1 Recoveries and activities of endo–PG I during the
purification procedure

Purification step	Volume (ml)	Protein (mg)	Total units (U)	Specific activity[a] (U/mg)	Recovery (%)
Starting solution	400	6800	94.34	0.014	(100)
DEAE–Sephadex A–50 (P–1)	960	1488	49.36	0.033	52.3
CM–Sephadex C–50 (P–1–2)	39.6	21.8	1.93	0.089	2.04
Bio–gel P–60 (P–1–2–2)	12.0	6.1	0.78	0.127	0.82
Bio–gel P–100 (endo–PG I)	4.5	1.8	0.34	0.184	0.36

a) Specific activity is defined as activity units/mg of enzyme protein. One unit is the pectic acid–saccharifying activity which produces a reducing power equivalent to 1.0 μmol of galacturonic acid in one min under the reaction conditions employed in the present work.

Table 2 Recoveries and activities of endo–PG II during the
purification procedure

Purification step	Volume (ml)	Protein (mg)	Total units (U)	Specific activity[a] (U/mg)	Recovery (%)
Starting solution	400	6800	94.34	0.014	(100)
1st DEAE–Sephadex A–50 (P–1)	960	1488	49.36	0.033	52.3
CM–Sephadex C–50 (P–1–3)	100	17	12.03	0.708	12.8
Bio–gel P–100 (P–1–3–2)	48	12	9.37	0.781	9.9
2nd DEAE–Sephadex A–50 (P–1–3–2–2)	36	4.3	3.49	0.808	3.7
Bio–gel P–100 (endo–PG II)	24	2.5	3.27	1.297	3.5

a) Specific activity and one unit are the same as in Table 1.

Table 3 Recoveries and activities of endo–PG III during the
purification procedure

Purification step	Volume (ml)	Protein (mg)	Total units (U)	Specific activity[a] (U/mg)	Recovery (%)
Starting solution	1600	27200	380.8	0.014	(100)
1st DEAE–Sephadex A–50 (P–2)	4320	9504	54.1	0.024	14.2
1st CM–Sephadex C–50 (P–2–2)	250	13.8	7.78	0.566	2.04
2nd CM–Sephadex C–50 (P–2–2–1)	150	4.5	6.23	1.384	1.64
Bio–gel P–60 (endo–PG III)	18	1.2	1.78	1.651	0.47

[a] Specific activity and one unit are the same as in Table 1.
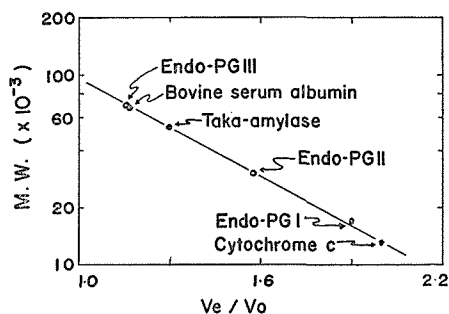

Fig. 8 Determination of the molecular weights of three
purified enzymes, endo–PG I, II, and III, by Bio–gel P–100
gel filtration.

V_e and V_o are the elution volume and void volume,
respectively. Column, 1.0×150 cm; flow rate, 3 ml/h;
fraction size, 3 ml; elution buffer, 0.1 M sodium acetate
buffer, pH 5.0.

over those of the starting solutions, respectively.

Molecular Weight by Gel Filtration. The three endo–PGs were each applied
to a Bio–gel P–100 gel column equilibrated with 0.1 M acetate buffer (pH 5.0).
Elution was carried out with the same buffer. Bovine serum albumin (M. W.
67,000), Taka amylase (M. W. 53,000), and cytochrome c (M. W. 13,000) were
used as standards. The void volume was determined using blue dextran 2,000.
The molecular weights of endo–PG I, II, and III were estimated to be 16,000,
32,000, and 70,000, respectively (Fig. 8).

Effect of Metal Ions. Table 4 shows the effect of various metal ions on the
enzyme activities. The activity of endo–PG I was slightly stimulated by Fe^{++},
Mg^{++}, or Mn^{++}, whereas those of the other enzymes were somewhat inhibited
by the same ions. Ag^+, Co^{++}, Cu^{++}, or Pb^{++} inhibited the enzyme activities to

Table 4 Effect of metal ions on enzyme activity[a)]

Metal ion	Concentration (mM)	Residual activity (%)		
		Endo–PG I	Endo–PG II	Endo–PG III
None	——	100	100	100
AgNO$_3$	1.58	71.9	73.5	64.2
BaCl$_2$	1.58	101.0	93.3	65.8
CoCl$_2$	1.58	79.4	57.1	47.6
CuSO$_4$	1.58	59.0	50.3	53.3
FeSO$_4$	1.58	127.8	91.0	67.1
HgCl$_2$	1.58	0	0	0
KCl	1.58	102.5	96.1	97.0
MgSO$_4$	1.58	113.0	68.0	72.3
MnSO$_4$	1.58	124.3	34.9	35.6
NaCl	1.58	103.4	92.2	97.6
Pb(CH$_3$COO)$_2$	1.58	71.6	70.9	64.6
ZnCl$_2$	1.58	96.1	89.2	93.7

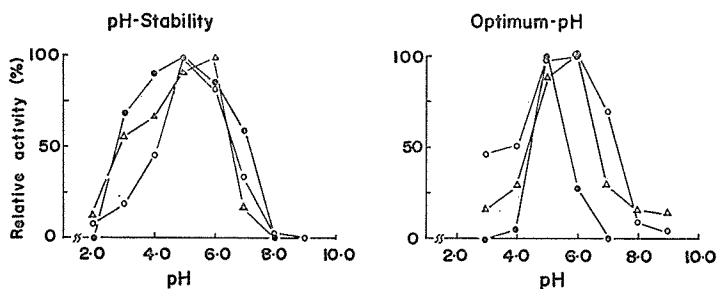a) Enzyme activity was determined as the pectic acid–saccharifying activity.

Fig. 9 pH stability and optimum pH curves for the pectic acid–saccharifying activities of three purified enzymes. —●—, endo–PG I; —○—, endo–PG II; —△—, endo–PG III.

some degree. The three enzymes were completely inhibited by Hg^{++}.

Effects of pH and Temperature on Enzyme Activity and Stability. The pH dependence of the purified enzyme activities was studied under the standard assay conditions with Britton-Robinson's wide range buffers (pH 2 to 10). As shown in Fig. 9, the optimum pH of endo–PG I is 5.0, while those of endo–PG II and III are 5.0–6.0. The effect of pH on the stability of the enzymes was examined after preincubation for 24 hr at 30°C at various pH values. Remaining activities were then assayed at pH 5.0. The endo–PG I activity was stable in the relatively wide pH range from 3.5 to 6.5, while the endo–PG II and III activities were stable only in the narrow range of pH 4.5 to 6.0. The effect of temperature on the
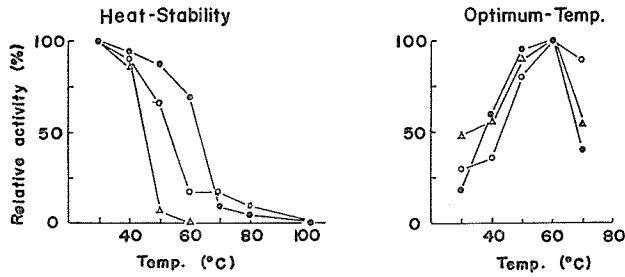
Fig. 10   Heat stability and optimum temperature curves for the pectic acid–saccharifying activities of three purified enzymes.

—●—, endo–PG I;   —○—, endo–PG II;   —△—, endo–PG III.

stability was studied by heating the enzymes at various temperatures for 20 min in acetate buffer (pH 5.0), cooling them quickly in ice water, and determining residual activities at 30°C by measuring the increase in the amount of reducing sugar. The optimum temperature and heat stability of each enzyme activity are shown in Fig. 10. The three enzyme activities each showed maxima at 60°C. The enzyme activities are stable at temperatures lower than 40°C but endo–PG III loses its activity remarkably at 50°C under the conditions employed.

   Macerating Activity.   The three purified enzymes each had macerating activity on potato disc. The macerating activities of endo–PG I, II, and III were 12.8, 22.4, and 16.2, respectively.
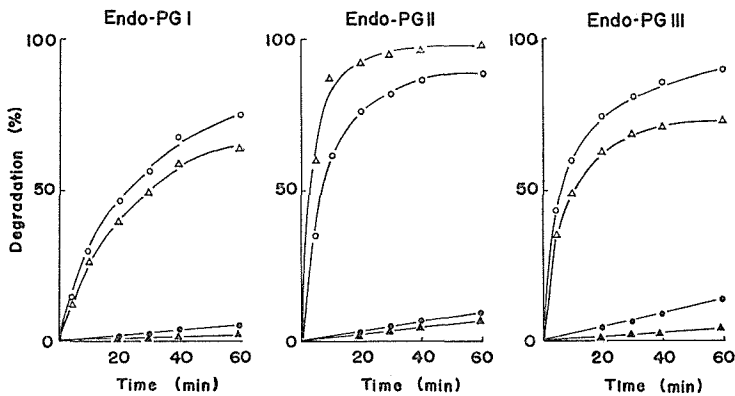

Fig. 11   Degradation of pectic acid and pectin by three purified enzymes.

Viscosity reduction (%): —○—, pectic acid; —△—, pectin.
Hydrolysis of glycosidic bond (%): —●—, pectic acid; ···▲···, pectin.

Actions on Pectin and Pectic Acid.   The three endo–PGs rapidly reduced the viscosity of pectin and pectic acid solutions at pH 5.0, although these enzymes were different from one another in the rate of reducing activity toward pectin and pectic acid. When the activities of the enzymes were followed by measuring the increase in the amount of reducing group, pectin and pectic acid each slowly underwent a hydrolysis, although the rates of pectic acid hydrolysis by these enzymes were all somewhat high as compared with those of pectin hydrolysis (Fig. 11).

Hydrolysis Products from Pectic Acid.   Hydrolysis of pectic acid by the three endo–PGs was first carried out under the standard conditions, and the products were identified by paper chromatography. Fig. 12 shows paper chromatograms in the time-course of hydrolysis of pectic acid by the three endo–PGs. At earlier stages, by endo–PG I it was hydrolyzed into higher galacturonic acids together with low galacturonic acids such as mono– to tetra–galacturonic acid in which di-galacturonic acid appeared to be most dominant.   On the other hand, by endo–PG II and III it was hydrolyzed progressively into higher galacturonic acids but low galacturonic acids were not detected. Higher galacturonic acids gradually disappeared as reaction proceeded, and mono–and di-galacturonic acids remained as the end products.

Hydrolysis of Oligo-galacturonic Acid.   The hydrolysis products from tetra-galacturonic acid by the three endo-PGs were also identified by paper chromato-
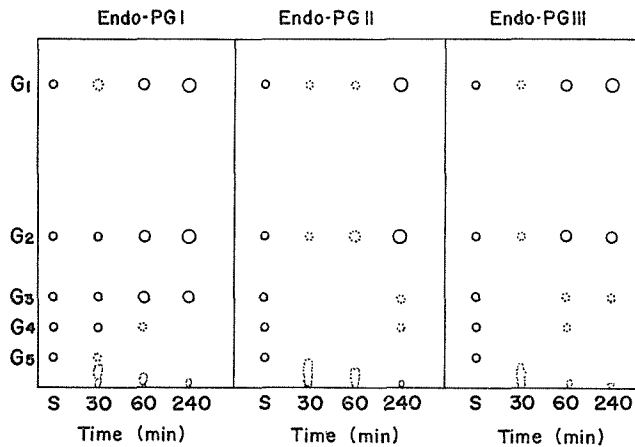

Fig. 12 Paper chromatograms showing products formed in course of hydrolysis of pectic acid by three purified enzymes.
S, standard; G_1, galacturonic acid; G_2, di–galacturonic acid; G_3, tri–galacturonic acid; G_4, tetra–galacturonic acid; G_5, penta-ga-lacturonic acid. Development time, 24 h.
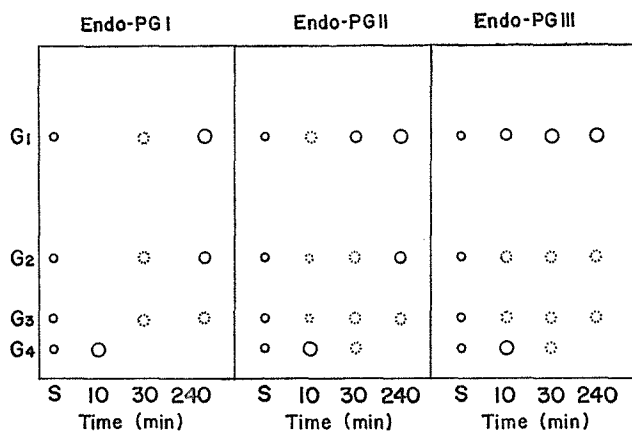
Fig. 13 Paper chromatograms showing products formed in course of hydrolysis of tetra–galacturonic acid by three purified enzymes. The symbols are the same as in Fig. 12. Development time, 24 h.

graphy as shown in Fig. 13. As expected, tetra–galacturonic acid was hydrolyzed into mono–, di–, and tri–galacturonic acids at early stages, and tri–galacturonic acid into mono–and di–galacturonic acids, whereas di–galacturonic acid was not further attacked.

Discussion

In the present study, three endo–polygalacturonase fractions (endo–PG I, II, and III) were obtained from Driselase, a commercial product of *Irpex lacteus* (*polyporus tulipiferae*), by successive gel filtration and chromatography on Sephadex ion exchangers. These endo–PG I, II, and III were purified approximately 13–, 93–, and 118–fold from the initial preparation, respectively. The purified enzymes were almost homogeneous on polyacrylamide disc electrophoresis.

The approximate average molecular weights of the three endo–PGs which were estimated by the gel filtration method, were considerably different from one another (16,000, 32,000, and 70,000 for endo–PG I, II, and III, respectively).

It is known that most PGs have optimal pHs in the weakly acidic region between pH 4.0 and 6.5.[15-18] The three endo–PGs show similar enzymatic properties in pH and thermal optima for pectic acid hydrolysis, while they are different from one another in pH and thermal stability.

Endo[19] presented that only one of three kinds of endo–PG from *Coniothyrium diplodiella* highly purified had macerating activity toward potato disc, the other two having no macerating effect on plant tissues. In the present study, all the

three endo–PGs from *Irpex lacteus*, like those from *Saccharomyces fragilis*,[21] were found to have macerating activity toward potato tissue, although their extents of activity were not remarkably different from one another. This suggests that purified three enzymes attack pectic substances in the natural state without any joint actions with other pectin hydrolases or hemicellulases.

The three endo–PGs of this fungus also cause a rapid decrease in viscosity of pectic acid or pectin solution, while the reducing powers in reaction media increase more slowly; concerning the hydrolysis of pectic acid, endo–PG I, II, and III were found to hydrolyze around 3, 2, and 2.5%, respectively, of the glycosidic bonds of pectic acid when the viscosity of the reaction mixture was reduced by 50%. The degrees of viscosity reduction of pectic acid solution by endo–PG I and III were more than those of pectin solution, while that by endo–PG II was in a reverse relationship. Moreover, the paper chromatographic analysis (Figs. 12 and 13) showed that the enzymes attack at random the inner glycosidic bond of pectic acid including galacturonic olygomers, although their modes of action were slightly different from one another at early stages; products formed from pectic acid and the olygomers, after prolonged incubation by the three endo–PGs, were determined to be mainly mono– and di–galacturonic acids but di–galacturonic acid was not further hydrolyzed. From these results, it is concluded that each of the three enzymes may be classified into a group of typical endo–polygalacturonase.

Thus, the endo–PG of *Irpex lacteus* seems to be composed of various kinds of endo–PG components. The existence of similar multiple components in this enzyme complex has already been reported for *Coniothyrium diplodiella*[20] and *Saccharomyces fragilis*.[21] Some of multiple components in the endo–PG of *Irpex lacteus* may be produced by secondary modification under the action of some factors from an endo–PG component of larger molecular weight to components of smaller molecular weight, as discussed on cellulase components from *Trichoderma viride* by Nakayama *et al.*[22] The investigation on these multiple components in this fungus, however, remains to be done.

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