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Some Properties of Polygalacturonase from *Rhizopus japonicus* IFO 5318

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Polygalacturonase (PG) from solid culture extract of *Rhizopus japonicus* IFO 5318 was purified by DEAE Sephadex A-50, CM Sephadex C-50 and Sephadex G-100 column chromatography. Finally the PG was purified approximately 121 folds based on its activity. The optimum pH and temperature on the PG activity were at pH 4.5 and 45°C, respectively and this enzyme was most stable at pH 7.5 and up to 35°C. The purified PG gives a molecular weight of 44,000 Da and Km value of 5.21 mg/ml. Furthermore, the results of the effect of some metal ions and inhibitors on enzyme activity showed that Zn^{2+}, Mg^{2+}, Cu^{2+}, Ca^{2+}, Mn^{2+} and Al^{3+} enhanced fairly the PG activity (1-9%) but K^{+}, Fe^{2+}, Ni^{2+}, Ba^{2+}, and Na^{2+} inhibited slightly the PG activity (1-6%) while Li^{+} and urea inhibited about 20% of PG activity.

INTRODUCTION

Pectic enzymes originating from microorganisms are industrially important because of uses for jute refining, vegetable purees production, fruit juice processing, etc.. In industrial practice, pectic enzymes are mainly obtained from *Aspergillus* strains and bacterial strains. There have been many observations on the maceration ability of pectic enzymes from *Rhizopus* strains so the strains tend to be highly potent source of PG and this enzyme is able to be applied in the beneficial way (Srivastava et al., 1959). After preliminary selection of pectic enzymes producing *Rhizopus* strains, *Rhizopus japonicus* IFO 5318 was selected as a good pectic enzyme producing strain. The objective of the present work is focused upon the purification and characterization of PG from *Rhizopus japonicus* IFO *5318.*

MATERIALS AND METHODS

Microorganism and media

Rhizopus japonicus IFO 5318 was used as a PG producing strain after preliminary selection. PDA medium was used for slant and solid culture medium in 1000 ml Erlenmeyer flask with cotton plug was composed of 30g wheat bran, lg pectin and 34 ml tap water. The solid medium was autoclaved at 121°C for 20 min.

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Cultivation

Rhizopus japonicus IFO 5318, cultured on a PDA slant for a week, was inoculated onto the solid medium and incubated at 30°C for 2 days.

Enzyme extraction

Enzyme was extracted with 200 ml tap water per flask at 4°C for 24 h. After filtration through celite bed, the filtrate was centrifuged at 4°C and 8,000 rpm. The clear centrifugate obtained was used as starting enzyme solution for further purification.

Ammonium sulfate fractionation

The crude enzyme was precipitated by salting out with 95% saturation of ammonium sulfate. The resultant precipitate was dissolved with small amount of 0.05 M Tris-HCl buffer (pH 7.5) and dialysed against distilled water for overnight at 4°C. The first dialyzate was dialyzed again against 0.05 M Tris-HCl buffer (pH 7.5) for 6 h. The second dialyzate was concentrated by ultrafiltration and then the concentrate was applied onto the column for purification.

DEAE-Sephadex A-50 column chromatography

The concentrated enzyme from the previous step was applied onto the DEAE-Sephadex A-50 column (4 cm in inside diameter and 40 cm in packed hight) equilibrated previously with 0.05 M tris-HCl buffer (pH 7.5) and then eluted with the same buffer. The flow rate of the elution buffer was 30 ml/h and each 14 ml fraction was collected. After fraction number 43, the column was eluted with a linear gradient of 0 to 2 M NaCl in the same buffer (1000 ml of total volume of gradient buffer).

CM-Sephadex C-50 column chromatography

The PG fraction from the previous column step was concentrated by ultrafiltration and then dialysed against distilled water for overnight and continuously dialyzed against 0.05 M phosphate buffer (pH 7.5) for 6 h. The concentrated and dialyzed enzyme was applied onto the CM-Sephadex C-50 column (2.5 cm in inside diameter and 80 cm in packed hight) previously equilibrated with 0.05 M phosphate buffer (pH 6) and eluted with the same buffer (pH 7.5). The flow rate of the buffer was 30ml/h and each 7ml fraction was collected.

Sephadex G-100 column chromatography

The PG fraction from the previous step was further purified by three steps gel filtration. The enzyme was applied onto Sephadex G-100 column chromatography (1. 6 cm in inside diameter and 85 cm in gel packed hight) and eluted with 0.05 M phosphate buffer (pH 7.5) at a 12ml/h flow rate and each 6ml fraction was collected.

Determination of pH optimum and stability

In the case of pH optimum determination, the PG was allowed to react with 1% pectic acid in various pH by using 0.2 M glycine-HCl buffer at the pH range from 2 to 4, 0.2 M acetate buffer from 4 to 5.5, 0.2 M phosphate buffer from 5.5 to 8 and 0.2 M glycine-NaOH buffer from 8.5 to 9.5. For the pH stability, PG activity was

determined by incubating the enzyme at 45°C for 20 min after treating the enzyme solution at the desired pH for 24 h at 4°C, using the same buffer as the previous step.

Temperature optimum and stability

The optimum temperature on activity was determined by allowing the enzyme to react with pectic acid at various temperature. For determination of temperature stability, the PG activity was assayed after incubation at various temperatures for 30 min.

Determination of molecular weight

Molecular weight of the PG enzyme was determined by gel filtration (Andrew 1965). The enzyme was applied onto the Sephadex G-75 column chromatography previously equilibrated with 0.05 M phosphate buffer (pH 7.5) and eluted with the same buffer at a flow rate of 12ml/h. Ovalbumin (chicken), myoglobin (house) and cyanocobalamin (Bio-Rad) were used as standard molecular weight makers.

Michaelis constant (Km)

The Km value was determined by using pectic acid as the substrate. Initial reaction velocity was determined at various concentration of the substrate and the reciprocal of initial reaction rate was plotted against the reciprocal of substrate concentration based on the Lineweaver-Bulk plot (Lineweaver and Burk 1934).

Effect of metal ions and inhibitors on PG activity

The enzyme was allowed to react with 1% pectic acid solution containing 1mM (as compound concentration) metal ion or inhibitor as follows; $ZnSO_4$, $MgCl_2$, $CuSo_4$, $CaCl_2$, $MnCl_2$, $AlCl_3$, KCl, $FeSo_4$, $NiCl_2$, $BaCl_2$, NaCl, LiCl, Tris and Urea.

Protein and PG activity determination

Protein was determined based on the absorbance at 280 nm and was assayed according to the method of Hartree (Hatree 1972). The reaction mixture was composed of 1ml pectic acid solution (1% pectic acid in 0.2 M acetate buffer (pH 4.5)) and 0.1ml enzyme solution. The reaction mixture was incubated at 45°C for 20 min and then the enzyme reaction was stopped by heating in boiling water for 10 min. Reducing sugar was determined from the reaction mixture by using galacturonic acid as a standard according to Miller method (Miller 1959). One unit of PG activity was defined as 1 μmol galacturonic acid released in the reaction mixture per min per ml of enzyme solution.

RESULTS

DEAE Sephadex A-50 CM Sephadex C-50 column chromatography

Figure 1 shows the elution profiles of DEAE Sephadex column chromatography. Before eluting the column with NaCl, one major protein peak was eluted and PG activity appeared in a peak at the fraction number from 9 to 40. These active fractions were pooled together for further purification in CM Sephadex C-50 column. After eluting the column with NaCl, the other major protein peak was eluted and the

PG activity was detected in this peak but the activity is too low as shown in Fig. 1.

Figures 2-A and -B show the elution profiles of the first and second CM Sephadex column chromatography. Only one protein peak was obtained in the first chromatography and the PG activity was detected in the fraction numbers of 30-60. These fractions were pooled together and applied onto the second column chromatography. The PG activity coincided in some part of the major protein peak and minor peak. The PG active fractions (numbers 20-45) were pooled together and used for the next step.

Sephadex G-100 column chromatography

Figures 3-A, -B and -C show the elution profiles by Sephadex G-100 column chromatography. As shown in Fig. 3-A, the PG activity was detected in the fraction numbers 12-17 being between the two protein peaks. These active fractions were pooled together for purification in the second step. Figure 3-B represents the 2nd elution profile on the same column as the previous step and fraction numbers 17-21 were collected. Figure 3-C also shows 3rd elution profile on the same column as 2nd step. In Fig. 3-C, only one protein peak was recognized and fraction numbers 14-17 were collected for further characterization of enzyme properties.

Summary of purification

Table 1 represents the summary of PG purification steps. The purified PG was about 121 folds (based on specific activity) purer than the crude enzyme and the

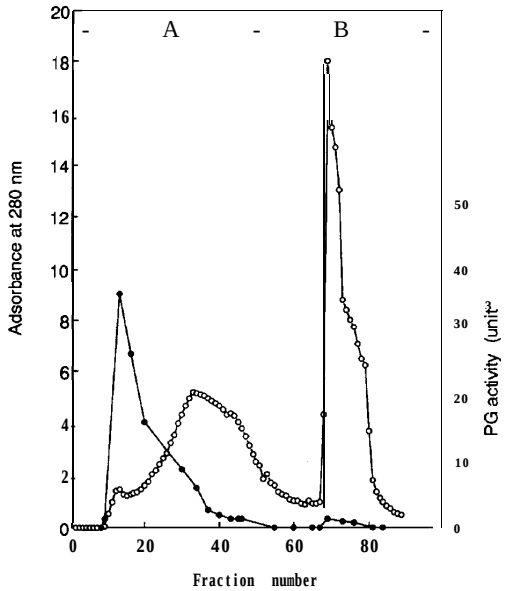

Fig. 1. Elution profile of PG on DEAE Sephadex A-50 column chromatography. A, the column was eluted with 0.05 M Tris-HCl beffer (pH 7.5); B, the column was eluted with liner gradient from 0 to 2 M NaCl in 0.05 M Tris-HCl buffer (pH 7.5).
0, adsorbance at 280 nm; ●, PG activity.

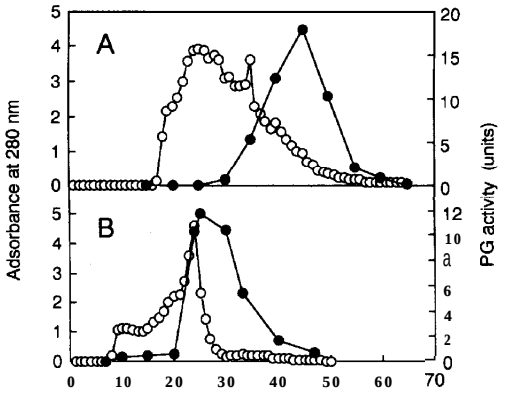

Fig. 2. A:Elution profile of PG activity on 1st CM Sephadex C-50 column chromatography. The column previously eluted with 0.05 M phosphate buffer (pH 6) was eluted with **0.05** M phosphate buffer (pH 7.5) after applying the enzyme onto the column. B: Elution profile of PG on 2nd CM Sephadex C-50. The column, previously eluted with 0.05 M phosphate buffer (pH 6), was eluted with 0.05 M phosphate buffer after applying the enzyme onto the column.
0, adsorbance at 280 nm; ●, PG activity

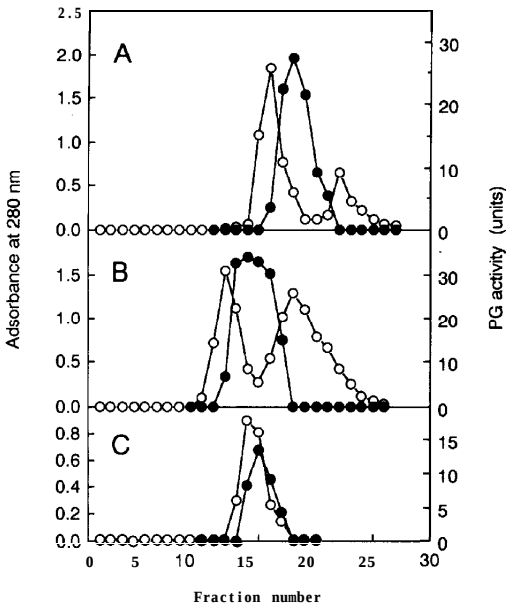

Fig. 3. A:Elution profile of PG on 1st Sephadex G-100 column chromatography. The enzyme was eluted with 0.05 M phosphate beffer (pH 7.5).
B: Elution profile of PG on 2nd Sephadex G-100 column chromatography. The enzyme was eluted with 0.05 M phosphate buffer (pH 7.5).
C: Elution profile of PG on 3rd Sephadex G-100 column chromatography. The enzyme was eluted with 0.05 M phosphate buffer (pH 7.5).
0, adsorbance at 280 nm; ● , PG activity

recovery based on specific activity was 2.7%.

Molecular weight determination

Figure 4 shows the plot of $V_e/_e$ against log-molecular weight of various standard proteins and the molecular weight of present PG was determined as 44,000 Da as shown in Fig. 4.

Effect of pH and temperature on activity and stability

Figure 5-A shows the effect of pH on enzyme activity and stability. The pH optimum of the activity was given at 4.5 and the PG was most stable at pH 7.5. As shown in Fig. 5-B, the temperature optimum of the activity was at 45°C and the enzyme was stable up to 35°C.

Michaelis constant (Km)

Figure 6 shows Lineweaver-Bunk plot of the PG with a substrate of pectic acid. As shown in Fig. 6, the Km value of the enzyme for pectic acid was determined as 5. 7 mg/ml.

Table 1. Summary of purification of PG.

Step	total volume (ml)	total activity (unit)	total protein (mg)	specific activity (unit/mg)	recovery (%)
crude extract	1000	12090	5000	2.4	100
$(NH_4)_2SO_4$	30	8983	1004	8.9	74
DEAE Sephadex A-50	522	8388	438	20.5	69
1st CM Sephadex C-50	298	2833	116	24.4	23
2nd CM Sephadex C-50	223	1712	81	21.3	14
1st Sephadex G-100	50	1200	36	33.9	10
2nd Sephadex G-100	30	660	7	95.7	5.5
3rd Sephadex G-100	24	329	1.1	291.2	2.7

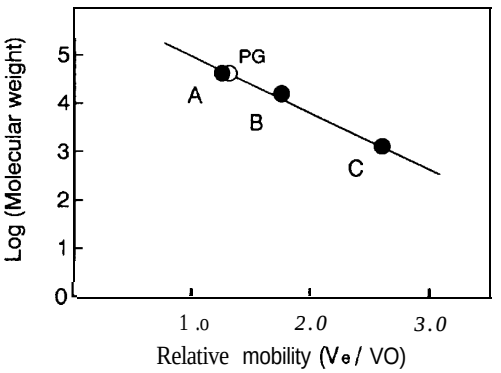

Fig. 4. Determination of molecular weight of PG by Sephadex G-75 column chromatography.

A, ovalbumin (chicken, MW 44,000); B, myogroblin (horse, MW 17,000); C, cyanocobalamin (MW, 1,350)

Effect of metal ions and inhibitors on PG activity

Table 2 shows the effect of some metal ions at 1mM and inhibitors on the PG activity. As shown in Table 2, every metallo-ions tested did not inhibit or enhance markedly the present PG activity except the Li-ion and urea which inhibited about 20% of the PG activity.

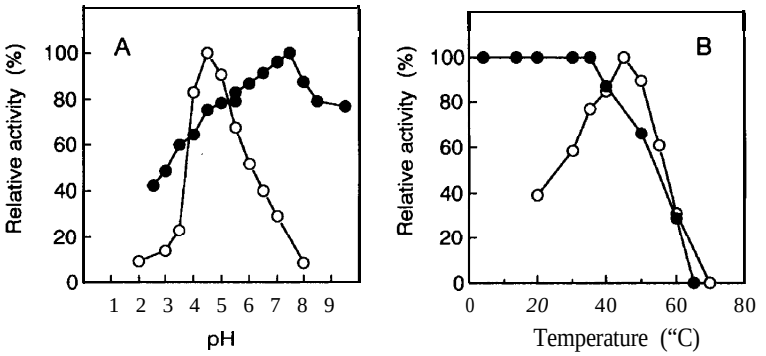

Fig. 5. A: The effect of pH on PG activity and stability.
○, pH activity; ●, pH stability.
B: The effect of temperature on PG activity and stability.
○, activity; ●, stability.

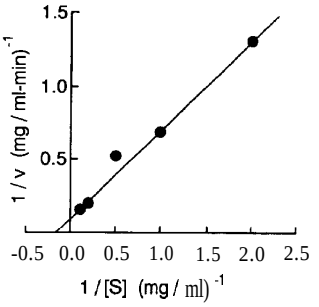

Fig. 6. Determination of Km value of PG by Lineweaber-Burk plot.

Table 2. Effect of metallo-ions and some inhibitors on PG activity.

Metallo-ion	None	Zu	Mg	Cu	Ca	Mn	Al	K	Fe	Ni	Ba	Na	Li
activity(%)	100	110	108	107	106	106	101	100	100	100	98	94	88

Tris	urea
108	82

DISCUSSION

Many researchers reported on the multiple forms of purified PG from fungi such as two forms of endo-PG and exo-PG from *Aspergillus niger* 35-1 (Hara et al., 1984; Hara et al., 1983) in different properties and three forms of PG from *Saccharomyces fragilis* (Lim *et al.*, 1980). This study found that PG from *Rhizopus japonicus* IFO 5318 was eluted into two PG active fractions by DEAE Sephadex A-50 column chromatography, i.e. one major activity fraction and the other minor activity fraction. The major activity fraction was collected for further purification by CM Sephadex C-50 column chromatography. The conditions for PG purification in every step had to choose pH being close to pH 7.5 since it was unstable at the pH above or below 7.5. Successively, the protein of PG enzyme was eluted behind the other major part of protein by eluting CM Sephadex C-50 column chromatography with 0.05M phosphate buffer at pH 7.5, (previously equilibrated with eluting buffer at pH 6). When phosphate buffer pH 7.5 flow through the column, it titrated with phosphate buffer pH 6 and then pH gradient was gradually formed in the column. The protein migrated through the column by different pI. After fractionation by different charges and pIs, it seems that the molecular weights of most proteins were not extremely different then three steps of gel filtration were used to exclude some part of the uninterested proteins little by little.

After purification, some properties of the purified enzyme were studied. The optimal pH on the PG activity was at pH 4.5, generally found in PG of fungi such as *Rhizopus stolonifer* (Trescott and Tampion 1974) and *Aspergillus niger* (Hara *et al.*, 1984; Hara *et al.*, 1983). The enzyme was most stable at pH 7.5. Alternatively, PG from *Aspergillus niger* (Hara *et al.*, 1984; Hara *et al.*, 1983) was stable in the range from pH 2.5 to 5 and that of *Rhizopus stolonifer* (Trescott and Tampion, 1974) was from pH 4 to 6. The optimum temperature on PG activity was 45°C while that of *Aspergillus niger* was higher (Hara *et al.*, 1984; Hara *et al.*, 1983). Furthermore, it was found that thermal stability of this PG was similar to that of *Rhizopus stolonifer* (Trescott and Tampion 1974) while that of *Aspergillus niger* was stable at higher temperature (Hara *et al.*, 1984; Hara *et al.*, 1983) than that of present PG. The molecular weight of PG was obtained as 44,000 Da by gel filtration. PG from *Rhizopus stolonifer* was unsuccessfully determined by gel filtration since the elution of enzyme in void volume has caused the association of the enzyme molecules themselves or the association of the enzyme with residual uronide material to form a complex with high molecular weight (Trescott and Tampion 1974). Exposure of present PG to Mg, Cu, Ca and Mn-ion increased slightly the PG activity while PG of *Rhizopus stolonifer* was inhibited slightly by these metal ions. Furthermore, the inhibitory effects of Fe, Ba and Na-ion on PG activity of present enzyme were found to be similar to that of *Rhizopus stolonifer* (Trescott and Tampion 1974).

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