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Some Properties of Pectinesterase from *Rhizopus juponicus IF05318*

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A pectinesterase(PE) as a hydrolase was purified and partially characterized from pectin- and cassava starch-supplemented wheat bran culture of *Rhizopus japonicus* IF05318, a strain selected among 25 isolates and authentic strains(from IFO). The PE was purified 162 folds with a recovery of 10.6% based on the total enzyme activity by batch extraction and column chromatography using SP-Sephadex C-50, CM-Sephadex C-50 and Sephadex G-75 gel filtration. The enzyme was stable at pH range of 5.0-8.5 and up to 40°C. The pH and temperature optima were 5.0 and 35°C, respectively. The K_m value was estimated as small value as 18.4mg/ml from the Lineweaver-Burk plot, comparing reported fungal K_m values. The molecular weight was determined as 33kDa by SDS-PAGE. The activity enhancers were Cu^{2+}, Ca^{2+}, Na^{+} and Mg^{2+} while the inhibitors were Pb^{2+}, Ba^{2+}, Zn^{2+}, Fe^{2+} and tannic acid.

INTRODUCTION

To convert farming products to more valuable one, *Rhizopus* strains have been isolated from fermented foods and other sources in order to saccharify straight cassava tuber or starch. As a first step, enzyme production by the *Rhizopus* isolates and some IFO authentic *Rhizopus* strains were tested on amylase, pectolytic enzymes, proteolytic enzymes and cellulolytic enzymes. Among of the extracellular hydrolytic enzymes, pectolytic enzymes are classified into several forms, namely the polygaracturonases, the pectinesterases and pectin lyases(Deuel and Stiltz, 1958). Studies concerning the purification of pectic enzymes of strains mostly dealt with polygaracturonase(PG) (Trescott and Tampion, 1974; Liu and Luh, 1987; Lee and West, 1981; Manachini et *al.*, 1987) in *Rhixopus* sp.(Archer, 1979), no purification work on PE has been done yet. Previously, we made some purification and characterization studies on polygaracturonase (Lourrungrung *et al.*, 1993) from *Rhizopus japonicus IF05318*. From an enzyme screening work from *Rhixopus* isolates and IFO authentic strains, we found out that *Rhizopus japonicus IF05318* was the most potent PE producer on solid wheat bran medium from jamong our isolates and authentic strains.

Present work deals with the purification and partial characterization of PE from *Rhizopus japonicus IF05318* as a hydrolase because there has been no characterization work of the PE yet.

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MATERIALS AND METHODS

Microorganisms and culture medium

Twenty five *Rhizopus* isolates and IFO strains were used for the screening work. They were maintained on potato dextrose agar(PDA) slants, making fresh culture every 3 months.

Cultivation

The flask cultures were macerated with five-folds of 0.05M acetate buffer(pH 5.0) and the enzyme was extracted for overnight at 4°C. Then, the crude enzyme extract was obtained by squeezing, vacuum filtration through Celite bed pre-coated on Toyo No. 2 filter paper and centrifugation at 15,000 X g for 20min.

PE purification

All purification steps were done at 4°C. PE was first extracted from the crude enzyme solution by batch treatment with SP-Sephadex C-50 at pH 5.0. The pH of the crude enzyme(2l) was adjusted to pH 5.0 with 2-N acetic acid and SP-Sephadex C-50(5g, pre-swollen in 0.05M acetate buffer(pH 6.0) was added. The mixture was stirred for 2h, then filtered and the residue was repeatedly washed with the same acetate buffer. The PE and other proteins, selectively adsorbed on to SP-Sephadex C-50, was eluted by mixing with 500ml of 0.5M NaCl dissolved in the same buffer, and filtered through Toyo No. 7 filter paper. Then the filtrate was dialyzed against the same acetate buffer for overnight. The dialyzate(680ml) was vacuum filtered through 0.02μm millipore filter and applied on to SP-Sephadex C-50 column chromatography(2.5 X 38cm), pre-equilibrated with the same acetate buffer. The column was eluted with a linear gradient of 0-0.3M NaCl in similar buffer at a flow rate of 30ml/h. The major active fractions were pooled, dialyzed against 0.05M acetate buffer(pH 6.0) for overnight and concentrated by ultrafiltration through a membrane with molecular cut-off of 10kDa with nitrogen pressurization at 3kg/cm^2. The enzyme concentrate(20ml) was charged next to CM-Sephadex C-50 column chromatography(2.5 ×38cm), pre-equilibrated with 0.05M acetate buffer(pH 6.0). Elution was done using a linear gradient of 0-0.3M NaCl dissolved in the same acetate buffer at a flow rate of 20ml/h. The PE active fractions were pooled, concentrated by ultrafiltration (10ml) and applied on to Sephadex G-75 gel filtration at a flow rate of 4.5ml/h and 3ml fractions were collected. Finally, the fractions having the protein peak that coincided with the PE activity, freed from PG activity, was collected and concentrated by ultrafiltration(6ml) as the purified enzyme.

Enzyme activity assays

PE activity assay was based on the release of methanol from pectin. The reaction mixture, composed of 4ml of 1% pectin and 0.1ml of enzyme solution was incubated at 35°C for 15min. The reaction was stopped by addition of 0.5ml 2-N H_2SO_4 and the methanol released was estimated by the method of Wood and Siddiqui(Wood and Siddiqui, 1971). One unit of PE was expressed as one micro mole methanol liberated per min-ml-enzyme solution. PG activity was assayed based on the release of garacturonic acid from pectic acid estimated using the dinitrosalicytic acid(DNS) method for reducing

sugar determination against alpha-galacturonic acid as a standard(Miller, 1959). A 0.9ml of 1% pectic acid solution (dissolved in O.lM acetate buffer to make pH 4.5) was reacted with O.lml of the enzyme solution at 40°C for 15min. One unit of enzyme activity was expressed as one micro-mole galacturonic acid liberated per min-ml of enzyme solution.

Protein determination

The protein content was estimated by reading the absorbance at 28Onm or by using the method by Lowry et al., as modified by Hartree(1972), against bovine serum albumin as a standard.

Electrophoresis

SDS-PAGE was performed using 12.5% polyacrylamide gel and Tris-glycine buffer(pH 8.4), according to the discontinuous buffer system method of King and Laemmli(1971). The gels were stained with 0.1% Coomassie brilliant blue R-250 and destained in 7.5% acetic acid.

Temperature and pH dependencies

The effect of temperature and pH activity was determined by varying the pHs and temperatures of activity assay. Thermo-stability and pH stability were determined by assaying the remaining activity after incubating at various temperature for 60min or various pHs for 24h at 4°C. The buffers used were 0.05M of Clark and Lubs buffer(pH l-2 and 8-10), McIlvane buffer(pH 3-7.5) and Na_2CO_3-$NaHCO_3$ buffer(pH 11-12).

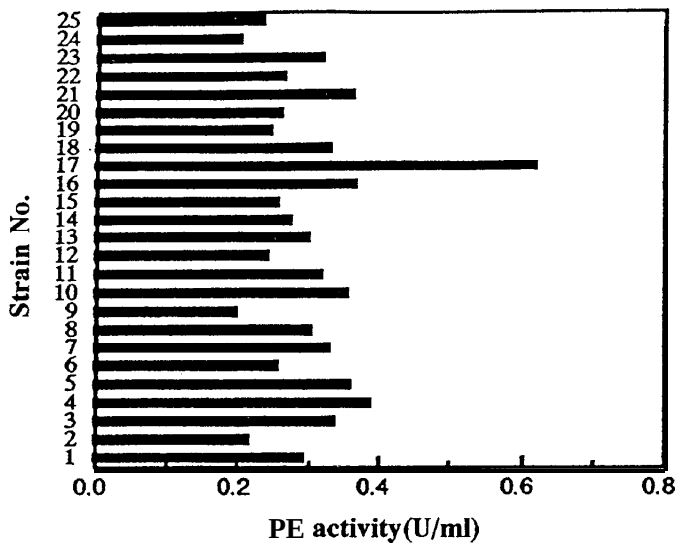

Fig. 1. PE activity assay of the wheat bran culture extract of 25 *Rhizopus* strains.

RESULTS AND DISCUSSION

Screening for best PE producing strain

Figure 1 shows the results of PE activity of the crude enzyme extracts of the strains grown on pectin and cassava starch supplemented wheat bran medium. *Rhixopus japonicus* IF053 18 gave the highest PE activity of 0.64U/ml of crude enzyme extract, about twice as much as that of other strains.

Table 1. Summary of purification of PE from *Rhizopus japonicus* IF05318.

Purification step	Volume (ml)	PE activity (U/ml)	Total activity (U)	Protein (U/mg)	Specific activity (U/ml)	Recovery (%)	Purification folds
Crude enzyme	2,000	0.37	740	0.62	0.60	100	1.0
SP Sephadex C-50(batch)	680	0.71	480	0.80	0.89	65	1.5
SP Sephadex C-50(column)	20	17.0	340	0.27	6.27	46	10.5
CM Sephadex C-50(column)	10	16.0	160	0.47	33.8	22	56.3
Sephadex G-75 gel filtration	6	13.0	78	0.13	97.0	10.6	162.0

PE purification

Table 1 shows the summary of the four chromatography steps done purified the enzyme by 162 folds and a recovery of 10.6% was obtained on the enzyme activity. Batch

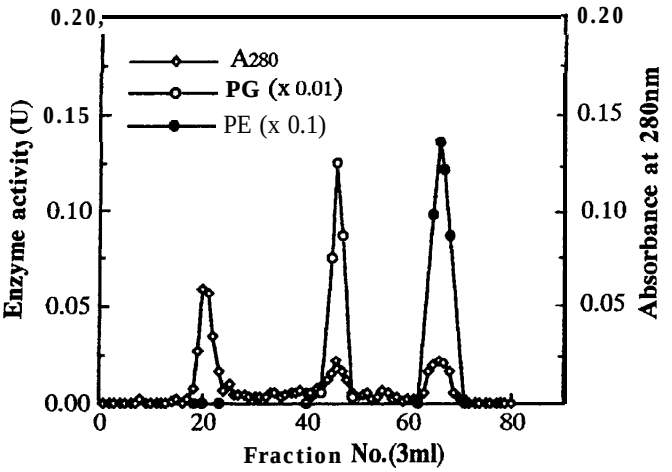

Fig. 2.

The enzyme was eluted with 0.05M phosphate buffer (pH 7.5) at a flow rate of 4.5ml/h.

extraction using SP-Sephadex C-50 adsorbed most of the PE and 65% was recovered upon elution with 0.5M NaCl. On the other hand, about 20% of the PG activity was retained. After SP-Sephadex C-50 chromatography, the major PE activity peak was separated from the major peaks by the conditions set in the column chromatography, although some minor PG activities remained. Further separation of the PE from the

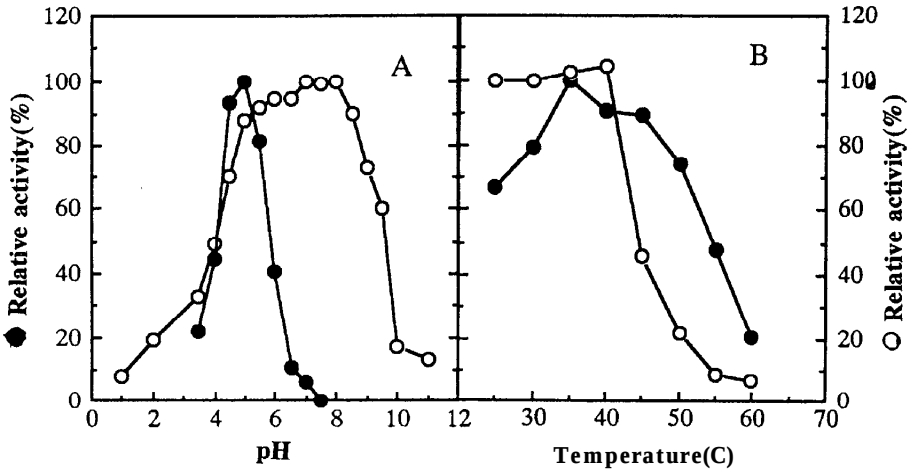

Fig. 3. pH and temperature optima (●)and stability *(0)* of *Rhizopus japonicus* IFO5318PE. The enzyme was incubated in various buffers for 24h at 4°C for pH stability determination and in desired temperature for 60min at pH 7.5 for temperature stability determination.

contaminating PG was made possible through chromatography in CM-Sephadex C-50 column. Figure 2 shows the result of Sephadex G-75 gel filtration that the PE activity coincided to a single peak, freed from the PG active fraction.

Effect of pH and temperature

Figure 3-A shows the effect of pH on the activity of the present PE. The enzyme was stable at pH 5-8.5 after incubation at 4°C while the optimum pH of the activity was pH 5.0. The pH optimum is typical of previously reported fungal PEs although pH stability was wider compared to *Aspergillus japonicus* PE(Ishii *et al.*, 1979) and *Coniothyrium diplodiella* PE(Endo, 1964) but narrower than that of *Aspergillus oryzae* PE(Lim *et al.*, 1983) and *Corticium rolfsii*(Yoshida et al., 1977). Figure 3-B shows the effect of temperature on the activity and stability of the present PE. The optimum temperature for the reaction was 35°C and rapid inactivation occurred upon incubation at temperature above 40°C for 60min. Such temperature characteristics are slightly inferior compared to that of most fungal PEs.

K_m value

Figure 4 shows Lineweaver-Burk plot of the PE and the K,,, value was determined as 18.4mg/ml. This value is only one third compared to the reoprted K_m value for the PE-II

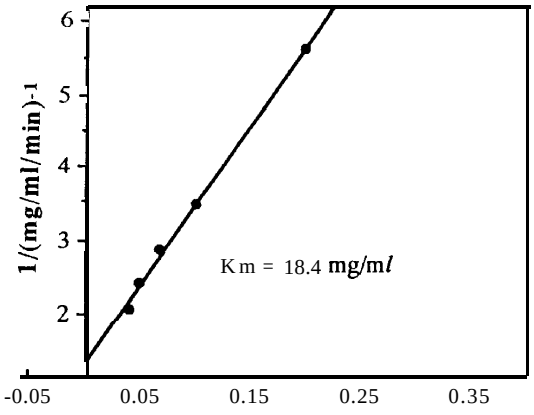

Fig. 4. Determination of K_m value by the Lineweaver-Burk plot.

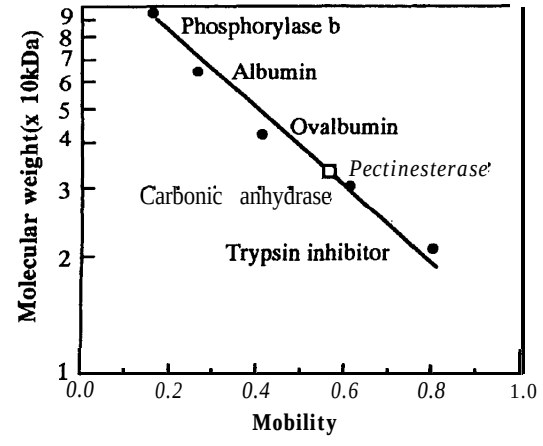

Fig. 5. Determination of the molecular weight of PE from *Rhizopus japonicus* IF05318.

of *Aspergillus oryzae* which was 50mg/ml(Lim et al., 1983). The K_m value of the present PE was so small that the hydrolysis rate of pectin substance by the PE can be more suitable enzyme than that of reported fungal PEs.

Molecular weight

Figure 5 shows logarithmic plot of the SDS-PAGE results using low molecular weight standard marker protein(BIO-RAD) and the molecular weight of the present PE was estimated 33kDa. The value is quite comparable to the molecular weight of *Aspergillus oryzae* PE(Lim *et al., 1983)* and *Aspergillus juponicus* PE(Endo. 1964).

Table 2. Effect of ionic compounds, phcnolic compounds and inhibitors on the activity of PE.

Compounds	Relative activity	Compounds	Relat ivr activity	Compounds	Relative activity
control	100	$CuSO_4$	156	EDTA	114
KCl	84	$CaCl_2$	144	PMSF	100
NaCl	127	$BaCl_2$	79	Gallic acid	98
$NiCl_2$	104	$MgCl_2$	12.5	Tannic acid	52
$LiCl_2$	112	$MnSO_4$	114	p-Coumaric acid	90
$ZnSO_4$	75	$AlCl_3$	93	Chlorogenic acid	121
$Pb(CHCOO)_2$	51	$Fe_2(SO_4)_3$	72	Cnffeic acid	98

Effect of cations, phenolic compounds and inhibitors

Table 2 shows the effect of some ionic, phenolic and protein inhibitory compounds at a concentration of 1mM on the PEs activity. Some cations enhanced the activity to as much as 20-50%, particularly Cu^{2+}, Ca^{2+}, Na^{+} and Mg^{2+}. On the other hand, the heavy metallic ions, Pb^{2+}, Fe^{2+}, Zn^{2+} and Ba^{2+} and tannic acid inhibited the activity from 30-50%. EDTA and PMSF did not cause any significant effect on the PE activity. The enhancers and inhibitors of *Rhizopus japonicus* IF05318 PE have almost similar actions as to that of other fungal PEs, except that of Cu^{2+} which activates the present PE but inhibits *Aspergillus oryzae* PE (Lim et al., 1983). In general, $CaCl_2$ and NaCl caused the most pronounced enhancement of most fungal PE activities. Tannic acid is a noted inhibitor of pectic enzymes (Liu and Luh, 1978) because of its high molecular weight. The mechanism of inhibition seems to be largely dependent on the random blocking of the active site by the large molecular sizes of the inhibitors.

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