Purification and Characterization of Three Extracellular Protopectinas with Polygalacturonase Activities from *Trichosporon penicillatum*

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In a culture filtrate of *Trichosporon penicillatum* B2, which is a γ-ray irradiation mutant induced from *T. penicillatum* SNO3, we found three kinds of pectin-releasing enzymes, protopectins SE1, SE2, and SE3, that have endo-polygalacturonase activity. These enzymes were purified to homogeneity with cation-exchange and size exclusion chromatographies. The major PPase in the culture filtrate was PPase SE1, which accounted for 75% of total activities in the culture filtrate, and the two others were 15% (PPase SE2) and 0.007% (PPase SE3). Their molecular masses were approximately 41, 41, and 42 kDa on SDS-PAGE, respectively. They had similar enzymatic properties but different PPase activity and pH- and thermostability. Antibody against PPase S, which is produced by strain SNO3, inhibited the activities of PPase SE1, SE2, and SE3. However PPase SE1 was completely inhibited by treatment with the anti-PPase S antibody, but the activities of PPases SE2 and SE3 remained at 20 and 50% of the original activity, respectively.

**Key words:** protopectinase; polygalacturonase; purification; *Trichosporon penicillatum*; enzyme properties

Pectin constructing a cell wall polysaccharide in plant tissues is composed of arabinan, galacturan, and rhamnogalacturan. Some microorganisms produce pectin-releasing enzymes (PPase), which release soluble pectin from insoluble pectic substances, protopectin. The first report of a PPase from *Trichosporon penicillatum* appeared in 1978. The species of *Kluyveromyces*, Yoshitake et al. reported that two PPase, PPase-F and -W, produced from *K. fragilis* and *K. wickerhamii*, respectively, have PGase activities but their PPase activities are quite different. They suggested that PPase-F and -W have different ratios of PPase activity to PGase activity (q_{PPase/PGase}), which may be due to their different molecular structures. We are keenly interested in the relation between the molecular structure of PPase and the PPase activity.

In our previous study, we isolated *T. penicillatum* strain B2, induced from strain SNO3 by γ-ray irradiation, to prepare the new PPases containing different PPase activities from PPase S. Strain B2 produced much larger amounts of PPases than strain SNO3. We found three different PPases in the culture filtrate of *T. penicillatum* B2, which have similar enzymatic properties but not immunological properties. We also found that they had the different q_{PPase/PGase}. This paper deals with isolation and characterization of these three PPases.

**Materials and Methods**

**Chemicals.** CM-Toyopearl 650M was obtained from Toosho Co. (Tokyo). Mono-S HR 5.5, Superose 12, and a standard protein kit for the estimation of molecular mass were purchased from Pharmacia Biotech K.K. (Tokyo). Polygalacturonite acid was prepared as follows: 1% solution of commercial polygalacturonic acid (Sigma Chemical Co., St. Louis) adjusted to pH 5.0 with NaOH was mixed with a cation-exchange resin. Dowex HCR-W2 (Murumachi Kagaku Kogyo K.K., Tokyo), for 12h with shaking, an equal volume of acetone was added to supernatant of resin-mixture, and precipitate formed was washed twice with ethanol, dried, and used as pure polygalacturonic acid. All other chemicals were from Wako Pure Chemical Industries Ltd. (Osaka) and were of certified reagent grade.

*Microorganism and cultivation conditions.* *T. penicillatum* B2 was used throughout this study. The organism was cultured in SGP medium composed of 2% glucose, 0.2% yeast-extract, and 0.4% peptone, pH 5.0, at 30°C, and maintained on agar slants of SGP medium. For purification of the PPases, the microorganism was cultivated aerobically in 8 liters of liquid SGP medium in a 10-liter fermentor (model ACID-14, Sanki Seiki Co., Osaka) at 30°C for 30h with aeration (0.5vol vol⁻¹ min⁻¹) and agitation (250rpm). Silicon KM70 (Shin-Etsu Chemical Co., Ltd.) was added, when necessary, as an antifoaming agent, and the pH was automatically adjusted to 5.0 with 0.1NaOH.

**Enzyme assay.** PPase activity (pectin-releasing activity) was assayed by measuring of pectic substances liberated from lemon protopectin by the carbazole-H$_2$SO$_4$ method. The reaction mixture contained 10mg of protopectin, 20mm sodium acetate buffer, pH 5.0, and an enzyme solution in a total volume of 1ml, and the reaction was done at 37°C for 30min. One unit of PPase activity was defined as the activity that liberates soluble pectic substances corresponding to 1μmol of δ-galacturonic acid in 1h. Protopectin was prepared by washing of the albedo layer of lemon peel with 2% sodium hexametaphosphate, pH 4.0, until the soluble substance that reacted with carbazole-H$_2$SO$_4$ were washed out. Protopectins of different plant tissues were prepared by the same procedure. Polygalacturonase (PGase) activity was assayed by measuring the release of reducing groups from polygalacturonic acid by the method of Somogyi. The reaction mixture was composed of 0.49ml of 0.1% polygalacturonic acid solution in 20mM acetate buffer, pH 5.0, and 0.01ml of enzyme solution with adequate dilution, and the reaction was done at 37°C for 1h. One unit of PGase activity was defined as the activity that forms reducing groups corresponding to 1μmol of δ-galacturonic acid in 1ml reaction mixture, pH 5.0, at 37°C in 1h.
Electrophoresis of proteins. The homogeneity and molecular mass of enzyme were measured by SDS-PAGE by the method of Laemmli. SDS-PAGE was done in a mixture of 0.1% SDS and 100 mM Tris glycine buffer (3 g/20 ml Tris base and 14.4 g/liter glycine), pH 8.3, at 20 mA for 5 h. The protein bands were stained with Coo massie Brilliant Blue R-250.

Estimation of molecular mass. The molecular masses of enzymes were estimated by SDS-PAGE and exclusion chromatography. Size-exclusion chromatography was done on a Superose 12 HPLC 10/30 column equilibrated with 20 mM sodium acetate, pH 5.0, containing 100 mM NaCl with an FPLC apparatus (ECC 500 with a P500 pump; Pharmacia Biotech K. K., Tokyo). Proteins were eluted with the same buffer at a flow rate of 0.75 ml/min. The apparent molecular mass was calculated from the mobility (or elution volume in size exclusion chromatography) vs. molecular mass plot of protein markers: phosphorylase b, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; soybean trypsin inhibitor, 20.1 kDa; and &-lactalbumin, 14.4 kDa.

Analysis of amino acid sequence. N-Terminal amino acids were sequenced by 25 mm² square PVDF membranes (Nippon Bio-Rad Laboratories K. K., Tokyo) being incubated in methanol for 10 min and then in water for 10 min at room temperature. Around 200 pmol of purified enzymes were adsorbed on the PVDF membrane, washed thoroughly with water, and dried at room temperature. The enzyme-adsorbed PVDF membrane was put into a protein sequencer PSQ-1 (Shimadzu Co., Kyoto).

Purification of enzymes. Three PPases were purified from the culture filtrate of T. penicillatum B2. The culture filtrate (6 liters) was concentrated at 40 °C under reduced pressure to about 300 ml (all other steps of purification were carried out at 4 °C). The concentrate was dialyzed against 20 mM sodium acetate buffer (pH 5.0), and used as the starting material for enzyme purification. The protein solution was put on a CM-Toyopearl 650M column (3.0 cm × 27 cm) equilibrated with the dialysis buffer and washed thoroughly with the same buffer. The adsorbed proteins were eluted with a linear gradient of NaCl (500 ml from 0 to 750 mm) in this buffer at a flow rate of 1 ml/min, and 6-ml fractions were collected. The fractions containing enzyme activity were pooled, dialyzed against 20 mM sodium acetate buffer (pH 5.0), and concentrated under reduced pressure. The obtained enzyme solution was put on a Mono-S HR 5.5 column equilibrated with 20 mM sodium acetate buffer, pH 5.0, in an FPLC apparatus. The elution was done with a linear gradient of NaCl (100 ml from 0 to 400 mm) in the buffer at a flow rate of 0.5 ml/min, and fractions of 0.5 ml were collected.

Immunological assay. Antiserum against the PPase S, which was obtained from T. penicillatum strain SNO3 (the original strain of strain B2), was prepared by the method described by Sakai et al. Western blotting was done as follows. Proteins were transferred to PVDF membrane (Immobilon; Nihon Millipore Ltd., Tokyo), using Holize-Blot (Atto Co., Tokyo), and analyzed by ELISA, using the anti-PPase S antibody as a first antibody, the peroxidase-labeled protein A (Cosmo Bio K. K., Tokyo) as a second antibody, and 3,3'-diamino-benzidine tetrahydrochloride as a stain.

The PPases were incubated with the anti-PPase S antibody by measuring the decrease of enzyme activity when the enzyme solution containing 1.5 unit of PPase was mixed with antiserum and incubated at 4 °C for 8 h, and then enzyme activity was measured.

Results and Discussion

Purification of PPases from T. penicillatum B2

PPases were purified from the fermentation broth of T. penicillatum B2. Purification was done by monitoring PGase activity as the PPases also have PGase activity. Two protein peaks having PGase activity (one major peak and one minor peak) were observed in a cation-exchange chromatography on a CM-Toyopearl 650M column (Fig. 1A). Based on the absorbance at 280 nm, more than 90% of the protein mass in the culture filtrate had PPase activity, and the PPase fraction that accounted for about 95% of total PPase activity in the chromatography fractions was named PPase SE1. This peak was shown to contain a single protein by SDS-PAGE. Beside the PPase SE1 peak, a minor peak containing about 5% of total PPase activity was recovered. The fractions containing PPase activity in the minor protein peak were collected and rechromatographed on a second cation-exchange column (Mono-S HR 5/5). Three peaks with PGase activity were found in the eluted fractions (Fig. 1B). First peak was identified as PPase SE1, and the second and the third peaks were named PPase SE2 and SE3, respectively. Each of these fractions was analyzed on SDS-PAGE and found to contain a single protein (Fig. 2). The yields of these enzymes in the different purification steps is summarized in Table I. PPase SE1, SE2, and SE3 accounted for approximately 74.5%, 0.15%, and 0.007%, respectively, of the total PPase activity in the starting material (Table I).

Fig. 1. Ion Exchange Chromatograms of PPase Samples.
A: Chromatogram obtained from a CM-Toyopearl 650M column. Samples were prepared from T. penicillatum culture filtrates. Bound proteins were eluted by a linear gradient of NaCl (500 ml from 0 to 0.75 M) at a flow rate of 50 ml/h. Open circles show the absorbance at 280 nm and closed circles show polygalacturonase activity. Arrows show PPase SE1 fraction and a fraction of a minor peak. B: Chromatogram of fractions from the minor peak shown in A were obtained with a Mono-S HR 5/5 column. Elution was done by a linear gradient of NaCl (10 ml from 0 to 0.4 M) shown by broken line at a flow rate of 0.5 ml/min. Thin line is a profile of absorbance at 280 nm and closed circles are polygalacturonase activities. Arrows show three PPase (SE1, SE2, and SE3) fractions.

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Enzymatic properties of the three PPases

The molecular masses of three PPases were analyzed by SDS–PAGE (Fig. 2). Two PPases, SE1 and SE2, appeared to have a similar molecular mass, approximately 41 kDa, and PPase SE3 appeared to have a molecular mass of 42 kDa. The molecular masses of these PPases were also measured by size-exclusion chromatography on Superose 12 HR. The three PPases eluted in the same fraction and their molecular masses were estimated to be about 30 kDa (Table II). No sugar residues were detected in the three PPases by the phenol-sulfuric acid method. Many extracellular enzymes are known to have sugar chains, and the difference in sugar chain length makes different size enzymes. Thus, the difference in molecular masses of PPases SE1, SE2, and SE3 was not due to a difference in sugar chains.

The effects of pH and temperature on the activity and stability of the PPases are shown in Table II and Fig. 3. All enzymes had their maximum activity at pH 5.0 and were inactive at pHs higher than 7.0 (Fig. 3A). The three enzymes were stable at pHs lower than 7.0 but unstable at alkaline pH: they lost activity almost completely at pHs higher than 8.0 (Fig. 3B). When PPase SE1, SE2, and SE3 were assayed at pH 5.0, their thermal activity profiles were similar, with highest activities at 55°C (Fig. 3C). The thermal stabilities of the three enzymes at pH 5.0 are shown in Fig. 3D. After treatment at each temperature for 30 min, these enzymes showed different thermal stability profiles. These enzymes were stable after exposure to temperatures lower than 50°C. But had different activities after exposure to temperatures higher than 55°C. PPase SE3 was inactivated completely.

Table II. Properties of Three Proteasecins from T. penicillium B2

<table>
<thead>
<tr>
<th>Properties</th>
<th>PPase SE1</th>
<th>PPase SE2</th>
<th>PPase SE3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular size</td>
<td>41,000</td>
<td>41,000</td>
<td>42,000</td>
</tr>
<tr>
<td>by SDS-PAGE</td>
<td>30,000</td>
<td>30,000</td>
<td>30,000</td>
</tr>
<tr>
<td>by gel filtration</td>
<td>up to 45</td>
<td>up to 45</td>
<td>up to 45</td>
</tr>
<tr>
<td>Thermostability (°C)(at pH 5.0)</td>
<td>3.0 to 7.0</td>
<td>3.0 to 7.0</td>
<td>3.0 to 7.0</td>
</tr>
<tr>
<td>pH-Stability</td>
<td>55</td>
<td>55</td>
<td>45</td>
</tr>
<tr>
<td>Optimum temperature (°C)(at pH 5.0)</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Optimum pH</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 2. Molecular Masses of Three PPases by SDS-PAGE.
Five μg of purified enzyme preparations were put on the gel. Lane 1, protein standard; lane 2, PPase SE1; lane 3, PPase SE2; lane 4, PPase SE3.

Table I. Separation of T. penicillium B2

<table>
<thead>
<tr>
<th>Step</th>
<th>PGase activity (U)</th>
<th>Proteins (mg)</th>
<th>Specific activity (U/mg)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture filtrate total</td>
<td>8,800,000</td>
<td>2,150</td>
<td>4,100</td>
<td>100</td>
</tr>
<tr>
<td>CM-Toyopearl 650M Major peak</td>
<td>6,512,000</td>
<td>40,7</td>
<td>160,000</td>
<td>74</td>
</tr>
<tr>
<td>(PPase SE1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minor peak</td>
<td>44,000</td>
<td>2.04</td>
<td>21,600</td>
<td>0.5</td>
</tr>
<tr>
<td>Mono-S HR 5.5°</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st Peak (PPase SE1)</td>
<td>28,000</td>
<td>1.2</td>
<td>23,000</td>
<td>0.32</td>
</tr>
<tr>
<td>2nd Peak (PPase SE2)</td>
<td>13,800</td>
<td>0.64</td>
<td>21,000</td>
<td>0.15</td>
</tr>
<tr>
<td>3rd Peak (PPase SE3)</td>
<td>642</td>
<td>0.005</td>
<td>128,000</td>
<td>0.0073</td>
</tr>
</tbody>
</table>

Protease activity is expressed as polygalacturonase activity towards purified polygalacturonic acids.

Minor peak fractions on CM-Toyopearl 650M column were fractionated on Mono-S HR 5.5° column by FPLC.
Fig. 3. Effects of pH and Temperature on Enzyme Activities.
Enzymes (0.25 U) were reacted with polysaccharide. Squares (connected with thin line), circles (thick line), and triangles (broken line) show the relative activities of PPAse SE1, SE2, and SE3, respectively. The highest activity was defined as 100%.
A: pH-Dependency of enzymes. Enzymes were assayed at 37°C for 30 min in 20 mM sodium acetate (pH 3.0-6.0) and sodium phosphate (pH 6.0-9.0) buffers. B: pH-Stability of enzymes. Enzymes were incubated at 4°C for 30 min in 20 mM sodium acetate (pH 3.0-6.0) and sodium phosphate (pH 6.0-9.0) buffers and then assayed at 37°C for 30 min in sodium acetate buffer (pH 5.0). C: Thermal activity of enzymes. Enzymes were assayed at test temperatures for 30 min in sodium acetate buffer (pH 5.0). D: Thermal stability of enzymes. Buffers used was same as C. Enzymes were incubated at test temperatures for 30 min and assayed at 37°C for 30 min.

After incubation at 55°C for 30 min but SE1 and SE2 retained approximately 50% and 20% of their original activities, respectively. PPAse SE1 was the most thermostable form of the enzymes, retaining 30% of its activity after treating at 75°C for 30 min.

These results may indicate that the molecular structures of these enzymes are different.

Immunological properties of the three PPAse

We compared immunological properties of PPAse SE1, SE2, and SE3. A Western blot of PPAse SE1, SE2, and SE3 using the antibody against PPAse S showed that the antibody reacted with all three enzymes (Fig. 4).

Fig. 4. Western Blot of Three PPAses.
Purified enzymes were electrophoresed on SDS polyacrylamide gel, transferred to a PVDF membrane and analyzed by ELISA using the anti-PPase S antibody. Lanes 1, 2, and 3 were PPAse SE1, SE2, and SE3, respectively.

We also studied the effects of anti-PPase S antibody on the three PPAse activities. Activity profiles of enzymes after treating with anti-PPase S are shown in Fig. 5. PPAse SE1 was completely inactivated when one-tenth volume of the antiserum was mixed with the reaction mixture. This result indicates that the molecular structure of PPAse SE1 is similar to that of PPAse S. On the other hand, the activities of PPAse SE2 and SE3 were not completely inhibited by the antibody under the same conditions: SE2 and SE3 retained around 20% and 50% of the original activities, respectively. These results suggest that three PPAse have different molecular structures.

Fig. 6. N-Terminal Amino Acid Sequences of PPass S and Three PPasses of T. penicillatum B2.
Underlined residues are different from those of PPass S.

Table III. The $\delta_{PPasePGase}$ of Three PPasses

<table>
<thead>
<tr>
<th>Origin of pectin</th>
<th>PPass SE1</th>
<th>PPass SE2</th>
<th>PPass SE3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lemon</td>
<td>1.18</td>
<td>0.78</td>
<td>0.93</td>
</tr>
<tr>
<td>Orange</td>
<td>0.06</td>
<td>0.02</td>
<td>0.01</td>
</tr>
<tr>
<td>Grapefruit</td>
<td>0.24</td>
<td>0.06</td>
<td>0.08</td>
</tr>
<tr>
<td>Sugar beet pulp</td>
<td>0.98</td>
<td>0.30</td>
<td>0.39</td>
</tr>
<tr>
<td>Carrot</td>
<td>0.09</td>
<td>0.10</td>
<td>0.19</td>
</tr>
<tr>
<td>Potato</td>
<td>0.31</td>
<td>0.07</td>
<td>0.01</td>
</tr>
<tr>
<td>Apple</td>
<td>0.09</td>
<td>0.03</td>
<td>0.05</td>
</tr>
</tbody>
</table>

$\delta_{PPasePGase} = \frac{activity\ on\ pectin}{activity\ on\ polygalacturonic\ acid}$

N-Terminal amino acid sequences of three PPasses

The N-terminal amino acids of the three PPasses were sequenced, and about 17 amino acid residues from the N-terminal were confirmed as shown in Fig. 6. The N-terminal amino acid sequence of PPass SE1 was identical with that of PPass S, as far as the first 17 residues. However, some differences were observed in the amino acid sequences of PPass SE2 and SE3: the 2nd, 7th, 10th, and 17th amino acid residues in PPass SE2 and SE3 are different from those of PPass S and SE1. This corresponds to a 20% diversity in the primary structure.

We assume that these differences in primary structures are the cause of the different pH stabilities and thermal stabilities of the enzymes.

PPass activity

The $\delta_{PPasePGase}$ values of PPass SE1, SE2, and SE3 were measured using protectins from various plant tissues (Table III). The three enzymes had different $\delta_{PPasePGase}$ values, and PPass SE1 had the highest values with all protectins except for carrot. Generally, $\delta_{PPasePGase}$ values for these enzyme were SE1 > SE3 > SE2 except for potato and carrot protopectin.

As the $\delta_{PPasePGase}$ values indicate the activities in insoluble pectin (protopectin), the above results indicate that these three enzymes have different activities on insoluble pectin.

As mentioned above, these three PPasses are a kind of PGase. Previously, Sakai and Takaoka isolated a PGase from the culture filtrate of Aureoasidium pullulans (PGase-AY), which degrades polygalacturonic acid strongly but has weak PPass activity. The enzyme has a lower affinity for protopectin from Citrus unshiu than does PPass S; the $K_m$ for protopectin is one order of magnitude higher than that of PPass S, although the two enzymes have almost the same affinity for polygalacturonic acid. The differences in affinity for protopectin seem to be one reason why different PGases have different PPass activities.

We hypothesize that the differences in PPass activities of PPass SE1, SE2, and SE3 is due to their molecular structure. Thus, these three PPasses will be very useful for elucidating the molecular structure characteristics of PGase that give rise to PPass activity. We are presently working on the gene cloning and molecular structures of these enzymes.

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References