Purification and Characterization of Thermostable Pectate Lyase with Protopectinase Activity from Thermophilic *Bacillus* sp. TS 47

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Received May 8, 2000; Accepted June 13, 2000

A strain of thermophilic bacterium, *Bacillus* sp., with pectolytic activity has been isolated. It produced an extracellular endo-polygalacturonate trans-eliminase (PL, EC 4.2.2.1) when grown at 60°C on a medium containing polygalacturonate (PGA). The PL was purified by hydrophobic, cation exchange, and size exclusion column chromatographies. The molecular mass of the enzyme was 50 kDa by SDS-PAGE. The isoelectric point of the enzyme was pH 5.3. The enzyme had a half-life of 13 and 1 h at 65 and 70°C, respectively, and showed optimal activity around at 70°C and pH 8.0. It had protopectinase activity, besides PL activity, on lemon proteopectin and cotton fibers. The first 20 amino acids sequence of the enzyme had significant similarity with that of PL from methylophilic *Bacillus subtilis*, with 50% identity.

**Key words:** thermophilic; *Bacillus*; pectate lyase; thermostable enzyme

An enzyme that liberates water-soluble pectic substances by restricted hydrolysis from water-insoluble protopectin in plant cell walls has been proposed to be called protopectinase. Sakai and Okushima found a protopectinase produced by a yeast strain in 1978, and other microbial protopectinases have been found since then. Recently, Sakai *et al*. reported that *Bacillus subtilis* IFO 3134 produces three protopectinases including PPase-C, PPase-N, and PPase-R. Among these protopectinases, PPase-C catalyzes splitting the α-1,5-linked arabinofuranoside linkage of the arabinan region in arabinogalactan, which attaches pectin to the cell wall constituents, so that releases pectin. On the other hand, PPase-N and PPase-R, split glycosidic linkages of pectic polymers in trans-elimination reaction, so that they release pectin. For application of these enzymes, Sakai *et al*. have developed a biochemical process for scouring of cotton fabrics, and named it “BioScouring”. To improve BioScouring in respect of enzyme reaction rate in the process, we have attempted to use a thermostable PPase that has a high Q10 value.

Bacterial mesophilic pectate lyases (PL) have been described with species in *Erwinia*, *Bacillus*, *Clostridium*, *Xanthomonas*, and *Pseudomonas*. Compared to the mesophilic PL, little knowledge is accumulated concerned with thermostable PL. Karbassi *et al*. described a thermostable PL, that shows optimal activity at 70°C and pH 8.0, produced by *B. stearothermophilus*, a thermophilic bacterium. However, details have not been cleared with the PL.

This paper deals with the screening of thermophilic bacteria producing thermostable PL, having protopectinase activity, and the purification and characterization of a thermostable PL.

**Materials and Methods**

**Chemicals.** Unless otherwise specified, all chemicals were from Wako Pure Chemicals Industries (Osaka, Japan), and were of certified reagent grade.

**Microorganism, media, and culture conditions.** A thermophilic bacterium isolated in this research, *Bacillus* sp. TS 47, was used throughout in this research. The strain was isolated from a soil sample collected in Nara City as follows; A medium containing 0.5% pectin (from apple, Sigma, St. Louis, Missouri, U.S.A) as the sole carbon source, 0.2% ammonium sulfate, 0.6% KH2PO4, 1.4% K2HPO4, and 0.02% yeast extract (Difco, Detroit, Michigan, U.S.A) (separately autoclaved; pH 7.0) was used for the isolation of organisms. In a test tube (15.5-mm), 3 ml of the medium was inoculated with a soil sample and incubated at 60°C for 48 h, on shaker (120 rpm,
A sample of the culture broth was transferred into fresh medium and incubated at the same conditions. Then, a sample of the culture broth in which organisms had grown was spread out onto the medium agar plates. The plates were placed in plastic bags and incubated at 60°C, microbial colonies were individually picked up and were transferred onto agar plates containing 1.0% polygalacturonate (PGA, from orange, Sigma), 0.5% Tryptone (Difco), 0.5% yeast extract, 1.0% NaCl, and 2% agar (pH 7.0). After the plates were incubated at 60°C for 24 h, the plates were flooded with 5 ml of 0.2 N HCl and left at room temperature. After 30 min, microbial colonies that make cloudy haloes were isolated. For production of PL from Bacillus sp. TS 47, the microorganism was cultured in a medium containing 0.5% PGA, 1% Tryptone, 0.5% yeast extract, and 1% NaCl, pH 7.0, at 60°C, for 8 h on a shaker (120 rpm, 7-cm stroke).

Identification of isolates. The isolate was identified by the methods used for the description of “Berger’s Manual of Determinative Microbiology.”

Purification of thermostable pectate lyase. The culture filtrate (1 liter) of the strain TS 47 was dialyzed against 20 mM Tris-HCl buffer, pH 7.5, and made up to 50% saturation of ammonium sulfate. The precipitated protein was dissolved in 20 mM Tris-HCl buffer, pH 7.5, containing ammonium sulfate of a 20%-saturation and put on a butyl-Toyopearl 650M (Tosoh, Tokyo) column (10×160 mm) equilibrated with the same buffer. The column was washed, thoroughly, with the same buffer, and the adsorbed proteins were eluted by a linear gradient of from 20 to 0%-saturation of ammonium sulfate in 20 mM Tris-HCl buffer, pH 7.5, at a flow rate of 2 ml/min, and 2 ml fractions were collected. The fractions containing PL activity were pooled, dialyzed against 20 mM acetate buffer, pH 5.0. The dialysate was put on a SP-Toyopearl 650M (Tosoh) column (6×100 mm) equilibrated with the dialysis buffer. The column was washed with the same buffer, and adsorbed proteins were eluted by a linear gradient of 0–500 mM NaCl (20 ml) in 20 mM acetate buffer, pH 5.0, at a flow rate of 200 µl/min, and 0.5 ml fractions were collected. The fractions containing PL activity were pooled and dialyzed against 20 mM Tris-HCl buffer containing 100 mM NaCl, pH 7.5. The fractions containing PL activity were concentrated using Centricron-10 (Millipore, Bedford, Missouri, U.S.A.) and put on a Superdex 75 HR 10/30 (Pharmacia Biotech, Uppsala, Sweden) column equilibrated with 20 mM Tris-HCl buffer containing 100 mM NaCl, pH 7.0. The enzyme was eluted with the same buffer at a flow rate of 250 µl/min, and fractions of 250 µl were collected. The active fractions were combined, dialyzed against 20 mM Tris-HCl buffer (pH 7.0), and used for the characterization as the final preparation of purified enzyme.

Enzyme assay. Polygalacturonate lyase activity was assayed by measuring the increase in absorbance at 235 nm of the reaction mixture. Polygalacturonic acid and pectin with degrees of esterification of 22, 67, and 87% from Sigma were used as substrate. A reaction mixture containing 1 ml of 0.1% PGA in 100 mM Tris-HCl buffer, pH 8.0 and 0.5 mM CaCl2, and an appropriate amount of enzyme solution was incubated at 70°C for 30 min. The reaction mixture was stopped by keeping the mixture in a boiling water bath for 5 min. One unit of enzyme activity was defined as the activity that liberates 1 µmole equivalent of unsaturated galacturonic acid in 1 ml of the reaction mixture under these conditions for 1 h, and the molar extinction coefficient of the unsaturated galacturonic acid at 235 nm was regarded to be 4,600 M−1 cm−1. Protease (PPase) activity was assayed by measuring the amount of pectin liberated from proteopin by the carbazole-sulfuric acid method. The reaction was done in a mixture containing 10 mg of proteopin (prepared from lemon peel), 980 µl of Tris-HCl buffer, pH 8.0, and 20 µl of enzyme solution in a total volume of 1 ml at 50°C for 30 min. One unit of enzyme activity was defined as the activity liberating pectin corresponding to 1 µmole of p-galacturonic acid in 1 ml of reaction mixture at 50°C for 1 h. Lemon peel proteopin was prepared by the method described previously.

Electrophoresis of proteins. The homogeneity and molecular mass of the enzyme were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli on 10% (W/V) polyacrylamide gel, and the protein bands were stained with Coomassie Brilliant Blue G-250. The molecular weight standards used were from Bio-Rad (Japan Bio-Rad Laboratories, Tokyo), which included phosphorylase b (97,400), serum albumin (66,700), ovalbumin (45,000), carbonic anhydrase (31,000), trypsin inhibitor (21,500), and lysozyme (14,000).

Isoelectric focusing. Isoelectric focusing was done using a Bio-Rad Model III mini IEF cell with 5% (W/V) acrylamide gel and Bio-Lyte 3/10 (Japan Bio-Rad Laboratories) according to the manufacturer’s instructions. After electrophoresing, protein bands were stained with a silver stain kit (Japan Bio-Rad Laboratories). The pl of the enzyme was estimated from the relationship between the mobilities of the standard proteins (Isoelectric Focusing Calibration kit, Japan Bio-Rad Laboratories) and their pls.

N-terminal amino acid sequence. Around 100 pmol of purified enzyme was electrophoresed on 10%
SDS-PAGE and electrophoretically transferred onto a polyvinylidene fluoride membrane (Immobilon-Psq, Millipore) in 100 mM N-cyclohexyl-3-aminopropanesulfonic acid (CAPS)-NaOH buffer, pH 11.0, at 50 V for 1.5 h. The enzyme-blotted membrane was put into a protein sequencer (ABI Model 475, Applied Biosystems, New York, U.S.A.).

**Protein concentration.** The protein concentration was determined spectrophotometrically at 280 nm, with the assumption that the absorbance of 1% enzyme solution is 10. The protein concentration was also measured using a Micro BCA protein assay reagent kit (Pierce, Rockford, Illinois, U.S.A.) with bovine serum albumin (BSA) as a standard.

**Results**

**Screening of thermophilic bacteria with pectolytic activity**

Thermostable PL producing microorganisms were isolated from soil samples by an enrichment technique using the medium described in Materials and Methods. Of the isolated strains, TS 28, 34, 35, and 47 made clear haloes on PGA agar medium. In Table 1, PL and PPhase activity of culture filtrates from these strains are shown. In all strains, both activities were detected and the ratios of PPhase activity to PL activity (q-value) were between 0.13 and 0.24. One of the objectives of this research is screening of thermostable proteopectin-solubilizing enzyme (proteopectinase) available for biological scouring of cotton fabric (BioScouring). As the main process of BioScouring is enzymatic solubilization of proteopectin in cotton fiber, the enzyme having high q-value is favorable. The q-value of strain TS 47 was the highest of the strain, and strain TS 47 was selected and used throughout in this experiment.

**Identification of the microorganisms**

The properties of strain TS 47 were investigated. The microorganism was Gram-positive, spore-forming, and rod-shaped (0.5 to 1.0 by 3.0 to 6.0 μm). It grew well under aerobic conditions. The isolate was positive for reduction of nitrate, hydrolysis of starch, utilization of citrate, and production of catalase and cytochrome oxidase, and negative for formation of acetoin (Voges-Proskauer test), formation of indole from tryptophan, and gas from glucose. It grew over a range of 45°C to 60°C, and at initial pH values between 7 and 9. Based on the results, strain TS 47 was defined as an obligate thermophilic strain belonging to the genus *Bacillus*, and was tentatively designated as *Bacillus* sp. TS 47.

**Purification of thermostable pectate lyase**

From 11 of culture filtrate of strain *Bacillus* sp. TS 47, the PL was purified by column chromatographies on butyl-Toyopearl 650M (Fig. 1), SP-Toyopearl 650M, and Superdex 75 HR 10/30 (Fig. 2). The final purification step, that involved Superdex 75 column chromatography, the enzyme activity was eluted as a single peak, in which enzyme activity was parallel to the protein concentration, as shown in Fig. 2. The molecular mass of the enzyme was determined to be 20 kDa by the analysis using Superdex 75 column chromatography. A typical purification steps of the enzyme is shown in Table 2. By this procedure, the
enzyme was purified approximately 10,449-fold from culture filtrate with about 11% yield. The homogeneity of the enzyme was analyzed by SDS-PAGE. As shown in Fig. 2, SDS-PAGE revealed a single protein band and molecular mass was determined to be 50 kDa. The isoelectric point of PL 47 was found to be pH 5.3.

**N-terminal amino acid sequence**

The first 20 amino acids from the N-terminus of PL 47 were sequenced and aligned with other PLs (Fig. 3). The sequence showed significant similarity (50% identity in a 20-amino-acid overlap) with the N-terminal region of PLs from *B. subtilis* SO113 (BsPel)\(^{26}\) and IFO 3134 (PPase-N)\(^{9}\) but less similar to the same region of PLs from Gram-negative bacteria of *Erwinia* species, such as PelC\(^{26}\) and PeIE\(^{20}\).

**Effects of pH and temperature**

The effect of pH on PL 47 activity towards PGA at 70°C in four different buffers covering the range from pH 2 to 11 is shown in Fig. 4(A). The enzyme was most active at pH 8.0, and was quite stable in various buffers at different pHs between 4 and 11 when incubated at 37°C for 16 h, as shown in Fig. 4(B). The optimal temperature for activity was around 70°C, as shown in Fig. 5(A). To measure the thermal stability, PL 47 was incubated at various temperature for 30 min in 20 mM Tris-HCl (pH 7.5) containing 50 µg/ml BSA. The enzyme was stable up to 70°C, as shown in Fig. 5(B), and its half-lives were 13 and 1 h at 65 and 70°C, respectively. The activation energy \(E_a\) was given from the slope of the log \(k\):1/T (Arrhenius plots), where \(k\) is initial reaction rate, and \(T\) is the absolute temperature. The activation energy of the enzyme reaction on 0.1% PGA
Fig. 6. Arrhenius Plots Showing Relationship of Temperature to \( V_{\text{max}} \).

Fig. 7. Course of Release of Pectic Substance from Cotton Fabrics by Purified PL 47.

Cotton fabrics (40 mg) were incubated at 65°C in 1 ml of 100 mM Tris-HCl buffer (pH 8.0) with purified PL 47 (0.55 μg). Samples were taken out, filtered, and pectic substance contents measured by the carbozole-sulfuric acid method. The percentage of released pectic substance from cotton fabrics was calculated on the basis of total galacturonic solubilized from cotton fabrics by treating at 100°C in 1 N NaOH for 100 min.

was 32.5 kcal/mole, and the \( Q_{10} \) (the value of increasing of reaction rate by increasing temperature in 10°C) value was calculated to be 4.51 in the temperature between 60 and 70°C (Fig. 6).

*Effects of metal ions*

Generally, bacterial PLs require Ca\(^{2+}\) for maximum activity. The effect of Ca\(^{2+}\) concentration on the PL activity of PL 47 was determined. For the PL 47, the addition of 100 mM ethylenediaminetetraacetate (EDTA) to the reaction mixture completely suppressed PL activity under the standard assay conditions without Ca\(^{2+}\), and it was fully recovered when CaCl\(_2\) was added. In the absence of EDTA, maximal PL activity was obtained in the presence of CaCl\(_2\) (between 0.5 and 1 mM, data not shown). The effects of different metal ions on of PL 47 activity were determined by adding the appropriate salts (1 mM) to standard reaction mixture. The enzyme activity was inhibited strongly by Ag\(^{+}\), Ba\(^{2+}\), and Zn\(^{2+}\), moderately by Cd\(^{2+}\), Cu\(^{2+}\), and Mn\(^{2+}\), and was not affected by Fe\(^{2+}\), Hg\(^{2+}\), Ni\(^{2+}\), Mg\(^{2+}\), Co\(^{2+}\), Na\(^{+}\), and K\(^{+}\).

*Substrate specificity*

The substrate specificities of the purified PL 47 were tested using PGA with different degrees of methoxylolation. The enzyme activity decreased with increasing methoxyl group content of the substrate (Table 3). The \( K_m \) for PGA (methoxylolation ratio was 0%) was determined as 850 μg/ml and \( V_{\text{max}} \) value for PGA (methoxylolation ratio was 0%) degrading reaction was 1.1 mmol unsaturated products per min per mg protein, respectively.

The PPase activity of PL 47 on lemon protopectin was assayed, and the results are shown in Table 4. From the results, PL 47 was revealed to be the same kind of PL as PPase-N from *B. subtilis* IFO 3134.\(^4\)

PL 47 has pectin-releasing activity besides PL activity. However, the ratio of PPase activity (activity on insoluble pectic substance) to PL activity (activity on soluble pectic substance) was lower than that of PPase-N (Table 4). The pectin-releasing activity from cotton fabrics by PL 47 was also examined; when cotton fabrics (40 mg) incubated at 65°C in 1 ml of 100 mM Tris-HCl buffer (pH 8.0) with purified PL 47 (0.55 μg), 92.1% of pectic substance present in the cotton fabrics was solubilized in 3 h (Fig. 7).

\[
\text{Table 3. Lyase Activity of PL 47 toward Polygalacturionate with Different Degree of Methoxylolation}
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<table>
<thead>
<tr>
<th>Substrate</th>
<th>Methoxylation (%)</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGA</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Me-PGA</td>
<td>22</td>
<td>93.5</td>
</tr>
<tr>
<td>Me-PGA</td>
<td>67</td>
<td>27.4</td>
</tr>
<tr>
<td>Me-PGA</td>
<td>89</td>
<td>8.2</td>
</tr>
</tbody>
</table>

Reactions were done at 50°C and pH 8.0 for 30 min using 0.32 μg of PL 47 in 0.025% of substrate. Me-PGA: Methoxylated pectic acid.

\[
\text{Table 4. Pectate Lyase and Protosectinase Activity of PL 47}
\]

<table>
<thead>
<tr>
<th>Activity (U/mg)</th>
<th>PL 47</th>
<th>PPase-N</th>
</tr>
</thead>
<tbody>
<tr>
<td>PL activity</td>
<td>139.7</td>
<td>2010</td>
</tr>
<tr>
<td>PPase activity</td>
<td>679.0</td>
<td>17455</td>
</tr>
<tr>
<td>PPase activity/PL activity</td>
<td>4.9</td>
<td>8.7</td>
</tr>
</tbody>
</table>

PL and PPase activities of both enzymes were assayed at 50°C and pH 8.0.
Fig. 8. Viscosity Changes and Degradation of Polygalacturonate by Purified PL 47.
PL 47 was added to standard reaction mixture in a final volume of 10 ml and, at intervals, viscosity was measured at 50°C. The initial viscosity of the reaction mixture without enzyme was taken as 100%. To stop the reaction, the samples was boiled for 10 min. The percentage of cleavage was calculated on the basis of total galacturonic acid in the substrate. ○, relative viscosity; △, extent of glycosidic linkage cleavage.

The profile of changes of the reducing value and of viscosity of the reaction medium are used to determine the action mode of PGA-degrading enzymes. In PL 47 reaction on PGA, when viscosity of the reaction mixture in 50%, about 20% of the glycosidic linkages of the PGA were split. The rapid decrease in viscosity of PGA solution with a small increasing in reducing groups suggests that the enzyme degrades the substrate in a random manner (Fig. 8). Thus, PL 47 is appeared to be an enzyme belonging to endo-polygalacturonate trans-eliminase (PL, EC. 4.2.2.1).

**Discussion**

In this study, a thermostable PL, designated PL 47, was purified to homogeneity from a culture filtrate of thermophilic *Bacillus* sp. TS 47 isolated from soil. The enzyme was an *endo-polygalacturonate* lyase that required Ca²⁺ for enzyme reaction, as well as PLs from other origins.

The PL 47 is thermostable comparing to PLs from methophilic *Bacilli*; PL 47 is most active at 70°C (pH 8.0) and the half lives for inactivation are 13 h and 1 h at 65°C and 70°C, respectively, whereas the PLs from *B. subtilis* IFO 3134 (PPase-N), *B. subtilis* IFO 12113 (PPase-M), and *B. licheniformis* IFO 14206 (PPase-L) are inactivated completely by incubating at 70°C within 5 min. On the other hand, the optimum temperature of PL 47 reaction is around 70°C (at pH 8.0), whereas those from *B. pumilus* and *B. subtilis* are at 60°C and that of *B. polymyxa* is at 45°C. Thus, PL 47 is outstanding not only in its thermostability but in its thermo-activity.

The activation energy of PL 47 on 0.1% PGA is 32.5 kcal/mol, and the Q₁₀ value was observed to be 4.51 when the reaction activity was determined at 60 and 70°C. These values are apparently higher than that of PL from *B. stearothermophilus*: its activation energy have been reported as 17.1 kcal/mol and Q₁₀ value as 2.35 (from 60°C to 70°C), respectively. As little knowledges accumulated with the character of thermostable PL, up to now, this research is the first dealing with thermostable PL, actually, and the PL 47 is the most thermostable and thermo-active PL in the Bacilli PLs, so far reported.

The PL 47 has PPase activity not only on lemon propectin but cotton fabrics, and released pectic substance. Considering together with its outstanding thermostability, PL 47 would be suitable for scouring of cotton fabrics.

On butyl-Toyopearl column chromatography (Fig. 1), a chromatography based on hydrophobicity of proteins, PL 47 is eluted at a low ammonium sulfate concentration: It is eluted with 5%-saturated ammonium sulfate. The fact may indicate that PL 47 is a protein having higher hydrophobicity comparing with PPase-N, that is eluted at concentration of about 40%-saturated ammonium sulfate on the same column chromatography. The molecular mass of PL 47 was 20 kDa by size exclusion column chromatography and to be 50 kDa by SDS-PAGE. Recently, we cloned the PL 47 gene and determined its nucleotide sequence. Based on the results, from the deduced amino acid sequence, putative molecular mass of PL 47 is 47 kDa. Thus, SDS-PAGE is better method to determine the molecular mass of PL 47. Concerning the difference of molecular mass, depending on the determination method, of PL 47, we assume that PL 47 might have compact and rigid structure so that it move slowly in size exclusion column chromatography comparing to standard molecular marker protein, and such a unique structure, together with its hydrophobicity, might related to its thermostability.

The pI value (pH 5.3) of PL 47 is much more acidic than similar enzymes from other species of the Bacilli. Although the pIs of PLs from bacteria vary dependent on their origins, they are alkaline pHs (between 9 and 10), except that PLa from *E. chrysanthemi* EC16 has pI at acidic side (pH 4.6). The PLa has much less macerating activity (a synonym of so called protopinase activity) than the other macerating factor of plant tissue (a synonym of protopinaise). Sakai et al. have proposed to indicate the degree of protopinase activity (maceration activity) by an index, so called g-value. Namely, g-value is expressed the activity on protopextin/the activity on polygalacturonate (or polymethylgalacturonate).
The PL 47 (pI is pH 5.3) have proteopectinase activity against lemon protocatechin, however, the q-value of the enzyme, 4.9, is not as high as that of PPase-N (pI is pH 9.2) from mesophilic *B. subtilis* (q-value is 8.7). Favey et al. have speculated that macerating activity of PL depended on their pI: The PL having higher pI shows the higher macerating activity. The difference in q-values with PL 47 and PPase-N supports their speculation.\(^{28}\)

The N-terminal amino acid sequences of PL 47 are homologous with that of PLs from *B. subtilis* IFO 3134 (PPase-N) and SO113, up to 20 amino acids sequences. Moreover, the polyclonal antibody against PPase-N was cross-reacted with PL 47 (data not shown). From these results, we assume that PL 47 is highly homologous with mesophilic pectate lyase on the criterion of their three-dimensional structure. The three-dimensional structures of PL from *E. chrysanthemi*\(^{30,31}\) and *B. subtilis*\(^{29}\) have been determined. These enzymes have unique three-dimensional structures, the “parallel β-helix”, that is generated by coiling a β-strand into a large, right-handed helix with an unusual stacking of asparagines on consecutive turns of the parallel β-helix core. We assumed that the PL 47 could have a similar structure as the PLs from mesophilic *B. subtilis*. With the PL 47, in order to elucidate the relationship between molecular structure and proteopectinase activity, we are currently working on its molecular structure.

References


