PURIFICATION AND PROPERTIES OF TWO PECTATE LYASES PRODUCED BY ERWINIA CAROTOVORA

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Pectate lyase of Erwinia carotovora Er was separated into two active protein fractions, pectate lyases I (pI 10.7) and II (pI 10.1), by electrofocusing. These enzymes could be distinguished by SDS slab gel electrophoresis. Although they showed many similar properties, the optimal temperatures of pectate lyases I and II were 50° and 60°, respectively, and their $K_m$ values were 0.12 mg/ml and 1.1 mg/ml. The amino acid composition was very similar in the two enzymes, but pectate lyase I contained a few more residues of lysine, valine, glycine and proline than pectate lyase II and fewer residues of isoleucine. The neutral sugar content of the lyases was 2.5% and 4.8%, respectively. From these results, pectate lyases I and II seem to be different enzyme proteins despite the fact that they have quite similar properties.

Pectate lyase, which splits the glycosidic bond of poly-α (1→4) galacturonic acid and produces unsaturated end, is found in many bacteria (1-4) and fungi (1, 5). It is divided into two groups, exotype and endotype, based on the nature of the cleavage of pectic acid (1). Oligogalacturonate lyase which cleaves digalacturonide and oligogalacturonides is also known in some soft-rot bacteria (6). Most of endo-pectate lyases which are produced extracellularly by bacteria have almost the same properties. The pH optima of endo-pectate lyases ranged between pH 8.0 and 9.5 and the calcium ion is required for their activity (1). Among the soft-rot bacteria endo-pectate lyase has been shown to play an important role in pathogenesis in plant (7, 8).

1 Abbreviation: SDS, sodium dodecylsulfate; Caps, 3-(Cyclohexylamino)-1-propanesulfonic acid.

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In a previous paper (9), we reported the properties of pectate lyase in the soft-rot bacteria *Erwinia carotovora* which was purified as a single protein determined by SDS polyacrylamide disc gel electrophoresis after four steps of column chromatography. The molecular weight of this enzyme was estimated to be about 36,000 to 38,000 and the optimal pH was 9.0 to 9.2.

Recently, the multiplicity of pectate lyase, separated by the difference in isoelectric points, was reported in some bacteria, and some of these isozymes were characterized (7, 10). In this study, we separated the pectate lyase from *Erwinia carotovora* into two forms by electrofocusing and examined their properties.

**MATERIALS AND METHODS**

*Organism and culture conditions.* *Erwinia carotovora* Er (formerly *E. aroideae* Er, AMS 6082) was used as the enzyme source. It was grown in M9 medium containing pectic acid (0.5%) prepared from citrus (Wako Pure Chemical Industries, Ltd., Osaka) and casamino acid (0.2%) (Nissui Seiyaku, Tokyo) at 30° for 8 hr with shaking.

*Enzyme purification.* Two fractions of pectate lyase were purified as described by Kamimiya et al. (9) with a slight modification. The culture supernatant (20 l) was concentrated to about one tenth of the original volume with a flash evaporator below 30°. The concentrate was dialyzed against 50 volumes of deionized water at 4° overnight and then against 50 volumes of 10 mM potassium phosphate buffer (pH 7.0) with two changes of the buffer at 4° overnight. All purification procedures thereafter were carried out at 4°, unless otherwise stated. The dialyzed solution was then purified by three steps of column chromatographies, using DEAE cellulose, CM-cellulose and Sephadex G-100 as described previously (9).

After the active fractions were pooled and dialyzed against a few changes of 100 volume of distilled water, the enzyme solution was subjected to column electrofocusing (type 8101, LKB, Bromma, Sweden). The column (110 ml) was prepared according to the LKB manual. A pH gradient of 7 to 11 was made with Ampholine 9–11 (2.4 ml), Ampholine 8–10 (0.1 ml) and Ampholine 6–8 (0.3 ml). A density gradient (0 to 50% (w/v)) of sorbitol (E. Merck, Darmstadt, West Germany) was used. After electrofocusing at 2° for 7 days at a maximum voltage of 850 V, 2 ml fractions were collected and both enzyme activity and pH were measured immediately. Active fractions were pooled, dialyzed against several changes of distilled water for 3 days, lyophilized and stored at −20° until used.

*Preparation of substrate.* Pectic acid prepared from apples (Wako Pure Chemical Industries, Ltd., Osaka) was washed three times with 70% (v/v) ethanol to remove soluble materials and washed with 80%, 90% and pure ethanol and with acetone, successively. Both partially and highly methylated polygalacturonates were prepared by the method of Manabe (11) and the degree of methylation of the substrate was determined according to the method of Hirota (12).
Enzyme assay. The reaction mixture for pectate lyase assay was composed of 0.01 g pectic acid, 0.05 M Tris-HCl buffer (pH 9.0), 1 mM CaCl₂ and 0.5 ml of enzyme solution in a total volume of 5 ml. Unless otherwise stated, after the enzyme solution was added, the mixture was rapidly transferred to a quartz cuvette and the absorbancy at 235 nm was continuously monitored in a Hitachi-124 spectrophotometer equipped with a cuvette holder held at 30°. One unit of pectate lyase activity was arbitrarily defined as the amount of enzyme which increases an absorbancy of 0.01 at 235 nm per min. To measure $K_m$, various concentrations of pectate ranging from 0.4 mg/ml to 8 mg/ml were used, and apparent $K_m$ values were determined by Lineweaver-Burk plots. Protein concentration was determined by the method of Lowry et al. (13) using bovine serum albumin as the standard.

Polyacrylamide gel electrophoresis. Polyacrylamide disc gel electrophoresis was performed by the method of Taber and Sherman (14). The protein (50 μg) in 50 μl of 8 mM glycine-KOH buffer (pH 10.3) containing 0.058% tetramethyl-ethylene diamine and 20% sucrose was layered on top of the concentration gel. A current of 2 mA per gel (5 x 90 mm) was applied and electrophoresis was carried out for 1 hr. The gel was stained for carbohydrate with periodate-fuchsin Schiff reagent according to the method of Zacharius et al. (15). The gel was scanned at 550 nm by a Beckman Spectrophotometer ACTA CIII. After scanning, the gel was stained with coomassie brilliant blue R250 for protein detection. Electrophoresis in 10% polyacrylamide slab gel in the presence of 0.1% SDS was carried out for molecular size determination as described by Weber and Osborn (16) using the gel system of Laemmli (17).

Analysis of amino acid and carbohydrate content. Amino acid content was analyzed according to the method of Moore and Stein (18) using a Hitachi KLA-5 amino acid analyzer. The content of tryptophan residue was estimated spectrophotometrically by the method of Edelhoch (19). Neutral sugar content was estimated by the phenol-sulfuric acid method (20) with glucose as the standard.

RESULTS

Separation of the pectate lyase into two fractions

Pectate lyase isolated as a single protein peak by Sephadex G-100 gel filtration gave, on Ampholine column electrofocusing, two protein peaks which agreed well with the peaks of pectate lyase activities (Fig. 1). The two peaks were pectate lyases I (pI 10.7) and II (pI 10.1). Both enzyme fractions were nearly homogeneous, as shown in Fig. 2. On SDS polyacrylamide slab gel electrophoresis, the two lyases migrated slightly differently, though they could not be separated from each other by SDS disc gel electrophoresis (9). The difference between the molecular sizes of the pectate lyases was estimated to be about 1,000, assuming that their migration was affected only by the molecular weight of the proteins.

Two pectate lyase fractions were subjected separately to disc gel electrophoresis
using an alkaline buffer system according to the method of TAKER and SHERMAN (14). The gel was stained with periodate-Schiff reagent to detect carbohydrates, and then stained with coomassie blue for protein. A single band in each gel which
was stained slightly by periodate-Schiff reagent corresponded exactly with the protein band (data not shown).

**Effects of pH and temperature**

The optimal pH was 10 for both pectate lyases I and II.

The activities of the pectate lyases were changed hardly at all when the enzymes were incubated at room temperature for 9 hr in 50 mM sodium phosphate (pH 6.0, 6.5 and 7.0), Tris-HCl (pH 8.0 and 9.0) or glycine-NaOH buffers (pH 9.0, 10.0 and 11.0).

On the other hand, optimal temperatures for activities of the two pectate lyases were 50° and 60°, respectively (Fig. 3). At temperatures higher than 52°, the specific activity of pectate lyase I was much higher than that of pectate lyase II. This was in contrast to the specific activities of these two enzymes at lower temperatures.

**Effect of divalent cations**

Like the pectate lyases isolated from various other sources, these enzymes were
also activated by calcium ions. At the ion concentration of 0.5 mM, pectate lyases I was activated about 7-fold and II about 4-fold. On the other hand, Sr\(^{2+}\) inhibited the activity of pectate lyases I and II by 28% and 63%, respectively, and other divalent cations (Zn\(^{2+}\), Ni\(^{2+}\), Cu\(^{2+}\), Cd\(^{2+}\), Mg\(^{2+}\) and Mn\(^{2+}\)) had a similar inhibitory effect.

**\(K_m\) for pectic acid**

Apparent \(K_m\) values for pectic acid of pectate lyase I and II were 0.12 mg/ml, and 1.1 mg/ml, respectively. Thus, the affinity of pectate lyase I was about ten times higher than that of pectate lyase II. The two lyases were most active toward the polygalacturonate whose carboxyl residues were methylated by 20 to 50% and by 20 to 40%, respectively (Fig. 4). However, both enzymes were distinguished from pectin lyase (21) which cleaves specifically highly esterified substrate.

**Amino acid composition and carbohydrate content**

The amino acid composition was quite similar in the two enzymes (Table 1). Pectate lyase I, however, contained extra residues of lysine, valine, glycine and proline, and pectate lyase II contained extra residues of isoleucine. The extra lysine

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Pectate lyase I</th>
<th>Pectate lyase II</th>
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<tbody>
<tr>
<td>Lys</td>
<td>27</td>
<td>24</td>
</tr>
<tr>
<td>His</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
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<td>Phe</td>
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<td>10</td>
</tr>
<tr>
<td>Trp</td>
<td>6</td>
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</table>

* The values for threonine and serine were extrapolated to the zero time of hydrolysis.
* The values for valine, isoleucine and leucine were the highest of all residue.
residues contained in pectate lyase I could be attributed to the higher isoelectric point of this enzyme.

As mentioned above, both pectate lyases contained carbohydrates. The neutral sugar content of pectate lyase II was about two times higher than that of pectate lyase I (Table 2).

**DISCUSSION**

In a previous paper, we reported the purification and the properties of pectate lyase from *Erwinia carotovora* as a single protein (9). Pectate lyase was always eluted as a single peak from both CM-cellulose and Sephadex G-75 columns. And only one band of protein had been observed on SDS polyacrylamide disc gel electrophoresis. However, when the purified pectate lyase was subjected to gel electrofocusing, it was separated into two bands each with almost the same amount of protein, as shown in Fig. 1.

These enzymes were not separated by gel filtration with Sephadex G-50, 75 and 100 (data not shown). The molecular weights of the two pectate lyases were almost the same, but there was a little difference in their migration on SDS slab gel electrophoresis as shown in Fig. 2. Inconsistent results observed between disc gel and slab gel electrophoresis of the lyases may be due to a difference in the resolution abilities of the gel systems.

The difference in the molecular weights of pectate lyases I and II was estimated to be around 1,000 based on the difference in mobilities of the two enzymes on SDS slab gel electrophoresis. But it is known that the migration on SDS gel electrophoresis is affected not only by the molecular weight but also by the electric charge of the protein (22), and an abnormal migration on SDS gel electrophoresis has been known in proteins containing carbohydrate moieties (23). Since both pectate lyases contain carbohydrates, it is difficult to estimate the exact difference in molecular weight between them. From the difference in the amino acid composition the difference in molecular weight was calculated to be 1,250.

Isozymes of pectate lyase have been found in some bacteria (7, 10). Garibaldi and Bateman (7) characterized the isozymes of pectate lyase of *Erwinia chrysanthemi*. The isozymes of *E. chrysanthemi* had isoelectric points of 9.4, 8.4, 7.9 and

<table>
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<tr>
<th>Neutral sugar content</th>
<th>% (w/w)</th>
<th>Nearest integer*</th>
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<tbody>
<tr>
<td>Pectate lyase I</td>
<td>2.0</td>
<td>4</td>
</tr>
<tr>
<td>Pectate lyase II</td>
<td>4.8</td>
<td>10</td>
</tr>
</tbody>
</table>

* Values are the number of residues as glucose per molecule of pectate lyase based on a molecular weight of the protein moiety of 37,000.
4.6. The first three of these isozymes could degrade and macerate intact plant tissues, but not the last one. Among those three isozymes, no significant difference in properties were reported. The two pectate lyases of *E. carotovora* reported in this paper, had properties similar to those isozymes of *E. chrysanthemi* which had isoelectric points in the alkaline region.

Most of the properties of pectate lyases I and II were not significantly different from each other and, on the whole, were almost the same as many pectate lyases reported from other bacterial sources (1). However, a small difference was observed between pectate lyases I and II with respect to the *Km* values for the substrate, optimum degrees of substrate methylation, and optimum temperatures.

There were also small differences in amino acid composition and carbohydrate content. But it is still not clear whether pectate lyases I and II are isozymes identified by distinct genes or by belonging to multiple forms produced by modification of one original protein.

In a previous paper, we reported that the optimum pH of pectate lyase was 9.0 using Tris–HCl buffer (9). However, in the present experiment we used three different buffer systems ranging from pH 7.2 to 10.8, and found that both pectate lyases have the optimum pH at 10.0. Therefore, the optimum pH reported previously should be revised accordingly. Previously, we reported that the pectate lyase activity decreased in proportion to the degree of methyl-esterification (9). However, in the present experiment, both pectate lyases were consistently most active toward partially methylated polygalacturonate. Since it is known that the most suitable substrate of many pectate lyases from various sources is partially methyl-esterified polygalacturonate (1), the previous report of the suitable substrate should be corrected.

REFERENCES

19) H. Edelhoch, Biochemistry, 6, 1948 (1967).