ENZYMIC DEGRADATION OF PECTIC ACID

IX. Limited Hydrolysis of Pectic Acids by Fungal Exopolygalacturonase

Chitoshi HATANAKA and Junjiro OZAWA

Several workers (1, 2) reported that fungal exopolygalacturonase (exo-PG), unlike carrot exopolygalacturonase (CPG), could degrade pectic acid completely. It seems likely, however, that these observations were due to the contamination of the enzyme preparation with endopolygalacturonase (endo-PG). The results in previous work (3), obtained by using unsaturated pectic acid and exo-PG preparations of Sclase (strong, Sankyo Co. Ltd.) containing a small amount of endo-PG, suggested the inability of fungal exo-PG to decompose pectic acid completely. Afterwards, we succeeded in preparing Sclase exo-PG free from endo-PG and, by the use of this enzyme preparation, conclusive evidence that Sclase exo-PG degrades pectic acid incompletely was obtained. In this paper these results are presented.

Miller (4) reported two exo-PGs of Aspergillus niger; one displayed its full activity in the presence of Hg ions and the other showed no requirement for metal activators. In our laboratory effect of Hg ions on activity of the Sclase exo-PG and CPG was investigated and the results obtained are also described in this paper.

MATERIALS AND METHODS

1. Substrates

Digalacturonic acid and acid-soluble pectic acid were prepared as described in previous papers (5, 6). Average degree of polymerization was found to be 14.9 for acid-soluble pectic acid. Pectic acid was prepared from commercial citrus pectinic acid by saponification with sodium hydroxide (0.05N) at 0°C for 90 min. (7).

 Unsaturated acid-soluble pectic acid. A crude preparation of unsaturated acid-soluble pectic acid (acid-soluble pectic acid having 4,5-unsaturated galacturonic acid unit at the non-reducing end of the molecule) was made by treating commercial citrus pectinic acid with alkali at 100°C for 5 min. (7). CPG was allowed to act on this preparation until no more galacturonic acid was liberated (3, 7).

2. Enzymes

 Exo-PG of Sclase. Sclase (100 g) was suspended in 0.02 M acetate buffer, pH 5 (300 ml), and brought into solution by adding 1 N acetic acid. After being adjusted to pH 6 with 1 N sodium hydroxide the solution was heated rapidly to 100°C and kept for 20 min. at 100°C. After cooling it was brought to pH 5 with 1 N acetic acid, saturated with ammonium sulfate and kept overnight in the ice chest. The mixture was filtered through diatomaceous earth to remove precipitate and dialyzed successively against tap water (several hours), distilled water (1 day)
and 0.02 M acetate buffer, pH 4.6 (2 days). The dialyzed solution was added to a Duolite CS-101 column (4 x 20 cm) prepared in 0.02 M acetate buffer, pH 4.6, and washed with the same buffer. The effluent having PG activity was collected and applied to a DEAE-cellulose column equilibrated with 0.02 M acetate buffer, pH 4.6. The column was washed with the same buffer and eluted with 0.2 M acetate buffer, pH 4.6. The eluate having PG activity was dialyzed successfully against water and 0.02 M acetate buffer, pH 4.6, pervaporated and dialyzed once more against the same buffer. This was called exo-PG I and found to be free from endo-PG.

From a Sclase preparation made at different date a PG solution (exo-PG I') was prepared by the same procedure as above. To 100 ml of this solution were added about 50 ml of 1 % rivanol solution and 0.8 g of fuller's earth (Katayama Kagaku Co. Ltd.) without being activated. After stirring the mixture was allowed to stand for a while and then centrifuged. The precipitate was washed with water, suspended in 40 ml of 1 M sodium acetate solution at 0°C, kept in the ice chest for about 30 min. and centrifuged to remove the precipitate. The pale yellow supernatant was perfectly decolorized by mixing with fuller's earth. The supernatant was adjusted to pH 4.6 with acetic acid and dialyzed successively against water (overnight) and 0.02 M acetate buffer, pH 4.6 (exo-PG II).

The remaining portion of the exo-PG I' above-mentioned was kept frozen for 10 months at -10~ -20°C and thawed. This was treated with rivanol and dialyzed as in the case of exo-PG II (exo-PG III).

Experiments with unsaturated acid-soluble pectic acid as substrate indicate that exo-PG I and exo-PG III are free from endo-PG like CPG (Fig. 1).

Fig. 1. Hydrolysis of unsaturated acid-soluble pectic acid by various exo-PG preparations. Reaction mixture: unsaturated acid-soluble pectic acid, 0.1 %; acetate buffer, pH 4.65, 0.04M; various units (PGU/ml) of heated Sclase extract*, Sclase exo-PG (exo-PG I, II, III, A(3)) or CPG per ml of reaction mixture. Incubation; 27°C, 15 hr. 0--0, Heated Sclase extract; ⧫--○, exo-PG I; ⧫--○, exo-PG II; ⧫--○, exo-PG III; ⧫--○, exo-PG A1; ○--○, CPG.

* Crude Sclase extract (pH 6) was heated at 100°C for 20 min.
Enzymic Degradation of Pectic Acid

CPG. This was prepared by the method previously described (8) but without the adsorption on calcium phosphate gel.

3. Analytical Methods

Uronic acid content (substrate concentration) was determined by the carbazole method (9) and hydrolysis rate of substrate was calculated from increased reducing power of reaction mixtures. The reducing power of reaction mixtures was determined by the micro Willstätter-Schudel method (10).

RESULTS

Degradation Limit of Pectic Acid by Exo-PG

Exo-PG III hydrolyzed citrus pectic acid incompletely (Fig. 2). The limit value of degradation is about 42% and it is almost the same as that of CPG.

![Figure 2](image)

**Fig. 2.** Limited hydrolysis of pectic acid by Sclase exo-PG and CPG. Reaction mixture: pectic acid, 0.05%; acetate buffer, pH 4.65, 0.02 M; various units of exo-PG III or CPG per ml of reaction mixture. Incubation: 27°C, 15 hr. •—•, Exo-PG III; ○—○, CPG.

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<th>TABLE 1</th>
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Influence of HgCl₂ and EDTA on the enzymic hydrolysis of acid-soluble pectic acid and digalacturonic acid

<table>
<thead>
<tr>
<th></th>
<th>Exo-PG I</th>
<th>CPG</th>
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<tbody>
<tr>
<td></td>
<td>Acid-soluble pectic acid</td>
<td>Digalacturonic acid</td>
</tr>
<tr>
<td>HgCl₂</td>
<td>95.7</td>
<td>101.3</td>
</tr>
<tr>
<td>EDTA*</td>
<td>98.5</td>
<td>103.0</td>
</tr>
<tr>
<td>Without HgCl₂ and EDTA</td>
<td>100(16.6)**</td>
<td>100(36.6)**</td>
</tr>
<tr>
<td>HgCl₂</td>
<td>95.2</td>
<td>102.3</td>
</tr>
<tr>
<td>EDTA*</td>
<td>100.7</td>
<td>100.2</td>
</tr>
<tr>
<td>Without HgCl₂ and EDTA</td>
<td>100(10.2)**</td>
<td>100(15.7)**</td>
</tr>
</tbody>
</table>

* Reducing power of reaction mixture was estimated by determining galacturonic acid by the naphthoresorcinol method (11).
** Percentage hydrolysis.
Fig. 3. Influence of pH on the enzymic hydrolysis of pectic acid and acid-soluble pectic acid. Reaction mixture: substrate, 0.25%; acetate buffer, 0.1M; suitable units of exo-PG I or CPG. Incubation; 35°C, 1 hr. Ex—Exo-PG I, pectic acid; ●—Exo-PG I, acid-soluble pectic acid; ○—CPG, pectic acid; □—CPG, acid-soluble pectic acid.

Effect of pH on the Activity of Exo-PG

Exo-PG I showed a rather broad pH optimum from 4.0 to 4.5 for pectic acid and from 4.45 to 4.75 for acid-soluble pectic acid. The optimum pH of CPG was found to be about 4.7 both for pectic acid and acid-soluble pectic acid (Fig. 3).

Effect of HgCl₂ and EDTA on the Activity of Exo-PG

The activity of exo-PG I and CPG was scarcely affected by HgCl₂ and EDTA the concentrations of which were 5 μM and 0.1% respectively (Table. 1).

DISCUSSION

Fungal exo-PG free from endo-PG was prepared from Sclase, a commercial pectinase preparation. It might be by mere chance that we succeeded in preparing endo-PG-free exo-PG of Sclase, because the elimination of endo-PG from exo-PG preparations seems to be affected seriously by the starting material and the conditions in the procedure. The procedure involved heat-treatment, fractionation with ammonium sulfate, treatment with Duolite CS-101, chromatography on DEAE-cellulose and rivanol-treatment. The enzyme preparations obtained showed no activity toward unsaturated pectic acid and hydrolyzed pectic acid incompletely, the limit value of degradation being about 42%. The value was almost the same as that obtained with CPG.

Since the exo-PG preparations obtained in the present study was found being devoid of the enzyme which hydrolyzes 4,5-unsaturated galacturonide linkage, absence of endo-PG in these preparations was ascertained by their inability to hydrolyze unsaturated acid-soluble pectic acid. When pectic acid is used as substrate,
its low limit of degradation is taken as evidence of absence of endo-PG. Inability to hydrolyze of unsaturated pectic acid and incompleteness of hydrolysis of pectic acid are considered as better criteria of purity of exo-PG than the apparent homogeneity in ultracentrifugation or in electrophoresis.

Exo-PG of Sclase and CPG were not activated by HgCl₂.

**SUMMARY**

1) The fungal exo-PG preparation free from endo-PG was made from Sclase. It hydrolyzed pectic acid incompletely, the limit value of degradation being about 42%. The value was almost the same as that obtained with CPG.

2) Neither Sclase exo-PG nor CPG was activated by HgCl₂.

**LITERATURE CITED**


