ENZYMIC DEGRADATION OFPECTIC ACID

XI. Identity of the Enzyme Hydrolyzing 4, 5-UnsaturatedGalacturonidic Linkage with Exopolygalacturonase

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Earlier reports from our laboratory demonstrated that carrot exopolygalacturonase (1) and an exopolygalacturonase preparation (2) made from Sclase (a commercial pectinase preparation of Coniothyrium diploidiella) were devoid of activity toward pectic acid having a 4, 5-unsaturated galacturonic acid unit at the non-reducing end of the molecule. Besides such enzymes there was observed in Sclase an enzyme hydrolyzing the 4, 5-unsaturated galacturonidic linkage (3). The preceding paper showed that similar enzyme was also produced by Aspergillus niger. In those studies, however, it was not possible to determine whether this enzyme activity is due to an exopolygalacturonase or to an enzyme specific for the 4, 5-unsaturated galacturonidic linkage. The present study provides evidence that this enzyme is a sort of exopolygalacturonase.

MATERIALS AND METHODS

Crude enzyme solution. According to the method described in the preceding paper, a mycelial extract of Aspergillus niger (isolated from a rotted root of sweet potato) was treated with Duolite CS-101 and chromatographed on a DEAE-cellulose column. The eluate containing the enzyme hydrolyzing 4, 5-unsaturated galacturonidic linkage [fraction No. 8 (4)] was used as crude enzyme solution.

Methods for preparation of substrates and for sugar analysis. These were the same as previously described (4).

RESULTS

Chromatography on a DEAE-cellulose column. About 20 ml of the crude enzyme solution just described was added to a column (2×6 cm) of DEAE-cellulose previously equilibrated with 0.02 M acetate buffer, pH 4.6. Elution was carried out with a linear concentration gradient of pH 4.6 acetate buffer: the mixer containing 250 ml of the 0.02 M buffer and the reservoir 250 ml of the 0.5 M buffer. Tube volume was 5 ml. As shown in Fig. 1, a fairly symmetrical peak was obtained. The peak was divided into three parts, which were lettered A, B and C.

Heat treatment of crude enzyme solution. The crude enzyme solution (see Materials) was chromatographed on a DEAE-cellulose column as above and the tubes corresponding to the peak were pooled. The enzyme so obtained was heated in 0.1 M acetate buffer, pH 4.0, at 50 and 60°C. After a fixed time this was
Fig. 1. Rechromatography of No. 8 on a DEAE-cellulose column. Assay conditions: acid-insoluble pectic acid, 0.25 %; acetate buffer, pH 4.6; about 0.1 M; incubation, 35°C, 30 min. (PGu)m: μmole of bonds split (determined by reducing power measurement) per ml of enzyme solution per hr. O---O (PGu)m/, •—• protein concentration, --- acetate buffer concentration.

cooled and assayed with pectic acid and 4,5-unsaturated digalacturonic acid as substrates. As shown in Fig. 2, the enzyme was stable at 50°C. It was labile, however, at 60°C: about 50% of its activity was lost at 60°C for 10 minutes. By the treatment under such conditions the ratio of activities toward pectic acid

Fig. 2. Rate of inactivation of rechromatographed No. 8 at 50 and 60°C. No. 8 (rechromatographed with DEAE-cellulose) was incubated in 0.1 M acetate buffer (pH 4.0) at 50 or 60°C and then the remaining activity was assayed at the same pH, using 0.2 % pectic acid and 4,5-unsaturated digalacturonic acid as the substrates. Conditions for incubation were the same as described for Fig. 1. The preparation, pretreated at 60°C for 10 min., was referred to below as the enzyme H. O—O Pectic acid, •—• 4,5-unsaturated digalacturonic acid, --- pretreated at 50°C, — pretreated at 60°C.
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and 4,5-unsaturated digalacturonic acid was not changed. The enzyme solution obtained by the treatment at 60°C for 10 minutes will be called enzyme solution H.

Effect of pH on activities of enzyme solutions A, B, C and H. Enzyme solutions A, B, C and H were allowed to act on pectic acid, digalacturonic acid and 4,5-unsaturated digalacturonic acid at varying pH. Fig. 3 shows the pH-activity curves obtained. pH optima for these substrates are at about 4, 5 and 4.6, respectively. There are no appreciable difference among A, B, C and H.

TABLE 1

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Enzyme</th>
<th>Aldehyde groups released (µmole/ml of enzyme/hr.)</th>
<th>Relative rate*</th>
</tr>
</thead>
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<tr>
<td>Pectic acid</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>A</td>
<td>1.456</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>B</td>
<td>1.832</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>C</td>
<td>1.784</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>H</td>
<td>1.720</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Digalacturonic acid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>8.85</td>
<td>6.08</td>
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</tr>
<tr>
<td>B</td>
<td>11.50</td>
<td>6.28</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>11.25</td>
<td>6.31</td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>10.95</td>
<td>6.37</td>
<td></td>
</tr>
<tr>
<td>4,5- Unsaturated digalacturonic acid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>0.129</td>
<td>0.089</td>
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</tr>
<tr>
<td>B</td>
<td>0.164</td>
<td>0.090</td>
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</tr>
<tr>
<td>C</td>
<td>0.163</td>
<td>0.091</td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>0.151</td>
<td>0.088</td>
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</tbody>
</table>

Assay conditions: substrate, 0.2%; acetate buffer, 0.05 M; pH 4.0 (pectic acid), pH 4.9 (digalacturonic acid), pH 4.6 (4,5-unsaturated digalacturonic acid); suitable amounts of enzyme; incubation, 35°C, 30 min.

* Based on the same enzyme concentration and on the initial linear portions of the rate curves.
Ratio of the activities toward pectic acid, digalacturonic acid and 4,5-unsaturated digalacturonic acid. In Table 1 a comparison is made of the activities of A, B, C and H toward pectic acid, digalacturonic acid and 4,5-unsaturated digalacturonic acid. Hardly any difference is observed among the four enzyme solutions. Ratio of the activity toward pectic acid, digalacturonic acid and 4,5-unsaturated digalacturonic acid, taking that toward pectic acid as unity, is about 1 : 6.3 : 0.09.

Effect of mercuric chloride on the enzymic activity. Effect of mercuric chloride on the enzymic activity was studied with digalacturonic acid as substrate. The results obtained are shown in Fig. 4. In all cases maximum activation was observed with 1~2 μM mercuric chloride. On the contrary, the presence of 10 μM mercuric chloride retarded enzymic hydrolysis.

Effect of enzyme concentration on the hydrolysis rate of pectic acid, digalacturonic acid and 4,5-unsaturated digalacturonic acid. The effect of varying the enzyme concentration was studied by using pectic acid, digalacturonic acid and 4,5-unsaturated digalacturonic acid as substrates (0.2%). So long as the percentage hydrolysis does not exceed a certain limit, the amount of product formed is proportional to the concentration of enzyme: the reaction is considered as being of zero order (Fig. 5). In the present study assay conditions were so chosen that a linear relationship could be found.

Activity of hydrolyzing 4,5-unsaturated galacturonidic linkage and of exopolygalacturonase. Relationship between velocity of enzymic reaction and concentration of substrates — The substrates used were digalacturonic acid and 4,5-unsaturated digalacturonic acid. The rate of hydrolysis increased with the substrate concentration, giving typical Michaelis-Menten plots (Fig. 6). Values
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Fig. 5. Relation of time and enzyme concentration to the hydrolysis of pectic acid, digalacturonic acid and 4,5-unsaturated digalacturonic acid. Assay conditions: substrate, 0.2%; acetate buffer, 0.1 M, pH 4.0 (pectic acid), pH 4.9 (digalacturonic acid), pH 4.6 (4,5-unsaturated digalacturonic acid); incubation, 35°C, 30 min. (1) 1.8, (2) 0.9, (3) 0.45, (4) 10.0 and (5) 5.0 units of enzyme B were used in 2 ml of reaction mixture. (A) Pectic acid, (B) digalacturonic acid, (C) 4,5-unsaturated digalacturonic acid.

for Km were estimated from these plots as the substrate concentration for one-half maximum velocity. The values of Km were $4.3 \times 10^{-4}$ M for digalacturonic acid and $9.23 \times 10^{-4}$ M for 4,5-unsaturated digalacturonic acid.

Inhibitory effect of galacturonic acid on the enzymic activity — Fig. 7 shows Lineweaver and Burk plots in the presence and absence of galacturonic acid. In this experiment digalacturonic acid and 4,5-unsaturated digalacturonic acid were used as substrates. The results in the figure indicate that enzymic activity toward digalacturonic acid and 4,5-unsaturated digalacturonic acid is decreased in the presence of galacturonic acid and that the inhibition is competitive. With digalacturonic acid as substrate the value of $K_i$ was found to be $5.15 \times 10^{-4}$ M. It was $5.88 \times 10^{-4}$ M in case of 4,5-unsaturated digalacturonic acid.

Effect of digalacturonic acid and 4,5-unsaturated digalacturonic acid on the enzymic activity — Activity toward digalacturonic acid is inhibited by 4,5-unsaturated digalacturonic acid (Fig. 7A). Since 4,5-unsaturated digalacturonic acid itself hydrolyzes under the enzyme action, the rate of inhibition appears to be difficult to determine on the basis of reducing power measurement. The effect of 4,5-unsaturated digalacturonic acid, however, is not very significant, because enzymic activity at pH 4.5 toward this compound is less than one-sixtieth that toward digalacturonic acid and decreases further in the presence of the latter. It is apparent that the two compounds inhibit competitively the hydrolysis of each other. The $K_i$ value for 4,5-unsaturated digalacturonic acid is calculated from the plots in the figure to be $8.67 \times 10^{-5}$ M. This value agrees fairly well with $K_m$ of this compound ($9.23 \times 10^{-6}$M).

When the enzyme is allowed to act on 4,5-unsaturated digalacturonic acid in the presence of digalacturonic acid, galacturonic acid is rapidly produced and increases in amount immediately after the start of reaction, and digalact-
Fig. 6. Influence of substrate concentration on the reaction rate. (A) Substrate: digalacturonic acid. Assay conditions: acetate buffer, pH 4.6, 0.1 M; enzyme, 0.3 unit of enzyme B per 2 ml of reaction mixture; incubation, 35°C, 20 min. (B) Substrate: 4,5-unsaturated digalacturonic acid. Assay conditions: acetate buffer, pH 4.6, 0.1 M; enzyme, 8.1 units of enzyme B per 2 ml of reaction mixture; incubation, 35°C, 30 min. \( v \): \( \mu \)mole of bonds split (determined by recucing power measurement) per ml of enzyme solution per hr. \( \circ -- \circ v, \bullet -- [S]/v \).

Uronic acid decreases more rapidly than 4,5-unsaturated digalacturonic acid, because enzymic activity is far greater toward digalacturonic acid than toward 4,5-unsaturated digalacturonic acid. For these causes the rate of inhibition of 4,5-unsaturated digalacturonic acid hydrolysis by digalacturonic acid becomes difficult to determine. Fig. 8 shows the results of an experiment made on the effect of galacturonic acid and digalacturonic acid on the activity toward 4,5-unsaturated digalacturonic acid. The rate of hydrolysis of 4,5-unsaturated digalacturonic acid was determined here by measuring the decrease in optical density.
Fig. 7. Effect of galacturonic acid and 4,5-unsaturated digalacturonic acid on the enzyme activity. (A) Substrate: digalacturonic acid. Assay conditions were the same as described for Fig. 6 A. (B) Substrate: 4,5-unsaturated digalacturonic acid. Assay conditions were the same as described for Fig. 6 B. •—• +4,5- Unsaturated digalacturonic acid 2.442 mM, ○—○ +galacturonic acid 2.338 mM.

Fig. 8. Effect of the concentration of galacturonic acid and digalacturonic acid on the enzyme activity toward 4,5-unsaturated digalacturonic acid. Assay conditions: substrate, 4,5-unsaturated digalacturonic acid, 1.344 mM: acetate buffer, pH 4.6, 0.1 M; enzyme, 4 units of enzyme B per ml of reaction mixture: incubation, 35°C, 30 min. Reaction rate was computed from the decrease in absorption at 232 m." O—O Galacturonic acid, •—• digalacturonic acid, •—• digalacturonic acid (expressed as galacturonic acid unit).

at 232 m.". The rate of inhibition by digalacturonic acid is higher than that by galacturonic acid when compared at the same molar concentrations. The reverse is the case, however, at the same concentrations of galacturonic acid unit. Fig. 9
Fig. 9. The enzyme activity toward 4,5-unsaturated digalacturonic acid and digalacturonic acid. Assay conditions: substrate, (1) digalacturonic acid (2.564 mM), (2) 4,5-unsaturated digalacturonic acid (2.688 mM), (3) digalacturonic acid (2.564 mM)+4,5-unsaturated digalacturonic acid (2.688 mM); acetate buffer, pH 4.6, 0.1 M; enzyme, 1.6 units of enzyme B per ml of reaction mixture; incubation, 35°C. Reaction rate was determined by reducing power measurement (ml of 0.002 N I₂ consumed per 0.5 ml of reaction mixture).

shows the rate of enzymic hydrolysis of digalacturonic acid or 4, 5-unsaturated digalacturonic acid or both. The rate of total hydrolysis in the mixture of the two substrates is far lower than the sum of hydrolysis rates of the individual substrates.

DISCUSSION

According to the method described in the preceding paper, fractions having an activity of hydrolyzing 4, 5-unsaturated galacturonic linkage were separated from the mycelial extract of Aspergillus niger by treatment with Duolite CS-101 and DEAE-cellulose chromatography. When one of these fractions was rechromatographed on DEAE-cellulose columns a single and symmetrical peak was obtained, which was arbitrarily divided into three parts A, B and C. From the peak obtained as above in another experiment an enzyme preparation H was made by heating it at 60°C for 10 minutes. Among A, B, C and H no significant difference in properties was observed. The results that A, B, C and H, and the enzyme preparations inactivated by heat to varying extent did not differ from one another in the ratio of the activity toward 4, 5-unsaturated digalacturonic acid and digalacturonic acid seem especially to indicate the homogeneity of these enzyme preparations.

For the purpose of obtaining further evidence that the enzyme hydrolyzing 4, 5-unsaturated galacturonicid linkage is a sort of exopolygalcturonase, experiments were made on whether 4, 5-unsaturated digalacturonic acid and digalacturonic acid inhibit competitively the hydrolysis of each other. Analysis of the
results by the method of Lineweaver and Burk showed that the inhibition of hydrolysis of digalacturonic acid by 4, 5-unsaturated digalacturonic acid was competitive. The value of $K_i$ for 4, 5-unsaturated digalacturonic acid was $8.67 \times 10^{-6}$ M. This agrees fairly well with the value of $K_m$ for this compound ($9.23 \times 10^{-6}$ M). The rate of total hydrolysis in the mixture of 4, 5-unsaturated digalacturonic acid and digalacturonic acid was found to be far lower than the sum of hydrolysis rates of the individual substrates. Galacturonic acid, a degradation product of these substrates, also acted as a competitive inhibitor. The values of $K_i$ for galacturonic acid obtained with digalacturonic acid and 4, 5-unsaturated digalacturonic acid as substrates coincided with each other: the values were $5.15 \times 10^{-4}$ M for digalacturonic acid and $5.88 \times 10^{-4}$ M for 4, 5-unsaturated digalacturonic acid.

From the above results it seems reasonable to consider that the enzyme hydrolyzing 4, 5-unsaturated digalacturonic acid is a sort of exopolygalacturonase, that is, the activities of hydrolyzing 4, 5-unsaturated digalacturonic acid and of exopolygalacturonase are attributed to one enzyme. Since the exopolygalacturonases have so far been reported to be devoid of activity toward pectic acid having a 4, 5-unsaturated galacturonic acid unit at the non-reducing end of the molecule, the enzyme of *Aspergillus niger* described in this paper is considered as being a new type of exopolygalacturonase.

**SUMMARY**

The present study has been undertaken to determine whether the activity on 4, 5-unsaturated galacturonicid linkage is due to a specific enzyme or to an exopolygalacturonase which hydrolyzes both saturated and unsaturated galacturonicid linkages. Ratio of activities on 4, 5-unsaturated digalacturonic acid and pectic acid was practically constant before and after heat treatment of the enzyme preparations. The competitive inhibition by galacturonic acid was observed when saturated or unsaturated digalacturonic acid was used as substrate. The latter two compounds also inhibited competitively the hydrolysis of each other. These results suggest that the enzyme hydrolyzing 4, 5-unsaturated galacturonicid linkage is identical with exopolygalacturonase.

**LITERATURE CITED**