A Saccharifying Pectate trans-Eliminase of *Erwinia aroideae*

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A saccharifying pectate trans-eliminase was found in the cells of *Erwinia aroideae*. This enzyme differs from known pectate trans-eliminase in the next two points. It degrades pectic acid liberating 4,5-unsaturated digalacturonic acid from the chain end of the molecule. It does not require calcium ion.

Some properties of 4,5-unsaturated digalacturonic acid, the main product of the saccharifying pectate trans-elimination, were also described in this paper.

In the past few years, it has become increasingly evident that uronic acid polymers are enzymically degraded to unsaturated oligouronides\(^1\). The trans-eliminases which decompose pectic substances are classified into two groups, pectin trans-eliminases\(^2\) and pectate trans-eliminases\(^3\). The former enzymes are inactive towards pectic acid, whereas the latter degrade pectic acid more rapidly than pectin. These enzymes cause trans-elimination reactions resulting in the cleavage of glycosidic linkages and the formation of double bonds between C-4 and C-5. Pectate trans-eliminases seem to be widely distributed in bacteria. Nagel and Vaughn have shown that the extracellular pectate trans-eliminase of *Bacillus polymyxa* catalyzes a random degradation of chain molecule. However, in our studies reported earlier\(^4\), saccharifying polygalacturonase activity was observed in the cells of *Erwinia aroideae*. Later investigation\(^5\) has shown the presence of a saccharifying pectate trans-eliminase responsible for a portion of this activity. Present paper describes the results of a more detailed investigation of this enzyme.

**MATERIALS AND METHODS**

**Preparation of Pectate trans-Eliminases.** A strain of *E. aroideae*, isolated from Japanese radish, was grown on a shaker at 27°C, potato extract (100 ml) containing 0.5% peptone, 0.1% KH\(_2\)PO\(_4\) and 0.5% Na\(_2\)HPO\(_4\)·12H\(_2\)O being used as medium. After 20 hours growth, the bacterial cells were harvested, washed with water and acetone-dried. The acetone-dried cells (0.1 g) were extracted overnight at 4°C. The extracts were passed through a column of Duolite CS-101 which had been equilibrated with 0.02M phosphate buffer, pH 7.0. The eluate was brought to a pH of 9.0 with 0.1 N-NaOH, kept at 45°C for 10 minutes and then used as enzyme solution of saccharifying pectate trans-eliminase\(^6\). This will be called enzyme solution A. Activity of forming DKGA\(^7\) or DKFA\(^8\) from pectic acid was not observed in this enzyme solution. In addition to this, another enzyme solution of saccharifying pectate trans-eliminase was obtained.

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8. The abbreviations used are: DKGA, 4-deoxy-5-keto-p-galacturonic acid (4-deoxy-L-threo-5-hexoseulose uronic acid); DKFA, 4-deoxy-5-keto-p-fructuronic acid (3-deoxy-D-glycero-2,5-hexodiulosonic acid).
prepared by the following method. Washed cells of *E. aroideae* were suspended in 0.02 M phosphate buffer, pH 7.5, containing a small amount of toluene. After standing two hours at 27°C, the suspension was centrifuged and the supernatant was discarded. The cells were again suspended in the buffer now without toluene and kept overnight at 4°C. This was centrifuged at 7000×g and the supernatant was passed through a column of Duolite CS-101, equilibrated with 0.02 M phosphate buffer, pH 7.0. The eluate was used as enzyme solution (enzyme solution B).

Enzyme solution B has a slight DKGA- or DKFA-forming activity at pH 7.5, but not at all at pH 9.56). The column, used for the preparation of enzyme solution B, was washed sufficiently with the buffer. The resin was transferred to a flask and suspended in 0.5 M-NH4HPO4. A 0.5N solution of sodium hydroxide was added with stirring at 4°C to raise the pH of supernatant to 7.5. The supernatant was dialyzed against 0.02 M phosphate buffer, pH 7.5, and used as enzyme solution of liquefying pectate trans-eliminase (enzyme solution C). Another enzyme solution of liquefying pectate trans-eliminase was prepared from the broth by the following procedures. The broth was centrifuged at 7000×g and dialyzed against 0.02 M phosphate buffer, pH 7.5, containing 0.5 mM-CaCl2. Duolite CS-101, equilibrated with the phosphate buffer (0.02 M, pH 7.0), was added to the dialysate. The mixture was stirred for 30 minutes at 4°C and then filtered. The residue was sufficiently washed with the buffer and suspended in water. The pH of supernatant was lowered to 4.5 by the addition of 0.2 N-HCl at 4°C. The supernatant was used as enzyme solution of liquefying pectate trans-eliminase (enzyme solution D), after the dialysis against 0.02 M phosphate buffer, pH 7.5, containing 0.5 mM-CaCl2.

Preparation of Substrates. 4,5-Unsaturated digalacturonic acid was prepared by the following method. *E. aroideae* was grown stationarily at 27°C on a medium containing 1% pectic acid, 0.4% (NH4)2HPO4, 0.2% KH2PO4, 0.5% Na2HPO4·12H2O and 0.05% MgSO4·7H2O. After 6 days incubation, the broth was centrifuged and concentrated under reduced pressure. It was then passed through a column of cation exchange resin (H-form). The eluate was neutralized with barium hydroxide and insoluble salts were filtered off. Alcohol fractionation (25 to 85%) was repeated three times for the filtrate and the final precipitate was dissolved in water. After treatment with cation exchange resin, the aqueous solution was placed on a column of Amberlite 410 which had been brought to OH-form. The column was washed with water and eluted with 0.3 M sodium carbonate solution. Fractions containing only thiobarbiturate-reacting material10 were collected. Sodium ion in the eluate was replaced with barium ion, and the crude salts of 4,5-unsaturated digalacturonic acid was precipitated with alcohol. The precipitate, after being dried, was dissolved in water and the pH was brought to 2.0 with sulfuric acid and ion exchange resin. The impurities were removed by 72 hours ether extraction in a continuous flow, liquid-liquid, extractor. The aqueous layer was neutralized with calcium carbonate and, after filtration, evaporated in the ice chest. Crystallization occurred in a few days. Two recrystallizations were carried out. Brucine salt of this compound was also prepared. After recrystallization from moist acetone, the m.p. was 180~182°C (decomp.). This compound was identified as 4-O-β-D-(4,5-dehydrogalacturonosyl)-D-galacturonic acid from the following results6). The molecular weight is 363. The ratio of carboxyl to aldehyde group is similar to that of digalacturonic acid. On hydrolysis it gives formic acid, 2-furancarboxylic acid, 5-formyl-2-furancarboxylic acid and n-galacturonic acid. It consumes 0.84 mol. bromine per mol. It shows an absorption maximum at 232 mµ, reacts with thiobarbituric acid to give a product which absorbs at 545~550 mµ and on treatment with ozone gives rise to the formation of oxalic acid. Periodate oxidation followed by treatment with iodine or bromine affords tartaric acid.

Pectin was prepared from "Citrus Pectin" (Nippon Kako Co. Ltd.). Other substrates were obtained as described previously.

Results

Mode of Action of Saccharifying Pectate trans-Eliminase. Saccharifying and liquefying pectate trans-eliminases were incubated respectively...
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FIG. 1. Changes in Reducing Power, Viscosity and Absorption at 230 m\(\mu\) of Pectic Acid Solution by the Action of Saccharifying Pectate trans-Eliminase*.

* Enzyme solution A was used.

FIG. 2. Changes in Reducing Power, Viscosity and Absorption at 230 m\(\mu\) of Pectic Acid Solution by the Action of Liquefying Pectate trans-Eliminase*.

* Enzyme solution D was used.

When incubated with the saccharifying pectate trans-eliminase, pectic acid solution showed a large increase in reducing power and a small decrease in viscosity. On the other hand, with the liquefying pectate trans-eliminase, the ratio of the increase of reducing power to the decrease of viscosity was found to be lower. Furthermore, the changes which occurred during incubation were followed with the aid of paper chromatography. In the case of the reaction mixture of the saccharifying pectate trans-eliminase, only one compound identified as 4,5-unsaturated digalacturonic acid was observed from the early stages of degradation. Other reducing substances could not be detected even after almost complete degradation, so far as the high-molecular-weight pectic acid was used as substrate. In the case of the liquefying pectate trans-eliminase digest, it was not until the perfect diminution of the viscosity that some reducing substances were detected. These substances were confirmed to be a variety of 4,5-unsaturated oligouronides.

As described in the preliminary report, the ratio of increased absorption at 230 m\(\mu\) to increased carboxyl value of the saccharifying pectate trans-eliminase digest, freed from polygalacturonic acid by the addition of barium chloride, was identical with that of absorption at 230 m\(\mu\) to carboxyl value of 4,5-unsaturated digalacturonic acid. These results indicate that the saccharifying pectate trans-eliminase ruptures the second linkage of pectic acid molecule from the chain end.
Properties of the Saccharifying Pectate trans-Eliminase. pH optimum for the saccharifying pectate trans-eliminase seems to be about 8.0~8.3. Optimum for the action of liquefying pectate trans-eliminase was somewhat higher and was at pH 8.8 (Fig. 3).

Calcium requirement of bacterial pectolytic enzymes has been reported by many workers\(^1\). Extracellular liquefying pectate trans-eliminase of *B. polymyxa* requires calcium ion\(^3\). The same result was obtained with the intracellular liquefying pectate trans-eliminase of *E. aroidae*. However, the saccharifying pectate trans-eliminase was found to be scarcely affected by calcium ion (Fig. 4).

It is known that liquefying pectate trans-eliminase degrades pectic acid more rapidly than pectin, in the case of that of *B. polymyxa*, ratio of the activities towards pectic acid and pectin being about 4\(^3\). As shown in

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Fig. 5, the saccharifying pectate trans-eliminase was almost inactive towards pectin.

Thermal Degradation of 4,5- Unsaturated Digalacturonic Acid, the Main Product of the Saccharifying Pectate trans-Elimination. Earlier investigation\(^{12}\) showed that exopolysaccharidase preparation from carrot hydrolyzed digalacturonic acid, though the reaction velocity was much lower as compared with pectic acid. However, this preparation was found later to be inactive towards 4,5-unsaturated digalacturonic acid. As described in the preliminary report\(^ {9}\), 4,5-unsaturated digalacturonic acid was converted into DKGA or DKFA by crude cell-free extracts of \textit{E. aroideae}.

Since little was known about non-enzymic hydrolysis of 4,5-unsaturated digalacturonic acid, the following experiments were carried out on the thermal degradation of this compound. A solution of 0.08 M 4,5-unsaturated digalacturonic acid, pH 3.0, was heated in an ampoule for 30 minutes at 120°C. Treatment of the heated solution with magnesium powder and diluted hydrochloric acid led to the formation of formaldehyde which gave a chromotropic acid test. This result indicates the presence of formic acid. Paper chromatograms also showed a corresponding spot. In addition to formic acid, 2-furancarboxylic acid, 5-formyl-2-furancarboxylic acid and D-galacturonic acid were found in the heated solution. These substances were identified by the methods described previously\(^ {9}\). Other acids and reducing substances were not detected chromatographically in the heated solution. Since an authentic sample of 5-formyl-2-furancarboxylic acid was found not to convert into 2-furancarboxylic acid under the same condition as the above (0.08 M, 120°C, 30 minutes), the following equations may be proposed for the thermal degradation of 4,5-unsaturated digalacturonic acid:

Measurements of reducing power and acidity were carried out with the above solution before and after heating. In addition, a

![Diagram](https://via.placeholder.com/150)

**TABLE I. CHANGES IN REDUCING POWER AND ACIDITY OF 4,5-UNSATURATED DIGALACTURONIC ACID SOLUTION DURING THERMAL DEGRADATION**

<table>
<thead>
<tr>
<th></th>
<th>Reducing power*</th>
<th>Acidity**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solution before heating</td>
<td>8.0</td>
<td>16.0</td>
</tr>
<tr>
<td>Heated solution</td>
<td>12.3</td>
<td>19.4</td>
</tr>
<tr>
<td>Ether extracts of heated solution***</td>
<td>4.3</td>
<td>11.3</td>
</tr>
</tbody>
</table>

* ml of 0.02 N-I\(_2\) consumed/ml.
** ml of 0.01 N-NaOH required to neutralize/ml.
*** Ether was evaporated and the residue was dissolved in the same quantity of water as that of the original heated solution.

portion of the heated solution was continuously extracted with ether for 72 hours, after being saturated with ammonium sulfate. Reducing power and acidity of this extracts were also estimated (Table I). By paper chromatography, no reducing substance other than 5-formyl-2-furancarboxylic acid was detected in the ether extracts. When an authentic sample of 2-furancarboxylic acid or 5-formyl-2-furancarboxylic acid was extracted with ether as above, the recovery was 99~100%. Formic acid was confirmed not to be reducing towards sodium hypoiodite. These results and the above equations suggest that the concentrations of formic acid (A), 2-furancarboxylic acid (B), 5-formyl-2-furancarboxylic acid (C) and D-galacturonic acid (D) in the heated solution may be calculated from the data given in Table I.

\[
\begin{align*}
C &= 4.3 \times 0.01 \text{ M} = 0.043 \text{ M} \\
A &= B = \frac{11.3 - 4.3}{2} \times 0.01 \text{ M} = 0.035 \text{ M} \\
D &= 0.043 \text{ M} + 0.035 \text{ M} = 0.078 \text{ M} \\
\text{Rate of degradation} &= \frac{7.8}{8.0} \times 100 = 97.5 \% 
\end{align*}
\]

**DISCUSSION**

Besides known liquefying pectate trans-eliminase, a saccharifying pectate trans-eliminase was found in the cells of *E. aroideae*. This enzyme liberates 4,5-unsaturated digalacturonic acid from the chain end of pectic acid molecule. The enzyme seems to be responsible for a large portion of saccharifying pectolytic activity of this microorganism. However, when the crude cell-free extracts of this microorganism were allowed to act on pectic acid, DKGA or DKFA was observed in the reaction mixture. Since neither liquefying pectate trans-eliminase nor saccharifying pectate trans-eliminase described above is capable of forming these compounds, some other specific enzyme may be present for this reaction. In order to clarify this point, further experiments are being conducted in these laboratories.

Non-enzymic hydrolysis of 4,5-unsaturated digalacturonic acid gives four compounds, formic acid, 2-furancarboxylic acid, 5-formyl-2-furancarboxylic acid and D-galacturonic acid. Our attempts to find out an enzyme which hydrolyzes 4,5-unsaturated digalacturonic acid have never been successful.

In the previous paper it was shown that cell suspensions of *E. aroideae* utilized pectic acid or digalacturonic acid more rapidly than galacturonic acid. This finding could be explained by the fact that, in the pectolytic enzymes of this microorganism, pectate trans-eliminases are predominant. A metabolic pathway of DKGA, the final product of pectate trans-elimination, has recently been demonstrated in a Pseudomonad by Preiss and Ashwell.

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