THE MECHANISM OF ACTION OF THE SACCHARIFYING PECTATE *TRANS*-ELIMINASE*

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In our studies reported earlier (1963), the presence of a saccharifying pectate *trans*-eliminase was observed in the cells of *Erwinia aroideae*. As this enzyme was found to be of exo-type unlike other polyuronate eliminase (polyuronate lyase) so far known, it was thought worth while to study the mechanism of its action. The present investigation was undertaken in order to determine whether the glycosidic bond of pectic acid is broken from the non-reducing or reducing end by the action of this enzyme.

EXPERIMENTAL AND RESULTS

Acid Soluble Pectic Acid. A solution of 2% pectin in 2% HCl was heated for two hours on a water bath. The precipitate containing the degraded pectic acid of higher molecular weight was centrifuged off and barium hydroxide solution was added to the supernatant. The resulting precipitate contained the degraded pectic acid of lower molecular weight. This was collected by centrifugation, washed with water and dissolved in dilute H_2SO_4, pH of the solution being adjusted to 2. 2. The precipitated barium sulfate was filtered away and barium hydroxide was added to the filtrate. These procedures were repeated until traces of low-molecular-weight oligouronides, examined by paper chromatography (Toyo paper, No. 52; n-butanol-acetic acid-water, 5 : 2 : 3), were removed. The final filtrate was neutralized with 0.1N-NaOH to pH 7. 0. To this solution, alcohol was added to precipitate sodium salt of acid soluble pectic acid. The precipitate was collected with a centrifuge and dried at room temperature. On the basis of reducing end determination, the average degree of polymerization (DP) of the acid soluble pectic acid was found being 11. 9.

Pectate trans-Eliminases. The saccharifying pectate *trans*-eliminase preparation possessing no activity of forming 4-deoxy-5-keto-D-glucuronic acid (or 4-deoxy-5-keto-D-fructuronic acid) previously described was used in this study. The liquefying pectate *trans*-eliminase preparation was obtained from the broth. *E. aroideae* was grown under the same conditions as described in the preceding

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paper (1964). The broth was centrifuged at 7000×g and dialyzed against 0.02M phosphate buffer, pH 7.5, containing 0.5mM-$CaCl_2$. Duolite CS-101, equilibrated with the phosphate buffer (0.02M, pH 7.5), was added to the dialyzate. 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. Dilute HCl (0.2N) was added with stirring at 4°C to bring the pH of the supernatant to 4.5. The supernatant was used as enzyme solution, after dialysis against 0.02M phosphate buffer, pH 7.5, containing 0.5mM-$CaCl_2$. This preparation may contain other pectolytic enzymes as impurities.

Oligouronides Produced from Acid Soluble Pectic Acid by the Action of Pectate trans-Eliminases

The saccharifying pectate *trans*-eliminase preparation were incubated with acid soluble pectic acid at 32°C. After four hours digestion, 4 m*l* of the reaction mixture was applied as a 40 cm band to Toyo No. 52 paper (40×40 cm) and run in n-butanol-acetic acid-water (5 : 2 : 3) for 17 hours. The paper was dried and sprayed with bromophenol blue reagent. Five yellow bands (1–5) were given in all. Bands 1 and 2 corresponded in position to D-galacturonic acid and 4, 5-unsaturated digalacturonic acid respectively. Band 5 contained the oligouronides which did not moved from the starting line. Band 2 was cut off together with band 1 which was faint and adjacent to band 2. The other bands were cut off separately. Each section was eluted with 8 m*l* of 0.2% ammonium oxalate solution. Naphthoresorcinol and thiobarbituric acid tests were carried out with these eluates. Table 1 shows that all the eluates from bands 3, 4 and 5 give naphthoresorcinol test and negative thiobarbituric acid

TABLE 1
Naphthoresorcinol and thiobarbituric acid tests on the oligouronides formed by the action of pectate *trans*-eliminases

Bands	Saccharifying pectate *trans*-eliminase Optical density		Liquefying pectate *trans*-eliminase Optical density	
	Naphthoresorcinol test	Thiobarbituric acid test	Naphthoresorcinol test	Thiobarbituric acid test
1+2	4.625	0.500	0.631	0.074
3	0.399	0.006	1.128	0.412
4	0.403	0.003	0.857	0.173
5	1.326	0.009	4.200	0.486
D-Galacturonic acid, 0.2μM	0.468			
4,5-Unsaturated digalacturonic acid, 0.2μM	0.425	0.260		

The incubation medium contained 2 m*l* of 1% acid soluble pectic acid or intact pectic acid solution and 2 m*l* of enzyme solution; pH of the medium, 8.0. Optical density was measured on Hitachi photoelectric photometer, EPO-B-type; No. 57 filter being used. Periodate oxidation in thiobarbituric acid test was for 15 min. at 80°C.

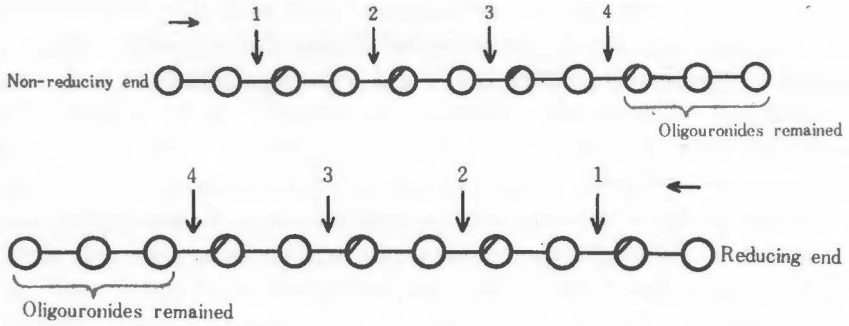
reaction. The recoveries from controls were found to be 97.5% for acid soluble pectic acid and 100% for 4, 5-unsaturated digalacturonic acid. These results suggest that oligouronides in bands 3, 4 and 5 are digalacturonic acid, trigalacturonic acid and oligouronides of DP≧4 respectively. It is unlikely that the formation of saturated oligouronides and D-galacturonic acid (Table 1) might have been caused by some contaminating polygalacturonate hydrolase in the saccharifying pectate *trans*-eliminase preparation, as 4, 5-unsaturated digalacturonic acid and continually shortening pectic acid chain are the only detectable products of the action of this enzyme preparation on intact pectic acid of high molecular weight. The liquefying pectate *trans*-eliminase was incubated with intact pectic acid and the oligouronides produced were examined as for the above reaction system of saccharifying pectate *trans*-eliminase and acid soluble pectic acid. In this case also, five bands including bands 1, 2 and 5, which corresponded to D-galacturonic acid, 4, 5-unsaturated digalacturonic acid and immovile oligogalacturonides respectively, appeared on paper chromatogram. The results in Table 1, however, shows that bands 3, 4 and 5 contain 4, 5-unsaturated oligogalacturonides of various molecular weights.

When the saccharifying pectate *trans*-eliminase was allowed to act on trigalacturonic acid under the same conditions as the above, there was produced 4, 5-unsaturated digalacturonic acid together with D-galacturonic acid, although not so readily as from pectic acid.

DISCUSSION

There are two glycosidic bonds in trigalacturonic acid molecule, α-D-GalpA1–4α-D-GalpA1–4-D-GalpA. If the saccharifying pectate *trans*-eliminase splits the first linkage of trigalacturonic acid molecule from the reducing end, digalacturonic acid and 4-deoxy-5-keto-D-glucuronic acid (or 4-deoxy-5-keto-D-fructuronic acid) must be formed. In contrast, the cleavage of the second linkage from the reducing end would result in the formation of D-galacturonic acid and 4, 5-unsaturated digalacturonic acid. The result described in the preceding section supports the latter mechanism, but not the former.

Let us consider the mode of action of the saccharifying pectate *trans*-eliminase on a acid soluble pectic acid. If the enzyme degraded this substrate in stepwise fashion starting from the non-reducing end, the products in the later stages of degradation would be digalacturonic acid, 4, 5-unsaturated oligouronides of DP≧2 and 4-deoxy-5-keto-D-glucuronic acid (or 4-deoxy-5-keto-D-fructuronic acid). On the other hand, if the enzymic degradation started from reducing end, the products would be 4, 5-unsaturated digalacturonic acid, saturated oligouronides of DP≧2 and D-galacturonic acid. The results shown in Table 1 agree with the latter mechanism. This permits the conclusion that the saccharifying pectate *trans*-eliminase ruptures the second likage of pectic



acid molecule from the reducing end. There are, to our knowledge, no exo-polysaccharases yet known which degrade the substrate molecules from the reducing end except for this enzyme.

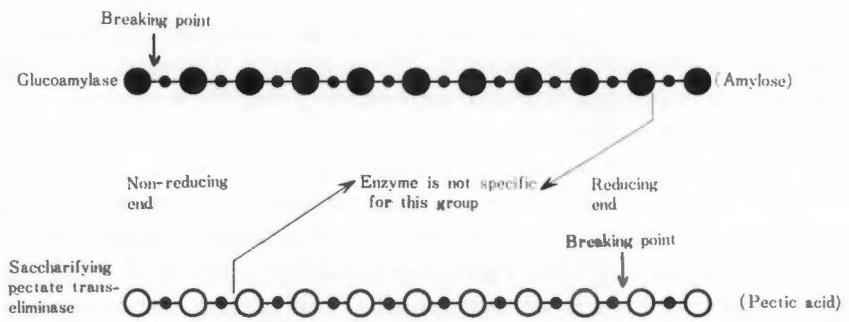
It is well known that polysaccharide hydrolases and transglycosidases split the glycosidic bonds between the carbonyl carbon atom and the oxygen bridge

Breaking point
A B
↓ ↓
R_1-O-R_2

R_1 arnd R_2 contain non-reducing end and reducing end respectively.

(A). Cohn expressed her opinion that the break of glycosidic bond comes on that side of the bridging atom which is nearest to the part of the molecule for which the enzyme is most specific, that is to say, it occurs as closely as possible to the group which is being transferred. Ludowieg et al. (1961) have clarified that bacterial hyaluronidase, belonging to a eliminase (lyase), acts to cleave a glycosidic bond on the alcohol side of oxygen bridge (B). Therefore, Cohn's mechanism also fits this case. From these facts and the finding in this study, the following hypothesis may be proposed for the mechanism of action of exo-polysaccharases:

The exo-polysaccharases splitting the gycosidic bonds between the carbonyl carbon atom and the oxygen bridge (A) degrade the substrate molecules from the non-reducing end, while those breaking the glycosidic bonds on the alcohol



side of oxygen bridge (B) decompose the substrates from the reducing end. For example, glucoamylase which splits R_1-O bond is most specific for the carbony group of non-reducing end glucose unit, but never specific for that of the second glucose unit from the reducing end. On the other hand, the saccharifying pectate *trans*-eliminase which breaks O-R_2 bond is specific for the group, -CH (OR)-CH(COOH)-, of the second galacturonic acid unit from the reducing end, but not for that of the third galacturonic acid unit from the non-reducing end.

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