SOME PROPERTIES OF THE SACCHARIFYING PECTATE TRANS-ELIMINASE OF ERWINIA AROIDEAE

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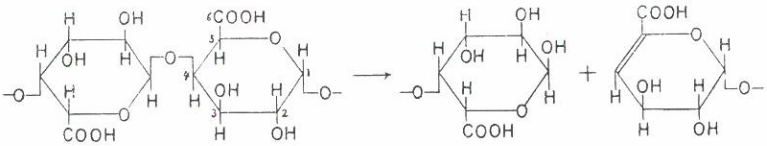
From the work of Linker et al. (1956) on bacterial hyaluronidases, it has become increasingly evident that uronic acid polymers are enzymically degraded to unsatnrated oligouronides. The eliminases which decompose pectic substances are classified into two groups, pectin *trans*-eliminases (Albersheim et al. 1960) and pectate *trans*-eliminases (Nagel and Vaughn 1951). The former enzymes are inactive towards pectic acid, whereas the latter degrade pectic acid more rapidly than pectin. Pectate *trans*-eliminases catalyze the reaction:



In this reaction, RO at C-4 and H at C-5 are eliminated to yield ROH. Moreover, the reaction results in the formation of double bond between C-4 and C-5. Since RO at C-4 and H at C-5 are in axial position, the reaction is a *trans*-elimination.

Pectate *trans*-eliminases seem to be widely distributed in bacteria. Nagel and Vanghn have shown that the extracellular pectate *trans*-eliminase of *Bacillus polymyxa* catalyzes a random degradation of chain molecule. However, in our studies reported earlier (Ozawa and Okamoto 1957), saccharifying polygalacturonase activity was found in the cells of *Erwinia aroideae*. Later investigation (Okamoto et al. 1963) have clarified that this activity can be attributed in part to a pectate *trans*-eliminase which ruptures the second linkage of pectic acid molecule from chain end, This enzyme is somewhat interesting in its mode of action, because most of exo-polysaccharases have been considered to split the first linkage of chain-molecules, the only one exception being β amylase. In the present paper, the results of a investigation on the properties of this pectate *trans*-eliminase are described.

MATERIALS AND METHODS

Preparation of Saccharifying Pectate trans-Eliminase. The strain of *E. aroideae* used in this study was isolated from Japanese radish in these laboratories. Potato extract (100 m*l*) containing 0.5% peptone, 0.1% KH_2PO_4 and 0.5% $Na_2HPO_4 12H_2O$ was taken in a 500 m*l*-flask and sterilized. *E aroideae* was inoculated and grown on a shaker at 27°C. After 20 hours growth, the bacterial cells were harvested with a centrifuge and washed with distilled water. The cells were suspended in 0.02M phoshpate buffer, pH 7.5, containing a small amount of toluene.

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After two hours standing at 27°C, the suspension was centrifuged and the supernatant was discarded. The cells were again suspended in the above 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 which had been equilibrated with 0.02M phosphate buffer, pH 7.0. The eluate was used as enzyme solution of saccharifying pectate *trans*-eliminase. This enzyme solution was found to have a activity of forming DKGA* or DKEA* (Preiss and Ashwell 1963) at pH 7.5. But this activity was not observed at pH 9.5. Therefore, when this enzyme solution was used for the saccharifying pectate *trans*-eliminase assay, incubation was carried out at pH 9.5.

Preparation of Liquefying Pectate trans-Eliminase. The above column used for the preparation of saccharifying pectate *trans*-eliminase was sufficiently washed with 0.02M phosphate buffer, pH 7.0. The resin was transferred to a flask and suspended in 0.5M-Na_2HPO_4. Dilute sodium hydroxide solution (0.5N) was added with stirring at 4°C until the supernatant had a pH of 7.5. The supernatant was dialyzed against 0.02M phosphate buffer, pH 7.5, and used as enzyme solution of liquefying pectate *trans*-eliminase

Preparation of Substrates

Pectin and pectic acid. — These were prepared from the commercial "Citrus Pectin" (Nippon Kako Co. Ltd.).

Degraded pectic acid. — This was obtained by the acid hydrolysis of pectin according to the method of Ehrlich and Schubert (1929). The average degree of polymerisation of this substrate was calculated at 21.8 on the basis of end group determination.

Oxidized pectic acids. — Pectic acid and degraded pectic acid were oxidized respectively with hypoiodite according to the method of Willstätter-Schudel.

Reduced pectic acid. — Pectic acid was reduced with sodium borohydride under the conditions similar to that described by McCready and Seegmiller (1954).

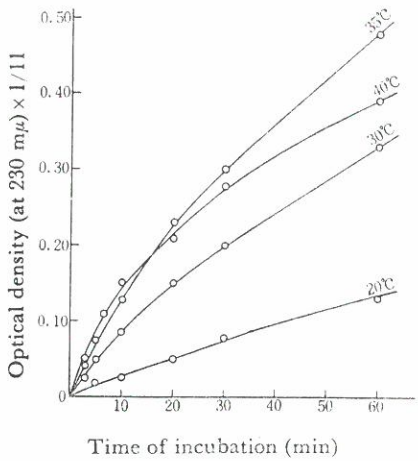
Other substrates were obtained as described previously.

Andlytical Methods. Thiobarbituric acid test was performed in the usual manner (Weissbach and Hurwitz 1959). Optical density at 230 mμ was measured on a Beckman DU spectrophotometer, cuvettes with a 1cm light path being used. both 4, 5-unsaturated digalacturonic acid and DKGA (or DKFA) are cleaved by periodate to form β formylpyruvate, which can be assayed with thiobarbituric acid. On the other hand, 4, 5-unsaturated digalacturonic acid alone can be measured in the ultraviolet absorption assay. Ascending paper cbromatography was carried out on Toyo No. 52 paper, n-butanol-acetic acid-water (4 : 1 : 2) being used as solvent system.

RESULTS

Effect of Temperature on Enzyme Activity. When the saccharifying pectate *trans*-eliminase preparation was incubated with pectic acid, higher enzyme activity was observed at 40°C rather than 35°C before 10 minutes incubation (Fig. 1). But thereafter the reaction proceeded

* The abdreviatins used are: DKGA, 4-deoxy-5-keto-D-glucuronic acid (4-deoxy-L-threo-5-hexoseulose uronic acid); DKFA, 4-deoxy-5-keto-fructuronic acid (3-deoxy-D-glycero-2, 5-hexo-diulosonic acid).



Fif. 1. Effect of temperature on the activity* of saccharifying pectate *trans*-eliminase. The reaction mixture contained 1 m*l* of 1% pectic acid, 3 m*l* of 0.02M borax-0.04N NaOH buffer. pH 9.5, and 1 m*l* of the enzyme solution. * In all Figures and Tables except Table 4, the activities of pectate *trans*-eliminases are expressed as optical density (at 230 mμ) ×1/11 of the reaction mixtues.

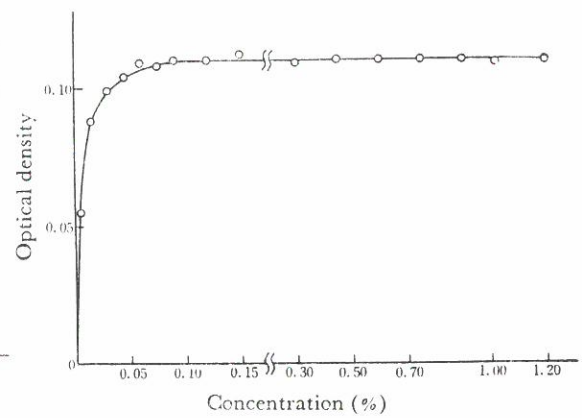


Fig. 2. Effect of concentration of pectic acid on the activity of saccharifying pectate *trans*-eliminase. The incubation were carried out at 30°C for 15 min.; 0.02 M borax 0.04N NaOH buffes, pH 9.5, being used.

slowly at 40°C. After 20 minutes incubation, the rate of degradation was found to be higher at 35°C than at 40°C. These results suggest that this enzyme would be inactivated at 40°C.

Effect of Substrate Concentration on Enzyme Activity. Pectic acid was employed as substrate and the concentration was varied from 0.005 to 1.2%. It was found that maximum reaction velocity was atained at a concetration of 0.05% and retained up to 1.2% (Fig. 2). With degraded pectic acid as substrate, maximum reaction velocity was obtained at 0.03% concentration (Table 1).

Table 1 Activity of saccharifying pectate *trans*-eliminase towards pectic acids and oxidized pectic acids.

Concentration of substrate (%)	Pectic acid	Pectic acid oxidized	Concentration of substrate (%)	Degraded pectic acid	Degraded pectic acid oxidized
0.01	0.063	0.056	0.01	0.068	0.046
0.02	0.076	0.065	0.03	0.078	0.059
0.03	0.080	0.070	0.05	0.078	0.058
0.04	0.084	0.075	0.08	0.079	0.059
0.05	0.088	0.080	0.12	0.077	0·059
0.06	0.088	0.079	0.16	0.080	0.058

The assay conditions were as in Fig. 2.

Comparison of Actiaities of Pectate trans-Eliminases towards Pectic Acids and Oxidized Pectic Acids. As shown in Tables 1 and 2, the degraded pectic acid oxidized was somewhat more resistant to the action of saccharifying pectate *trans*-eliminase than the original degraded pectic acid. In the other cases, little difference was observed between the substrates oxidized

Table 2 Activities of pectate *trans*-eliminases towards pectic acids and oxidized pectic acids

Substrates	Time of reaction (min.)	Saccharifying pectate *trans*-eliminase			Liquefying pectate *trans*-eliminase		
		Oxidized a	Not oxidized b	a/b	Oxidized c	Not oxidized d	c/d
Pectic acid	30	0.209	0.223	0.937	0.378	0.371	1.020
	60	0.363	0.377	0.963	0.583	0.596	0.979
	90	0.459	0.472	0.973	0.703	0.729	0.965
Degraded pectic acid	30	0.269	0.387	0.695	0.525	0.526	1.000
	60	0.432	0.565	0.765	0.732	0.722	1.014
	90	0.560	0.700	0.800	0.920	0.901	1.021

The reaction systems were as follows: 1m*l* of 1% substrate solution + 2 m*l* of 0.02 M borate buffer, pH 9.5 + 2 m*l* of the enzyme solution: $CaCl_2$ added for liquefying pectate *trans*-eliminase assay (0.5 mM). Temp 35°C.

and not oxidized.

Comparison af Activities of Pectate trans-Eliminases towards Pectic Acid and Reduced Pectic Acid. With both enzymes, difference was scarcely observed between pectic acid and reduced pectic acid (Table 3).

Table 3 Activities of pectate *trans*-eliminases towards pectic acid and reduced pectic acid

Time of reaotion (min.)	Saccharifyiog pectate *trans*-eliminase			Liquefying pectate *trans*-eliminase		
	Pectic acid			Pectic acid		
	reduced a	not reduced b	a/b	reduced c	not reduced d	c/d
30	0.279	0.285	0.979	0.479	0.461	1.038
60	0.488	0.491	0.975	0.691	0.669	1.035
90	0.666	0.688	0.969	9.909	0.931	0.977

The assay conditions were as in Table 2.

Degradatiou Limit of Pectic Acid by the Action of Saccharifying Pectate trans-Eliminase. As can be seen from the data in Table 4, 100% degradation of pectic acid was obtained with the saccharifying pectate *trans*-eliminase. This result seems to contrast with that of a earlier investigation on carrot exopolygalacturonase (Ozawa 1955).

Table 4 Degradation limit of pectic acid by the action of saccharifying pectate *trans*-eliminase

Time of reaction (hr.)	0.02 N-I_2 consumed/1.1 m*l* (m*l*)	Rate of degradation (%)
0.5	0.15	78.1
1.0	0.18	93.7
2.0	0.185	96.4
3.0	0.19	99.0
4.0	0.20	104.2

The reaction system was as follows: 0.1 m*l* of 0.795% pectic acid solution + 1 m*l* of the enzyme solution; incubaon being at pH 9.5 and 32°C.

pH *Stability of Saccharifying Pectate trans-Eliminase at* 45°C. The enzyme solution of saccharifying pectate *trans*-eliminase was adjusted to the required pH with 0.1 N-HCl or 0.1 N-NaOH at 4°C for 10 minutes, cooled to 4°C and brought back to pH 7.5 with 0.1 N-NaOH or 0.1 N-HCl. The samples were incubated respectively with pectic acid. After four hours, polygalacturonate was removed by adding 0.1 M $BaCl_2$ solution, containing N/30 HCl, to the reaction mixtures in the proportion of 4 to 3. Thiobarbituric acid test and ultraviolet absorption measurement were carried out with these polygalactnronate-free reaction mixtures. As shown in Table 4, the activity of forming DKGA (or DKFA) was almost completely destroyed by the treatments at pH values higher than 8.0. The activity of forming 4, 5-unsaturated digalacturonic acid was considerably stable in the pH range 5.0 to 9.0 at 45°C. Below pH 4.5 both activities were lost rapidly.

Preparation of Saccharifying Pectate trns-Eliminase Possessing No DKGA (or DKEA)-Forming Activity. On the basis of the observations in the preceding paragraph, we attempt to separate the saccharifying pectate *trans*-eliminase from the DKGA (or DKFA)-forming enzyme. The enzyme solution of saccharifying pectate *trans*-eliminase was adjusted to pH 9.0 at 4°C with 0.1 NaOH and incubated at 45°C. Samples were removed every five minutes, cooled to 4°C and braught to pH 7.5 with 0.1 N-HCl. These were assayed for enzyme activities (Fig. 3). The saccharifying pectate *trans*-eliminase retained 45% of its activity after 20 minutes incubation, whereas DKGA or DKFA-forming activity was almost completely lost before five minutes. Thus, the enzyme preparation of saccharifying pectate *trans*-eliminase having no DKGA (or DKFA)-forming activity was obtained by subjecting the enzyme solution of saccharifying pectate *trans*-eliminase, used throughout the foregoing expriments, to the conditions: pH, 9.0; tempereature, 45°C; time of incubation, 10 minutes.

Table 5 pH stability of saccharifying pectate *trans*-eliminase at 45°C

pH	Ultraviolet absorption Optical density at 230 mμ × 1/11	Thiobarbituric acid test*	
		Optical density at 550 mμ, found	Optical density at 550 mμ, corresponding to DKGA (or DKFA)
3.4	0.048	0.027	0.002
4.1	0.048	0.027	0.002
4.5	0.090	0.071	0.025
5.0	0.219	0.397	0.285
6.0	0.288	1.807	1.660
7.0	0.298	2.327	2.175
7.3	0.299	1.272	1.120
7.5	0.304	0.445	0.290
7.7	0.309	0.358	0.200
8.0	0.304	0.205	0.050
8.2	0.289	0.157	0.009
9.0	0.260	0.140	0.007
4, 5-unsaturated digalacturaonate, 1.5 mμM	0.196	0.100	
4-deoxy-5-keto-D -fructuronic acid, 0.05 mμM	0.015	0.318	

* This was carried out on 0.4 m*l* of polygalacturonate-free reaction mixture; Beckman DU spectrophotometer used for optical density measurement.

The reaction systems were as follows: 0.5 m*l* of 1% pectic acid + 0.45 m*l* of M/15 phosphate buffer + 0.55 m*l* of the treated enzyme solution. Temp. 30°C.

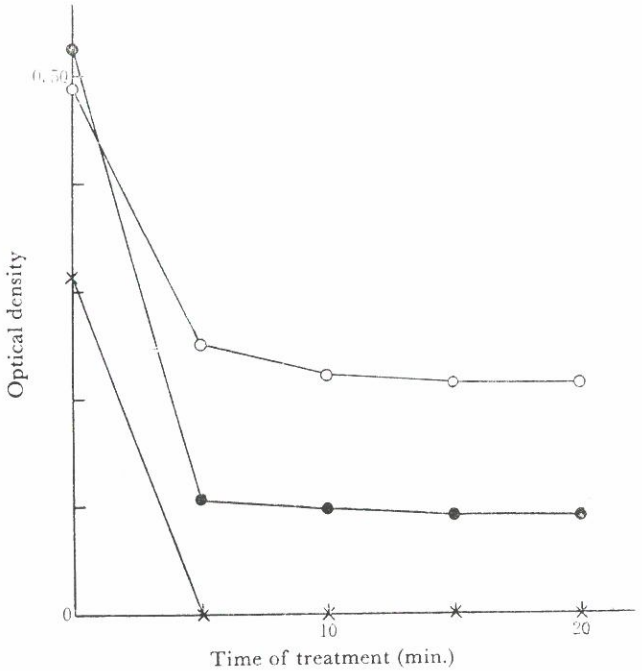


Fig. 3. Changes in enzyme activities during the treatment of the enzyme solution of saccharifying pectate *trans*-eliminase. pH 9.0, at 45°C. The experimental conditions were as described in Table 5. ○, optical density at 230 mμ; ●, optical density at 550 mμ, corresponding to DKGA (or DKFA). Optical density

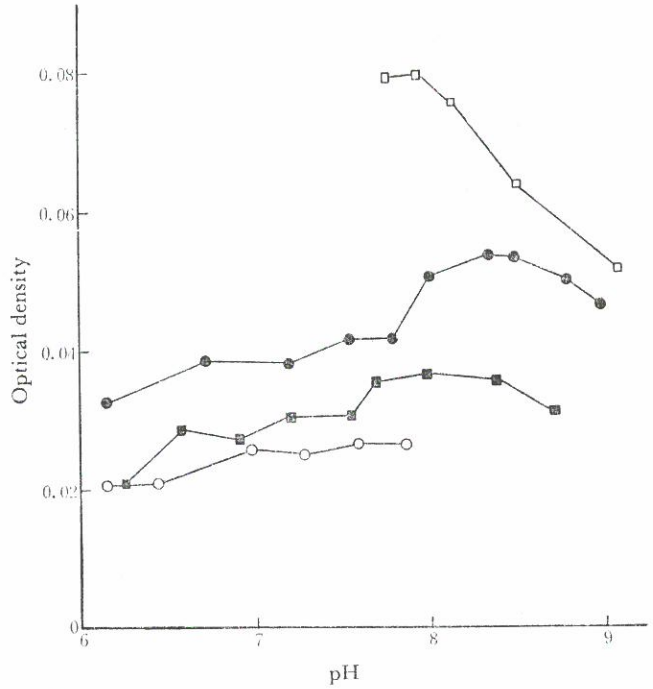


Fig. 4. Effect of pH and buffers used on the activity of saccharifying pectate *trans*-eliminase. The reaction mixture contained 0.1 m*l* of 1% pectic acid, 0.3 m*l* of buffer and 0.1 m*l* of the enzyme solution. ○, M/15 phosphate buffer; ●, 0·05 M borax-0.1N HCl buffer; □, 0.2 M NaOH; ■, 0.05 M borax 0.1M KH_2PO_4 buffer.

Effects of pH *and Buffers Used on the Enzyme Activity.* The enzyme solution of saccharifying pectate *trans*-eliminase having no DKGA (or DKFA)-forming activity was used in this experiment. The pH-activity curves varied with the buffer employed. In borate buffers, activity was greater than in phosphate buffer. The optimum pH found in borax-HCl buffer was about 8.3 (Fig. 4).

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