Increase in Viscosity and Gel Formation of Fruit Juice by Purified Pectinesterase

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The pectinesterase produced by *Sclerotinia libertiana* was purified by the column chromatography employing cation exchange resin Amberlite CG 50 and CM cellulose. The specific activity per mg protein was increased 266-fold. The homogeneity of the enzyme preparation was confirmed by ultracentrifugal analysis and starch zone electrophoresis. The purified pectinesterase was found to remarkably increase the viscosity of pectin solution and fruit juice, and, in a pectin solution of appropriate concentration (about 0.5~1 %) and some fruit juices, it finally formed the gel, even if Ca salt was not added. The viscosity thus increased and gel formed by the enzyme action did not change for a long time.

INTRODUCTION

The studies on purification of pectinesterase (PE) from some microbial origins were contributive to explaining the role of PE in the clarification of fruit juice.^{1~5)}

However, the effect of PE on viscosity of fruit juice has never been so far described. The pectic enzyme produced by *Sclerotinia libertiana* can also be used for clarification of fruit juice, and has a tolerably powerful PE activity.⁶⁾ The authors attempted the purification of PE of the fungus, and, as a result of investigating the mode of action of the purified PE, it was found that the viscosity of fruit juice is remarkably increased by PE and, in some juices, gelling of the solution occurs without addition of Ca salt. This fact could be used to estimate the contamination of polygalacturonase (PG) in PE. In the present paper, some physical and enzymatic properties of the purified enzyme are also

described.

EXPERIMENTAL AND RESULTS

Citrus Pectin. A commercial product of pectin (California Fruit Grocer Exchange product) was purified repeating the precipitation with ethanol (80% v/v), before use. It contained about 1.0% of ash, 0.67% of nitrogen and 5% of pentosan, and its esterification degree was 69%.

Fruit Juice. Juices were freshly prepared from apples ("Kokko" and "Indo"), strawberry ("Hoko") and orange ("Hassaku") by crushing with a grater and pressing through gauzes, and, after the heat-treatment at 90°C for 20 min. and centrifugation at 3000 r.p.m. for 10 min., the supernatants were used.

PE Activity. The reaction mixture consisting of 5 ml of 2% pectin solution, 1 ml of enzyme solution, 1 ml of 1.5 M NaCl solution and 3 ml of water (adjusted to pH 5.0 with 1/10 N NaOH, the total volume 10 ml) was incubated at 30°C for 60 min. and carboxyl residue liberated during the incubation was titrated by N/50 NaOH using a glass electrode. One unit of PE activity was defined as that which produces 2×10^{-5} moles of free carboxyl group under the condition.

1) A. Endo, This Journal, 28, 757 (1964).

2) M. Yamasaki, A. Kato, K. Shu, T. Yasui and K. Arima, Ann. Meeting of Agr. Chem. Soc. of Japan, 1964.

3) A. Endo, This Journal, 29, 129 (1965).

4) M. Yamasaki, T. Yasui and K. Arima, This Journal, 28, 779 (1964).

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6) Y. Satomura, J. Agr. Chem. Soc. of Japan, 26, 486 (1952).

Viscosity. The viscosity was measured at intervals with the Ostwald viscosimeter and expressed as relative viscosity. Before the measurement, pH of pectin solution was adjusted to 5.5 with N/10 NaOH. With fruit juice, pH was unadjusted.

TABLE I. PURIFICATION PROCEDURE OF PECTINESTERASE

Procedure	Volume (ml)	Specific Activity (unit/mg)	Total Activity (units)
Extracts of bran culture	2000	0.178	2500
↓			
Ppt. with 0.55 satur. of $(\text{NH}_4)_2\text{SO}_4$ and dialyzed against water for one day	400	0.5	2300
↓			
Adjusted to pH 3.2 with 1.0 M acetate buffer and centrifuged			
↓			
Supernatant	450	0.6	1800
↓			
Duolite A2 buffered with 0.1 M Acetate at pH 3.2			
↓			
Effluent	800	1.00	1600
↓			
Ppt. with 0.75 satur. of $(\text{NH}_4)_2\text{SO}_4$ and dialyzed against water for one day	114	1.21	1420
↓			
Adsorbed on Amberlite CG 50 buffered with 0.1 M Acetate at pH 3.2			
↓			
Eluted with 0.1 M acetate buffer (pH 4.0)			
↓			
Eluate	200	6.00	1240
↓			
Ppt. with 0.9 satur. of $(\text{NH}_4)_2\text{SO}_4$ and dialyzed against water for one day	60	6.50	1160
↓			
Adsorbed on CM cellulose column buffered with 0.05 M Acetate buffer at pH 3.6			
↓			
Eluted stepwise with 0.1 M Acetate buffer (pH 3.6~5.8)			
↓			
Eluate at pH 3.9	44.4	47.3	730
↓			
Rechromatography on CM cellulose column			
↓			
(Purified Pectinesterase fraction)			

Purification of PE. Purification procedure is summarized in Table I. The bran culture of *Sclerotinia* fungus was extracted with 5 times its weight of distilled water. The extract was brought to 0.55 saturation with ammonium sulfate and allowed to stand overnight at 4°C. The resulting precipitate was collected by centrifugation at 4,000 r.p.m. for 20 min., dissolved in a small volume of distilled water, and dialyzed against distilled water. To the dialyzed solution, acetate buffer (pH 3.2) was added to M/10 concentration. The precipitate formed was removed by centrifugation and the supernatant was passed down through Duolite A2 resin column (5.5 cm × 54 cm) which had been bufferized at pH 3.2 with M/10 acetate buffer, whereby the decolorized enzyme solution was obtained. To the enzyme solution ammonium sulfate was added to 0.75 saturation. The resulting precipitate was collected by centrifugation, and, after dissolving in a small volume of distilled water, dialyzed against distilled water. The dialyzed solution was adjusted at pH 3.2 with acetate buffer (M/10 in final), and passed through Amberlite CG 50 resin column (5.5 cm × 45 cm) which had been bufferized pH at 3.2 with M/10 acetate buffer, whereby the PE was adsorbed on the resin. Then, the PE was eluted with M/10 acetate buffer (pH 4.0) and again precipitated with 0.9 saturation of ammonium sulfate. The precipitate was dissolved in a small volume of distilled water and dialyzed. The dialyzed solution was adjusted at pH 3.6 with acetate buffer (M/20 in final), and subjected to a chromatography on CM cellulose column which had been equilibrated with M/20 acetate buffer at pH 3.6. The column was eluted with M/10 acetate buffer whose pH was changed stepwise from 3.6 to 5.8. As presented in Fig. 1, the PE was eluted at nearly pH 4. The PE fraction obtained was subjected to rechromatography on CM cellulose, as shown in Fig. 2. In this step, the activity of PE was attained to 266-fold per mg protein.

FIG. 1. Chromatography of PE on CM Cellulose Column, (2.1×54 cm).

FIG. 2. Rechromatography of PE on CM Cellulose Column, (2.1×60 cm).

Some Properties of the purified PE

i) **Electrophoresis.** Starch zone electrophoresis was performed according to the method of

Kunkel,⁷⁾ using a starch block encased in

7) H. G. Kunkel, "Methods of Biochemical Analysis", Interscience Publishers Press, (1954), 1, p. 156.

plastic case ($30 \times 3 \times 1$ cm) and $m/50$ tris buffer (pH 8.2). About 4.3 mg of the purified PE was, after dissolving in 2 ml of the tris buffer and dialyzing against the same buffer overnight, placed on the starch block in 1.0 cm wide and at the distance of 10 cm from the cathode side. After electrophoretic movement was conducted at 250 volt, 8 m amp. and 4°C for 8 hrs, the starch block was segmented to 1 cm intervals with a spatula. To each segment, 5 ml of water was added, followed by stirring at 4°C for 20 hrs. and then the extracts obtained by filtration were analyzed for the enzyme activity and optical density. The result is shown in Fig. 3. The PE exhibited a single main peak, the center of which was present at 2 cm from the original point, though a small amount of impurities was recognized at 8~10 cm. from the point.

ii) **Ultracentrifugal Analysis.** Prior to analysis, the PE was dissolved in $m/10$ phosphate buffer (pH 7.0) and dialyzed against the same buffer overnight. The dialyzed enzyme solution containing about 6.33 mg of protein per ml was subjected to the sedimentation with a Hitach UCA-1 ultracentrifuge at 60,000 r.p.m. and 20.5°C . As shown in Fig. 4, in each

FIG. 3. Starch Zone Electrophoretic Pattern of PE.

Starch block; $1 \times 3 \times 30$ cm,
condition ; 250 V, 8 mamp.,
Temperature; 4°C ,
Time ; 8 hrs.,

Fig. 4. Sedimentation Pattern of PE by Ultracentrifugation.

Protein concentration; about 6.33 mg/ml
Revolution ; 60,000 r.p.m.
Temperature ; 20.5°C
Bar angle ; 55°

FIG. 5. pH-Activity.

case at 15 and 31 min. after reaching the maximal speed of revolution, presence of a single component was proved. The sedimentation coefficient was calculated to be 4.41 S. iii) **Opt. pH, pH Stability and Thermostability.** As shown in Fig. 5 and 6, the optimum pH was about 4.8~5.2 in acetate buffer, and in a range of pH 4.0~5.0 the enzyme was most stable. Also, as shown in Fig. 7, after the incubation at pH 4.0 and 55°C for 5 min. a half of the enzyme activity still remained.

iv) **Hydrolysis Rate of Pectin.** The hydrolysis

FIG. 6. pH-Stability.
(Incubated at 30°C, for 22 hrs.)

FIG. 7. Heat-Stability.
(Incubated at pH 4.0 for 5 min., in 0.1 mol. Acetate)

TABLE II. EFFECTS OF METALS AND SOME
SURFACE ACTIVE AGENTS

Addition	Concn.	Relative Activity (at pH 5.0)
Control	—	100
NaCl	0.15 (M)	127
	0.02	110
	0.01	100
KCl	0.05	100
	0.01	95
CaCl ₂	0.02	74
	0.005	110
MnCl ₂	0.02	74
	0.005	87
MgCl ₂	0.02	67
	0.005	100
CoCl ₂	0.02	20
	0.005	70
NiCl ₂	0.02	45
	0.005	95
ZnCl ₂	0.02	20
	0.005	80
MgCl ₂	0.02	67
	0.005	80
Na-Desoxycholate	0.005	85
	0.002	123
Na-Taurocholate	0.005	90
	0.002	90
Saponin (Soy bean)	0.01%	81
	0.001%	100
EDTA	M/800	50

FIG. 8. Hydrolysis Curves of Pectin by PE.

●—● crude PE pH 5.5
○—○ crude PE pH 4.4
▲—▲ PE (ca. 2~3 units) pH 5.5
△—△ PE (ca. 2~3 units) pH 4.4

of pectin by the purified PE at pH 4.4 and 5.5 is shown in Fig. 8. At pH 5.5, the hydrolysis by PE with 2 units of the activity reached to 30% in 2 hrs. and finally 38% in 24 hrs. At pH 4.4, the hydrolysis rate was at first a little lower than that with pH 5.5, but, afterwards, became higher and finally extended to 58.3% in 24 hrs.

v) **Effect of Metals.** As shown in Table II, the activity of PE was increased by NaCl (0.02~0.15 M) and inhibited by Co^{++} , Ni^{++} , Mn^{++} , Mg^{++} , and Zn^{++} salts (0.02~0.005 M). Ca^{++} salt was also inhibitive, but rather stimulative in a dilute concentration (0.005 M).

Some surface active agents such as Na-desoxycholate, Na-taurocholate and saponin were all inhibitive, but Na-desoxycholate in the concentration of 0.002 M was stimulative. Ethylenediaminetetra-acetate (EDTA, M/800) inhibited about 50% of PE activity.

Viscosity Gel Change and Gel Formation of Pectin Solution and Fruit Juice by PE

i) **Pectin Solution.** The reaction mixture, as described above in the measurement of PE activity (pectin; 1%, NaCl; 0.15 M, in final, and PE; 4 U.), was incubated at 30°C, and kept at pH 5.5 with N/10 NaOH. As presented in Fig. 9. viscosity of the pectin solution increased gradually at first, then rapidly, and, finally, the viscous solution gelled in about 3 hrs. With 0.5% pectin solution the gel formation also occurred in 18 hrs. (Fig. 10). The rate of hydrolyzed ester bonds at that time was about 20% and did not so proceed, afterwards. At pH 3.6 and in NaCl-free

FIG. 9. Viscosity Change and Gel Formation of Pectin Solution by PE.

○—○ PE: at pH 5.5
●---● crude PE: at pH 5.5
×---× PE+PG: at pH 5.5
○---○ PE: NaCl-free at pH 3.6

medium the viscosity-increase was slight and the gel formation did not occur even after 24 hrs., but here the addition of NaCl (0.15 M in final) immediately raised the viscosity and formed the gel. If a small activity of polygalacturonase (PG) was added to PE, the vis-

TABLE III. VISCOSITY INCREASE AND GEL FORMATION OF VARIOUS FRUIT JUICES BY PE

Juices	Pectin added	PE (unit)	pH	Relative Viscosity Time (min.)						18 (hrs)
				0	60	75	135	200		
Apple ("Kokko")	0	0	3.6	1.8	1.8	1.8	1.8	1.8		1.8
	0.1%	2 u	3.6	2.2	2.35	—	2.95	3.8		4.0
	0.2%	2 u	3.6	2.4	2.6	—	3.70	6.0		7.5
	0.5%	2 u	3.6	3.0	3.5	—	7.0	11.0		∞ gelled
	0	2 u	3.6	2.0	2.05	—	2.4	2.7		2.75
Apple ("Indo")	0	2 u	4.4	4.3	—	3.7	5.4	∞ gelled		
	0.2%	2 u	4.4	5.4	—	5.4	8.7	∞ gelled		
	0.5%	2 u	4.2	7.1	—	7.1	7.7	∞ gelled		
Strawberry	0	0	4.0	11.7	—	11.7	11.5	10.9		10.0
	0	2 u	4.0	11.7	—	10.0	8.5	12.3		∞ gelled
	0.2%	2 u	4.0	12.3	—	12.0	13.3	16.8		∞ gelled
Orange	0	2 u	3.8	1.4	1.4	1.4	1.4	1.4		1.4
	0.2%	2 u	3.8	1.9	1.89	1.88	2.15	3.7		∞ gelled
	0.5%	2 u	3.8	3.0	2.95	2.90	3.0	3.2		∞ gelled

cosity of the pectin solution at first increased but soon decreased gradually. The crude PE, which contained substantial activity of PG, immediately decreased the viscosity.

ii) **Fruit Juice.** To 10 ml. of fruit juice, 0.1 ml. of PE (about 2 units) solution was added and incubated at 30°C. During the incubation, pH was unadjusted, and the results are shown in Table III. With apples, the viscosity of juices gradually increased in 2 or 3 hrs, and, with the variety "Indo", the juice soon gelled without addition of pectin. While, the juice from the variety "Kokko" did not gel without external pectin. The addition of pectin to these juices (0.2~0.5% in final) promoted the viscosity-increase and gel-formation. A photograph of the gel is shown in Fig. 10. With strawberry, though the viscosity rather fell for a time (about 2 hrs), but soon rose, and the gel formation occurred in 18 hrs. With orange juice, the viscosity unchanged but, by adding pectin, increased, finally forming the gel in 18 hrs. The gels thus formed from various juice by PE did

not degrade after the storage for 6 months at room temperature.

DISCUSSION

Lately, two PE fractions were isolated from the culture extract of *Coniothyrium diplodiella* by A. Endo, and no significant difference was observed in the characteristics of the two fractions.¹¹ In the present work, though one PE fraction has been purified from the *Sclerotinia* fungus, there was actually another PE fraction corresponding to 10~20% in the total activity, which was not adsorbed on cation exchange resin Amberlite CG 50 at pH 3.2. The characteristic of the latter seems to differ from the purified PE.

Formerly, it had been known that the presence of a certain bimetallic ion such as Ca^{++} , Ba^{++} or Sr^{++} is necessary for the gel formation of pectin solution by PE, because such bimetallic pectate forms the gel. However, the above purified PE did not need these metal ions for the gel formation of pectin solution. In this case, the gel must be formed from a low methoxyl pectin (pectinic acid) and not from pectic acid, because the hydrolysis rate of pectin by the PE is very low. The viscosity of pectinic acid (esterification degree 35~50%) increases with the dropping of NaOH, and shows the maximum at around pH 6. Then it seems reasonable that the optimum pH of PE for increasing the viscosity and gelling of pectin solution was about 5.5 (opt. pH of PE for hydrolysis of pectin, 4.8~5.2). Also, it seems of significance that the addition of NaCl (0.15 M in final) caused the gelling of pectin solution by PE even at pH 3.6. With fruit juice, a certain protein or amino acid may be involved in the gel formation, and also, the pH of fruit juice may have a close relation to this.

When PE decreases the viscosity of pectin solution, it seems necessary to examine the contamination of PG.

FIG. 10. Gel-Formation of Apple Juice, and Pectin Solution by PE.

P: 0.5% Pectin Solution

A: Apple Juice ("Indo") + 0.2% Pectin in final