Pectin *trans*-Eliminase with Fruit Juice Clarifying Activity

Shigetaka Ishii* and Tamotsu Yokotsuka

Pectolytic enzymes produced by *Aspergillus sojae*, which contained hydrolytic and *trans*-eliminative enzymes, showed a high fruit juice clarifying activity. Significant correlation between clarifying activity and pectin *trans*-eliminase was found in ammonium sulfate fractionation, heat stability, and gel filtration pattern on Sephadex G-100. Two pectin *trans*-eliminases of this fungus were purified until activities of hydrolytic pectinases, hemicellulase, and protease were not detected. Cloudy juices of apple and grape were completely clarified by purified pectin *trans*-eliminase alone. Both purified pectin *trans*-eliminases showed similar clarifying activity to the crude enzyme when pectin *trans*-eliminase activity was adjusted to the same level. We conclude that fruit juice clarifying activity of *Aspergillus sojae* is due to pectin *trans*-eliminase.

Pectolytic enzymes have long been used for clarification of fruit juices (Joslyn *et al.*, 1952; Neubeck, 1959). Clarification seems to be a result of degrading pectin, since the colloidally suspended pectin may hold other substances in suspension.

Many attempts have been made to determine the nature of the enzyme(s) responsible for clarifying fruit juice. Endo (1965) and Yamasaki *et al.* (1967) showed that cloudy apple juice was completely clarified by adding purified pectinesterase (PE) and *endo*-polygalacturonase (*endo*-PG), although each of them had no effect on the clarification when added alone. These authors proposed that only the degradation of glycosidic linkages in pectin is enough for the clarification. They ruled out other enzymes such as protease, amylase, cellulase, and hemicellulase as agents for the enzymatic clarification.

The lack of correlation between enzyme activity and the rate of juice clarification has been often reported (Joslyn *et al.*, 1952; Neubeck, 1959; Endo, 1961; Arima *et al.*, 1964). Therefore, the question as to which enzymes are involved in juice clarification remains unsolved.

Pectolytic enzymes of *Aspergillus sojae* No. 48 contain mainly PE, *endo*-PG, and pectin *trans*-eliminase (PTE) (Ishii *et al.*, 1970). PTE (E.C. 4.2.99) splits α-1,4-methylgalacturonide linkages in pectin by an eliminative mechanism forming 4,5-unsaturated galacturonides (Albersheim *et al.*, 1960). A crude enzyme of this fungus is capable of clarifying fruit juice. However, the correlation between hydrolytic pectinases and clarifying activity was not found at all.

We report on the nature of the enzyme from *Aspergillus sojae* responsible for the clarifying fruit juice.

MATERIALS AND METHODS

Apple Juice. Apple juice was prepared by grating commercially available apples and squeezing them with cotton cloth. Freshly prepared apple juice of "Jonathan" was used as substrate for the assay of clarifying activity.

Pectic Substances. Pectin and sodium polypectate were obtained from Sunkist Growers, Inc. They were washed three times with 70% (v/v) ethanol to remove soluble sugars. The degree of esterification of pectin was about 68%.

Crude Enzyme Preparation. *Aspergillus sojae* No. 48 was grown on 20 g of wheat bran moistened with 12 ml of water in 500-ml Erlenmeyer flasks at 30° C for 3 days. The enzyme in bran culture medium was extracted with 100 ml of wate at room temperature for 2 hr and filtered with cotton cloth Solid ammonium sulfate (60 g) was added to 100 ml of clear extract obtained by filtration. Resultant precipitates were collected by centrifugation and dried *in vacuo*.

Assay of Enzymatic Activities. CLARIFYING ACTIVITY. Clarifying activity was determined by the turbidimetric method of Endo (1964). Because it is difficult to calculate the accurate and reproducible values of clarifying activity, values relative to control were used. To 10 ml of apple juice, 1.0 ml of appropriately diluted enzyme solution was added, and the mixture was incubated at 40° C for 60 min. After heating for 5 min in a boiling water bath, the mixture was centrifugated at 3000 rpm for 5 min and transmittance at 660 nm of the supernatant was measured using a Hitachi Model 101 spectrophotometer. The transmittance of completely clarified apple juice was above 95.00%. The control test (blank) was performed by the same procedure with 1.0 ml of water instead of enzyme solution.

PECTINESTERASE. PE activity was determined by titration (Jansen and MacDonnell, 1945). Reaction mixtures containing 0.5 ml of enzyme solution, 1.0 ml of 1% pectin, and 1.0 ml of 0.05 *M* acetate buffer, pH 5.5, were incubated at 40° C for 3 hr. Carboxyl residue in pectin, liberated by PE, was titrated by 0.05 *N* NaOH using an automatic titrator. The activity of PE was expressed as titration values (ml of 0.05 *N* NaOH).

endo-POLYGALACTURONASE. *endo*-PG was determined by a Ostwald viscosimeter (Roboz *et al.*, 1952). Reaction mixtures contained 1.0 ml of enzyme solution, 3.0 ml of 1% sodium polypectate, and 3.0 ml of McIlvaine buffer, pH 4.5. The activity of *endo*-PG was expressed as the reciprocal of the time for 50% loss in viscosity of the reaction mixture at 40° C.

PECTIN *trans*-ELIMINASE. PTE was determined by spectrophotometry. Reaction mixtures containing 0.5 ml of enzyme solution, 1.0 ml of 1% pectin, and 1.0 ml of McIlvaine buffer, pH 5.5, were incubated at 40° C for 60 min. The activity of PTE was expressed as the increase in extinction at 235 nm.

PROTEIN CONCENTRATION. Protein concentrations were determined by the method of Lowry *et al.* (1951) by using bovine serum albumin as a standard.

SALTING OUT WITH AMMONIUM SULFATE. To clear filtrate of bran culture, solid ammonium sulfate was added with stirring up to the indicated saturation. After standing overnight at 4° C, each resultant precipitate was collected by centrifugation and dissolved in original volume of distilled water. Undissolved material was removed by centrifugation.

Partial Purification of PTE's from *Aspergillus sojae* No. 48.

Central Research Laboratories, Kikkoman Shoyu Co., Ltd., 399 Noda, Noda-shi, Chiba-ken, Japan.

Purification of PTE was directed toward the removal of hydrolytic pectinases, hemicellulase, and protease. It was previously concluded that PTE of this fungus was composed of two components (Ishii *et al.*, 1970). Two PTE's were purified by the following procedures.

To crude extracts of bran culture, solid ammonium sulfate was added up to 0.4 saturation. The precipitates formed were discarded and to the supernatant was added ammonium sulfate up to 0.75 saturation. The resultant precipitates were collected by centrifugation and dissolved in a small amount of water. By this step, most of *endo*-PG was removed. It was previously found that a cellophane tube was dissolved even at 4° C by the crude enzyme of this fungus if cellulase activity was contained (Ishii *et al.*, 1969). The removal of salt, therefore, was carried out by gel filtration or dialysis with fish bladder. The enzyme solution was passed through a Sephadex G-25 column to remove ammonium sulfate, and enzyme fractions obtained were freeze-dried. The enzyme was dissolved in 0.01 *M* acetate buffer, pH 4.0, and undissolved materials were removed by centrifugation. The enzyme solution was applied on a CM-cellulose column previously equilibrated with 0.01 *M* acetate buffer, pH 4.0. One component of PTE was adsorbed on CM-cellulose and eluted with 0.1 *M* acetate buffer, pH 5.0. The eluted PTE was collected and dialyzed against 0.01 *M* acetate buffer, pH 4.0, with cellophane tube. CM-cellulose column chromatography was again carried out under the above condition. Nonadsorbed fraction dried by freeze-drying was dissolved in a small amount of 0.01 *M* phosphate buffer, pH 6.5, and dialyzed against the same buffer with fish bladder. The dialyzed enzyme solution was applied on a DEAE-Sephadex column.

RESULTS AND DISCUSSION

Relation between Pectinase and Clarifying Activity. Apple juice was completely clarified by adding crude enzyme in a final concentration of 0.001–0.0015% as protein at 40° C for 60 min (Figure 1).

Table I shows pectinase and clarifying activities in the precipitates salted out with ammonium sulfate. PE was not salted out at 0.3 and 0.4 saturation and about 10% of original activity was precipitated at 0.5 saturation. *endo*-PG was not salted out at 0.5 saturation and even at 0.6 saturation only 4% of its activity was precipitated. On the other hand, PTE was more easily salted out than these hydrolytic pectinases. Clarifying activity was not detected in the precipitate formed at 0.3 saturation. It was obviously detected in the precipitate at 0.5 saturation, in which *endo*-PG was not detected at all.

Table II shows the heat stability of the above enzymes. Enzyme solutions dissolved in 0.1 *M* acetate buffer, pH 5.5, were heated at various temperatures for 10 min. PE and *endo*-PG were considerably labile by heat treatment. Both were completely inactivated by the treatment at 55° C for 10 min. However, about 50% of original PTE remained active. Clarifying activity was not detected in the enzyme solution heated at 70° C, but in both enzyme solutions heated at 55 and 60° C the activity remained active for juice clarification. These results suggested that neither PE nor *endo*-PG were involved in juice clarification. Good correlation between PTE and clarifying activity was found in both ammonium sulfate fractionation and heat stability.

Elution patterns of *Aspergillus sojae* pectinase on Sephadex G-100 column are shown in Figure 2. PE and *endo*-PG appeared as one peak at fractions No. 37 and 36, respectively. However, PE might be two components, as a shoulder of its

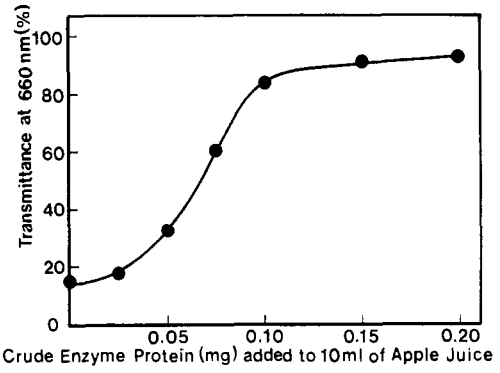

Figure 1. Apple juice clarification by *Aspergillus sojae* crude enzyme

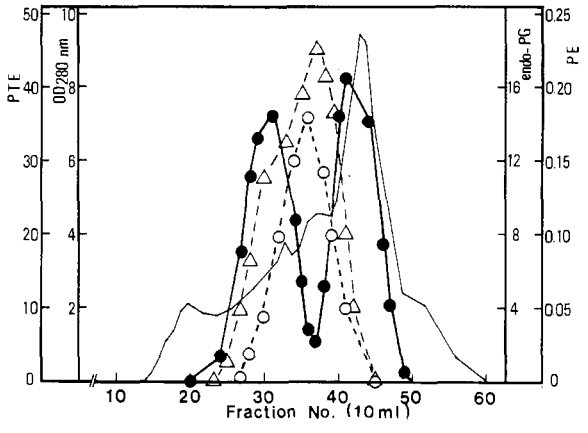

Figure 2. Elution patterns of *Aspergillus sojae* pectinase on Sephadex G-100

Column, 4 × 36 cm
——— o.d. 280 nm
—●— PTE
Eluent, distilled water
-- ○ -- *endo*-PG
—△— PE

Table I. Salting Out of Juice-Clarifying and Pectinase Activities with Ammonium Sulfate

	Activities in the precipitates			
	Clarifying[a]	Activity/ml		
Saturation	(blank 27.00)	PE	*endo*-PG	PTE
0.3	27.00	0	0	0
0.4	47.00	0	0	1.4
0.5	62.25	0.022	0	7.0
0.6	86.00	0.072	0.58	15.0
0.7	89.75	0.147	2.32	24.0
0.8	94.00	0.178	7.40	26.8
Original	95.75	0.206	14.51	28.0

[a] Used with 20-fold diluted enzyme solution.

Table II. Heat Stabilities of Enzymes

	Residual activities			
	Clarifying[a]	Activity/ml		
Temp., °C	(blank 15.75)	PE	*endo*-PG	PTE
40	85.75	0.066	6.8	26.6
45	83.75	0.055	4.1	24.5
50	79.00	0.025	0.8	18.6
55	58.00	0	0	13.2
60	29.50	0	0	8.2
70	14.75	0	0	0
Control	85.50	0.078	7.4	27.2

[a] Used with 20-fold diluted enzyme solution.

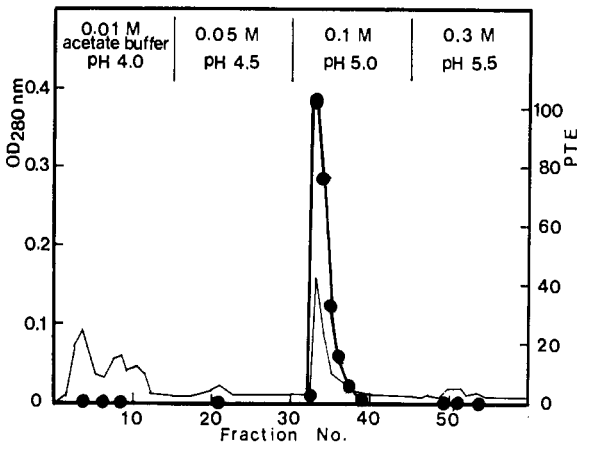

Figure 3. CM-Cellulose column chromatogram of PTE
——— o.d. 280 nm —●—●— PTE

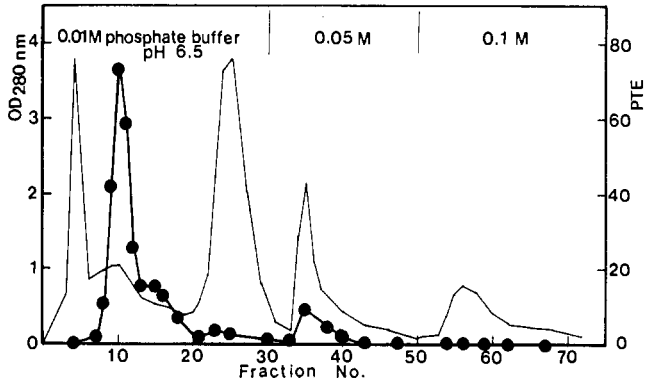

Figure 4. DEAE-Sephadex chromatogram of CM-cellulose nonadsorbed PTE
——— o.d. 280 nm —●—●— PTE

Table III. Juice-Clarifying and Pectinase Activities of Several Fractions Obtained by Gel Filtration on Sephadex G-100

Fraction no.	Clarifying[a] (blank 20.75)	Activity/ml PE	*endo*-PG	PTE
20	20.75	0	0	0
24	56.75	0.009	0	3.44
26	97.75	0.028	0	13.60
28	98.00	0.084	1.6	27.76
31	98.75	0.149	5.5	35.92
36	89.25	0.217	15.0	6.96
37	88.25	0.224	13.3	5.52
39	96.50	0.182	8.2	23.68
41	98.75	0.102	4.0	41.12
45	98.75	0	0	28.80
47	70.00	0	0	10.32
50	21.00	0	0	0

[a] Used with 10-fold diluted solution.

activity curve appeared around fraction No. 30. On the other hand, PTE separated into two components, which were represented by two peaks at fractions No. 31 and 41.

Table III shows clarifying and pectinases activities of several fractions obtained by gel filtration. Clarifying activity was extensively detected from fractions No. 24–47. But the activity might be composed of two components, because the activity was reduced around fraction No. 37. Fractions No. 36 and 37 showed highest PE and *endo*-PG activities, but a low PTE activity. On the contrary, fraction No. 45, in which a high clarifying activity was found, indicated a high PTE activity but no PE and *endo*-PG activities. Thus, the gel filtration pattern of clarifying activity agreed well with that of PTE.

These results strongly suggest that PTE is the enzyme responsible for the clarification in this fungus.

Clarification of Fruit Juice by Purified PTE. Column chromatograms of CM-cellulose and DEAE-Sephadex are shown in Figures 3 and 4, respectively. CM-cellulose adsorbed PTE (fractions 33–35) was purified about 110-fold, and CM-cellulose nonadsorbed PTE (DEAE-Sephadex fractions 9–11) was purified about 13-fold. These two PTE's were completely free from PE and *endo*-PG and almost completely free from hemicellulase (Table IV). It was also found that protease and cellulase activity were not detected in the purified enzymes. Table IV shows that both purified PTE's showed similar apple juice clarifying activity to the crude enzyme when PTE activity was adjusted to the same level. These results confirmed that apple juice clarifying activity of this fungus was principally contributed by PTE.

To 20 ml of each fruit juice, two units of purified PTE were added and the mixture was incubated at 40° C. Apple juice of Jonathan (pH 3.40) and Golden Delicious (pH 3.84) was completely clarified within 1 hr. Grape juice (Delaware, pH 3.35) was more slowly clarified than apple juice. More than 2 hr was necessary for complete clarification. Orange juice (pH 3.55), however, was not completely clarified by PTE in the prolonged incubation. The addition of water or heat-inactivated PTE could not change the cloudy fruit juice (Figure 5). It was proved that PTE independently possessed an ability of clarification not only of apple juice but also grape juice.

Commercial pectinases were tested for their abilities of apple juice clarifying and pectinase activities (Table V). Preparation B showed most active in juice clarification. Preparations D and G had similar PE and *endo*-PG activity to preparation B, but they did not show such a high clarifying activity. The lack of correlation between hydrolytic pectinases and clarifying activity corresponded with the results obtained by former investigators (Joslyn *et al.*, 1952; Neubeck, 1959; Arima *et al.*, 1964). It was noteworthy that all preparations tested contained no or only a negligible amount of PTE activity.

Table IV. Comparison of Apple Juice Clarifying Activity between Crude Enzyme and Purified PTE's

	Activity/ml PTE	PE	*endo*-PG	Hemicellulase	Clarification Jonathan (28.25)	Delicious (45.50)
Crude enzyme	1.20	0.012	0.037	3.115	90.25	92.00
Purified PTE (CM-cellulose)	1.20	0	0	0	97.00	95.25
Purified PTE (DEAE-Sephadex)	1.20	0	0	>0	88.50	97.25

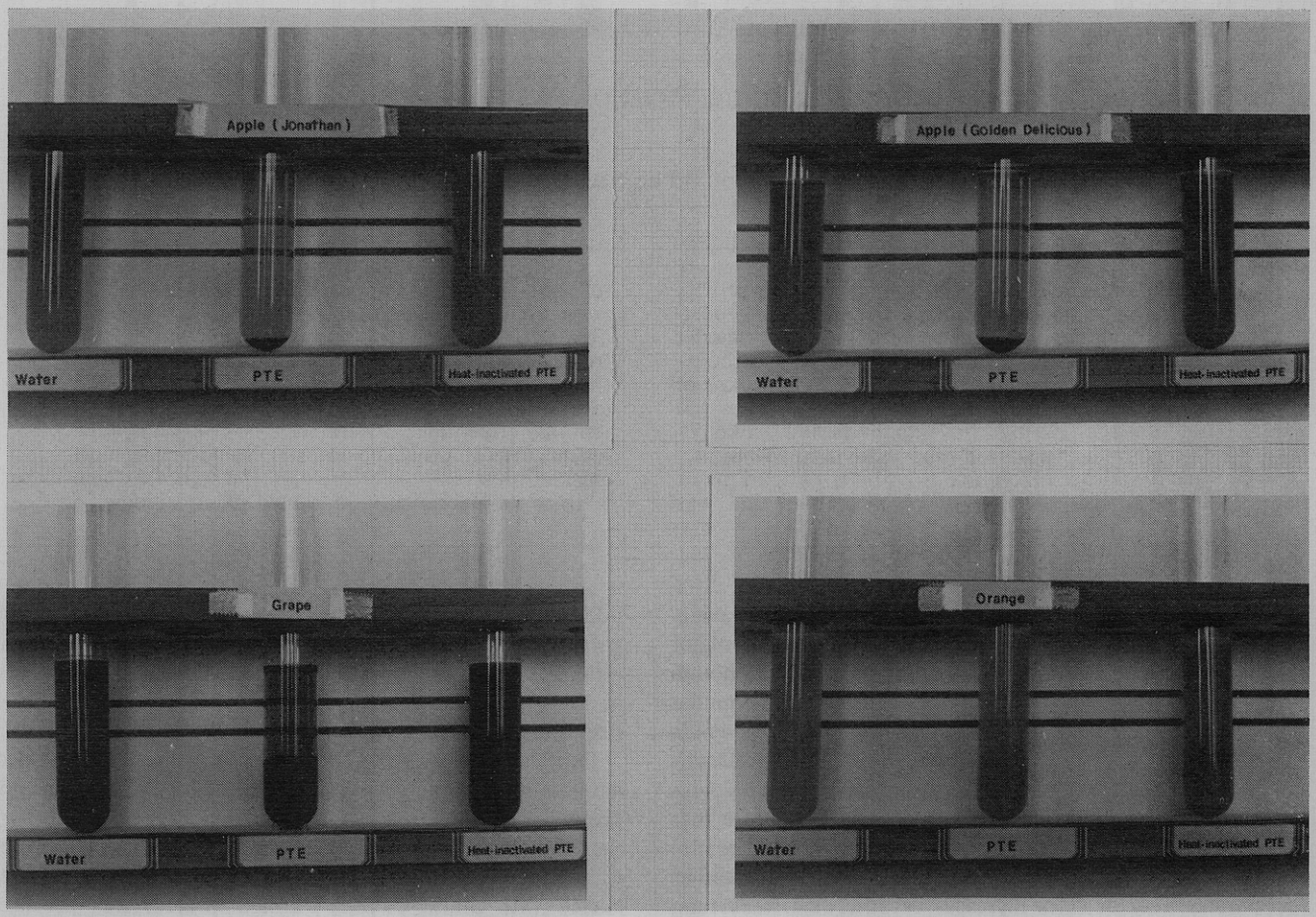

Figure 5. Clarification of fruit juice by purified PTE

Table V. Juice-Clarifying and Pectinase Activities of Commercial Pectinases

Pectinase	Clarification 0.05%[a]	Clarification 0.005%[a]	Activity/ml of 1% enzyme solution PE	*endo*-PG	PTE
A	95.00	47.00	0.25	25.0	0
B	98.00	95.25	1.74	218.0	>0
C	98.00	53.75	1.05	15.6	>0
D	98.00	54.75	1.75	200.0	0
E	92.00	44.50	0.02	23.2	0
F	46.00		0.10	26.7	0
G	98.00	44.50	2.42	266.6	0
H	87.00		0.82	1.4	0
	(blank 42.00)				

[a] Final concentration in apple juice.

The present investigation made it clear that (1) the enzyme responsible for fruit juice clarification is PTE and (2) only the degradation of pectin is enough for the clarification of apple and grape juice. However, it seemed that clarification of orange juice was not accomplished with only the degradation of pectin. Other factors might be necessary for the complete clarification of orange juice (Okada *et al.*, 1969; Mizrahi and Berk, 1970).

It is thought that PTE has not been used widely for the clarification of fruit juice because no or only a negligible amount of PTE activity was found in several commercial pectinase preparations. But PTE may be a useful enzyme used for fruit juice clarification because of the following reasons. (1) Clarification may be accomplished with a small amount of enzyme because PTE is most active to highly esterified pectin such as fruit pectin. (2) PTE does not produce methyl alcohol during clarification of fruit juice, because it directly splits α-1,4-methylgalacturonide linkages in pectin. On the other hand, the usual clarifying enzymes which are composed of PE and *endo*-PG produce methyl alcohol during clarification by the action of PE (Ishii, 1971).

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