might increase the membrane permeability of those cells in the separation layer and thereby increase the translocation of sucrose through this barrier. Such an effect might possibly explain the lack of an effect of EDTA on detached ears (Amir *et al.*, 1971) where no sucrose is available for translocation. If this assumption were true, exogenously supplied sucrose (by injection) should be transported to the endosperm more rapidly in the presence of EDTA. Results of such an experiment show that EDTA treatment significantly increased sucrose uptake, in comparison to the same treatment without exogenously supplied sucrose (Table II). It is suggested from these data that EDTA enchances sucrose translocation and accumulation in corn kernels, probably by changes in cell membrane permeability in the separation layer. In this regard it is of interest that PP_i increased sucrose uptake to a small extent, as well as inhibiting the metabolic loss of sucrose.

Spraying EDTA on the plant foliage has the same general effect on sucrose accumulation as injection into the ear (Table III).

To determine whether the EDTA response was a result of nonspecific chelation, other chelating agents were tested at the same concentration. At least two other chelators (nitrilotriacetic acid and iminodiacetic acid) are as effective as EDTA in this respect (data not shown).

Our results show that various chelators can be used under practical conditions to greatly augment sucrose accumulation in sweet corn. Furthermore, it appears likely that a comparable amount of both EDTA and PP_i treatment would have a combined effect on the sucrose level. It is thought that accumulation of sucrose in leaves may inhibit photosynthesis (Neales and Incall, 1968; Eastin *et al.*, 1969). Therefore, it is possible that the application of various chelators to cereal crops, as well as to other agronomic plants, might greatly increase carbohydrate yield.

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Clarification of Fruit Juice by Pectin *trans*-Eliminase

Shigetaka Ishii* and Tamotsu Yokotsuka

One milligram of purified pectin *trans*-eliminase from *Aspergillus sojae*, having 76.5 units of activity, was capable of clarifying 30–40 l. of apple juice within 1 hr at 40°C, while only one-fifth of the efficiency was shown in the case of grape juice under the same conditions. Optimal conditions for clarifying fruit juice by the enzyme existed in the range of pH 3–4, and 45–50°C. At the point when apple juice was completely clarified, one-half of the pectin in the juice was converted to a soluble form in 75% ethanol. A remarkable distinction between pectin *trans*-eliminase and hydrolytic pectinases was observed in the formation of carboxyl groups and methanol during clarification. Pectin *trans*-eliminase does not produce methanol but the ordinary hydrolytic pectinases do.

Pectin in fruit juice may suspend other materials in a colloidal system. For the clarification of fruit juice, therefore, pectolytic enzymes produced by molds have long been used (Joslyn *et al.*, 1952; Neubeck, 1959).

Pectin is composed mainly of α-1,4-linked polygalacturonide in which carboxyl groups of the galacturonic acid are mostly esterified with methanol (Deuel and Stutz, 1958; Pilnik and Voragen, 1970). Generally, there are supposedly three ways in which enzymatic degradation of α-1,4-linkages can occur in pectin, as shown in Figure 1 (Neukom, 1969): combined action of pectin esterase (PE) and polygalacturonase (PG); hydrolytic polymethylgalacturonase (PMG) reaction (Seegmiller and Jansen, 1952); and pectin *trans*-eliminase (PTE) (EC 4.2.99) reaction. Most of the ordinary pectinases used for clarifying fruit juice belong to the first case. In this case methanol is produced from pectin by the action of PE. The existence of PMG has never been clearly proved (Edstrom and Phaff, 1964). Unlike hydrolytic pectinases, PTE forms methylgalacturonides with unsaturated bonds between carbon atoms 4 and 5 in the anhydromethyl-galacturonosyl residues of nonreducing ends (Albersheim *et al.*, 1960). In addition to pectin *trans*-eliminase, similar enzymes (pectic acid *trans*-eliminase) which are specific for polygalacturonic acid have been reported in bacterial cultures (Nagel and Vaughn, 1962; Macmillan and Vaughn, 1964; Nasuno and Starr, 1967; Nagel and Wilson, 1970). However, these enzymes will not be useful for the clarification of fruit juice because they show the activity in an alkaline side.

Endo (1965a) ascribed apple juice clarifying activity in the crude enzyme of *Coniothyrium diplodiella* to the combination of PG's and PE when he mixed all purified enzymes in the same ratio as in the crude enzyme. Yamasaki *et al.* (1967) also reported that a purified preparation of *endo*-PG from

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Table I. Purification of PTE from *Aspergillus sojae* No. 48

Purification step	Total Volume, ml	Total PTE, units	Total Protein, mg	Specific activity	Yield, %
Extraction	34,000	26,690	327,760	0.081	100
Ammonium sulfate fractionation	1,353	19,930	74,415	0.268	74.7
Batchwise treatment with DEAE-Sephadex	480	12,720	17,808	0.714	47.6
CM-Cellulose column chromatography	200	7,216	1,278	5.65	27.0
DEAE-Sephadex column chromatography	120	3,900	148.8	26.2	14.6
SE-Sephadex column chromatography (first)	80	1,900	34.0	55.9	7.1
SE-Sephadex column chromatography (second)	40	1029.6	14.2	72.5	3.9
Gel filtration with Sephadex G-100	35	397.8	5.2	76.5	1.5

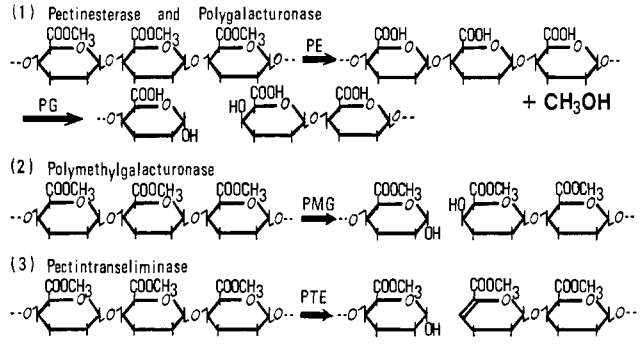

Figure 1. Enzymatic degradation of pectin

Aspergillus saitoi could clarify apple juice only in the presence of a PE preparation of *Sclerotinia arachnidis*, but that each preparation could not by itself. Recently, Ishii and Yokotsuka (1971) found that PTE was independently capable of clarifying fruit juices such as those of apple and grape.

In this paper, details of the clarification of fruit juices by PTE are reported using purified PTE from *Aspergillus sojae*.

MATERIALS AND METHODS

Fruit Juice. Four varieties of apple (Kokko, Jonathan, Golden Delicious, and Starking Delicious) and a variety of grapes, Delaware, were used in this study. Apple and grape juices were prepared by grating or crushing commercially available fruits and squeezing them through cotton cloth. Freshly prepared fruit juices were used without any treatment. The pH's of these fruit juices were as follows: Kokko, 3.90; Jonathan, 3.40; Golden Delicious, 3.85; Starking Delicious, 4.05; and Delaware, 3.35.

Pectin. Pectin N. F. (citrus pectin, No. 3442) was purchased from Sunkist Growers, Inc. It was washed three times with 70% ethanol to remove soluble sugars. The degree of esterification and anhydrogalacturonic acid content of pectin was 68 and 78%, respectively.

Purified Pectin *trans*-Eliminase. Purified PTE was obtained by the following procedures. *Aspergillus sojae* No. 48 was grown on moistened wheat bran at 30°C for 3 days. The culture medium was incubated with 5 vol of water at room temperature for 2 hr, and filtered through cotton cloth. Solid ammonium sulfate was added to the clear extract obtained by filtration with Celite. The precipitates formed between 0.4 and 0.75 saturation were collected by centrifugation and were dissolved in water. The solution was passed through a Sephadex G-25 column to remove ammonium sulfate and active fractions were collected and lyophilized. The powder was dissolved in 0.01 *M* acetate buffer, pH 4.0, and undissolved materials were removed by centrifugation. The solution was treated with DEAE-Sephadex batchwise at pH 4.0. The treated solution was applied on a CM-cellulose column previously equilibrated with 0.01 *M* acetate buffer, pH 4.0. After washing with the same buffer the column was eluted successively with 0.05 *M* acetate buffer, pH 4.5, and 0.1 *M* acetate buffer, pH 5.0. The active eluate was lyophilized, redissolved in distilled water, and dialyzed against 0.01 *M* phosphate buffer, pH 6.5 at 4°C for 24 hr. The dialysate was then applied on a DEAE-Sephadex column previously equilibrated with 0.01 *M* phosphate buffer, pH 6.5. The active fractions eluted with 0.05 *M* phosphate buffer, pH 6.5, were collected, lyophilized, redissolved in 0.1 *M* acetate buffer, pH 4.0, and finally dialyzed against 0.1 *M* acetate buffer, pH 4.0. The dialysate was applied on a SE-Sephadex column previously equilibrated with 0.1 *M* acetate buffer, pH 4.0. The column was eluted with a linear gradient of concentration of NaCl in the same buffer. The active fractions were collected and dialyzed against 0.1 *M* acetate buffer, pH 4.0. Final preparation was obtained by gel filtration with Sephadex G-100 of active fractions from rechromatography with SE-Sephadex.

Final preparation showed 940-fold purification over the crude extract and was almost homogeneous on disc electrophoresis. Specific activity of purified PTE was estimated to be 76.5 units per mg of protein. The purification of the enzyme is summarized in Table I.

The details of purification of PTE from *Aspergillus sojae* No. 48 will be presented elsewhere (Ishii and Yokotsuka, 1972).

Assay of Pectin *trans*-Eliminase Activity. PTE was determined by the method of Albersheim and Killias (1962) with slight modification. 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 a definite period. Sample (0.5 ml) was withdrawn from the reaction mixture and diluted tenfold with 0.01 *N* HCl or distilled water. Absorbance change at 235 nm against control in parallel system using boiled enzyme was measured in a Hitachi Model 101 spectrophotometer. One unit of PTE was defined as an in-

crease in absorbance at 235 nm by 1.0 in the reaction mixture per min. Absorbance at 235 nm increased linearly with relative enzyme concentration only to about five times and then fell (Figure 2).

Measurement of Clarification of Fruit Juice. To 10 ml of fruit juice 1.0 ml of enzyme solution (or water) 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 centrifuged at 3000 rpm for 5 min and the transmittance at 660 nm of the supernatant was measured.

Measurement of Viscosity. Changes in viscosity of apple juice were measured by an Ostwald viscosimeter in which the water blank value was 22.7 sec at 40°C. To 10 ml of apple juice in a viscosimeter 1.0 ml of enzyme solution was added, and the mixture was incubated at 40°C. Flow time readings were made at intervals, and the reducing rate of viscosity was calculated.

Measurement of Reducing Sugar. Reducing sugar was determined by the hypoiodite method of Willstätter and Schudel (1918) by using galacturonic acid as a standard.

Measurement of Carboxyl Group. Formation of carboxyl groups during clarification was determined by a titration method using a Radiometer autotitrator. To 5 ml of apple juice 0.5 ml of enzyme solution was added, and the pH of the mixture was immediately adjusted to that of the original apple juice. Newly appeared carboxyl groups were continuously titrated with 0.05 *N* NaOH to maintain the original pH. The number of carboxyl groups formed was calculated from the titration values.

Determination of Pectin in Apple Juice. To 25 ml of apple juice 75 ml of 99.5% (v/v) ethanol was added and the mixture was stirred for 10 min. The jellified materials were collected by centrifugation at 10,000 rpm for 10 min, washed three times with 75% (v/v) ethanol, and then dissolved in 25 ml of distilled water. The pectin content of the juice was estimated by determining anhydrogalacturonic acid of the above solution by the carbazole method (McComb and McCready, 1952).

Determination of Methanol in Apple Juice. The measurement of methanol content in apple juice was as follows. Twenty-five milliliters of apple juice was steam-distilled, and 50 ml of distillant was collected. Methanol in distillant was determined by the chromotropic acid method of Yamamura and Matsuoka (1954). To 1.0 ml of distillant in a test tube 0.2 ml of a solution of 5% potassium permanganate in 5% phosphoric acid was added. After 20 min at room temperature the mixture was shaken momentarily with 0.2 ml of 20% sodium sulfite. After 10 min the mixture was shaken vigorously with 3.0 ml of a solution of 0.05% sodium chromotropate in 75% sulfuric acid. The solution was placed in a boiling water bath for 45 min, cooled, and the optical density was measured at 580 nm with a Hitachi Model 101 spectrophotometer.

RESULTS AND DISCUSSION

Amount of PTE Required for the Clarification of Fruit Juice. Table II shows the relation between the amount of PTE used and the clarification of fruit juices. The addition of 0.018 to 0.024 units of PTE to 10 ml of apple juice was enough for complete clarification within 1 hr at 40°C, although there were slight differences according to juice source. The result is that 1 mg of PTE, having 76.5 units of activity, is capable of clarifying 30–40 liters of apple juice within 1 hr at 40°C.

More than five times the amount of PTE, however, was necessary for the complete clarification of grape juice. The difference in the clarification between apple juice and grape juice may be due to the degree of esterification of pectin in the two, since PTE is more active to highly esterified pectin. It was reported that the degree of esterification of apple pectin was 87–90% (Endo, 1965b), but that of grape pectin was only 45–55% (Lapsker *et al.*, 1970).

The amount of PTE to be used for the clarification of each fruit juice was not always constant. Empirically such a variation was found to be most remarkable upon clarifying Starking Delicious juice. Misawa *et al.* (1968) also reported that clarification of Starking Delicious juice was more difficult than that of other varieties tested.

The effect of pretreatment of Starking Delicious juice on the clarification by PTE was examined as follows. Freshly prepared apple juice of Starking Delicious was divided into two groups. One was preincubated at 30°C for an arbitrary time, and the other was immediately heated at 90°C for 5 min, rapidly cooled, and then preincubated at 30°C. The rates of clarification of the two samples by PTE were determined at intervals by the standard assay method. The result is shown in Figure 3. In the case of nonheat-treated juice, clarification became more difficult with increasing preincubation time at 30°C. Ten milliliters of freshly prepared juice without pretreatment was completely clarified by the addition of 0.017 units of PTE. But when the juice was preincubated for 24 hr at 30°C, even the addition of 0.085 units of PTE was not enough for complete clarification. On the other hand, the clarification of heat-treated juice was not affected by preincubation time.

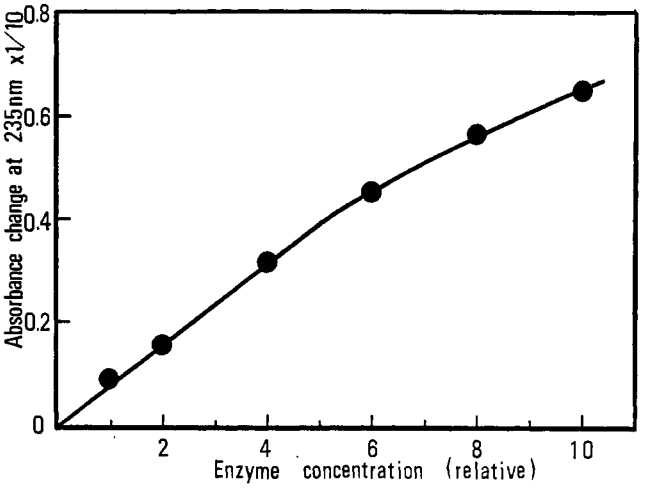

Figure 2. Relation between enzyme concentration and absorbance change at 235 nm

Table II. Relation between Amount of PTE and Clarification of Fruit Juice

Amount of PTE added to 10 ml of fruit juice, units	Clarification of fruit juice after 1 hr at 40°C, transmittance at 660 nm (%)				
	Apple				
	Kokko	Jonathan	Golden Delicious	Starking Delicious	Grape, Delaware
0	61.50	72.50	42.25	24.75	9.00
0.006	77.50	91.75	82.25	30.00	9.25
0.012	88.50	97.00	96.00	75.25	9.00
0.018	94.00	97.00	97.00	92.00	8.50
0.024	94.00	96.75	97.25	95.50	10.25
0.048				95.50	43.50
0.072					85.25
0.120					97.00

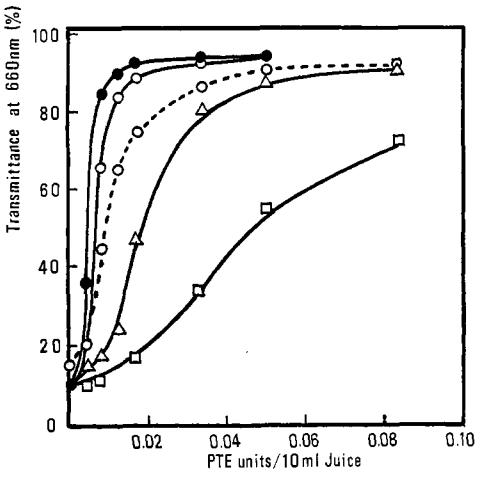

Figure 3. Effect of pretreatment of apple juice on clarification. —●— no treatment; —○— preincubation at 30°C for 1 hr; —△— preincubation at 30°C for 5 hr; —□— preincubation at 30°C for 24 hr; – –○– – heat treatment at 90°C for 5 min and preincubation at 30°C for 0, 1, 5, and 24 hr

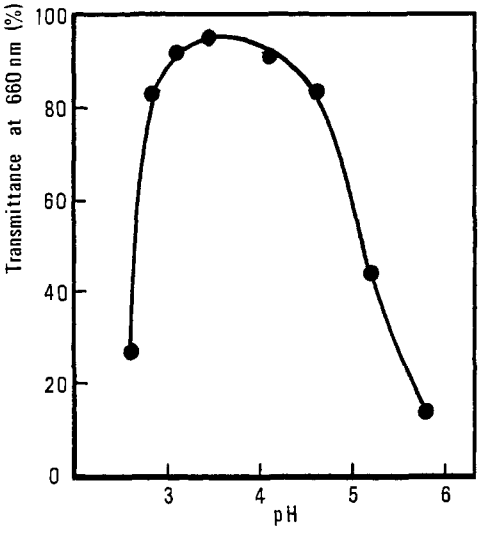

Figure 4. Effect of pH on clarification

The result suggests that such enzymatic activity as pectin esterase (Pollard and Kieser, 1951) in Starking Delicious tissue might make the juice difficult to clarify by PTE during preincubation. The treatment at 90°C for 5 min may cause the inactivation of such activity.

Optimal Condition for Clarifying Fruit Juice by PTE. The effects of pH and temperature on clarification of fruit juice by PTE are shown in Figures 4 and 5, respectively. Juice of Jonathan apples (pH 3.40) was adjusted to each pH by adding solid citric acid or sodium carbonate. As shown in Figure 4, the pH optimum for clarification lies between 3 to 4. Unexpectedly, it was difficult to clarify fruit juice above pH 5.0 although the pH optimum of PTE for citrus pectin (68% esterified) in buffer solution was 5.5 (Ishii *et al.*, 1970). This disagreement might be due to the fact that in clarification of fruit juice nonenzymatic coagulation of suspended materials (flocculation) resulting from degradation of pectin hardly occurred above pH 5.0, as pointed out by Yamasaki *et al.* (1964). When the cloudy juice after enzymation at pH 5.2 was returned to its original pH value (3.40), rapid flocculation followed by clarification occurred.

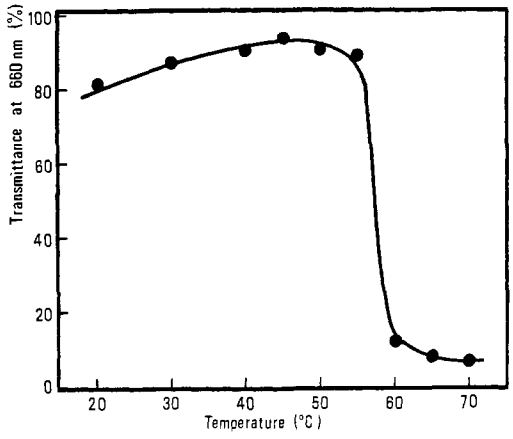

Figure 5. Effect of temperature on clarification

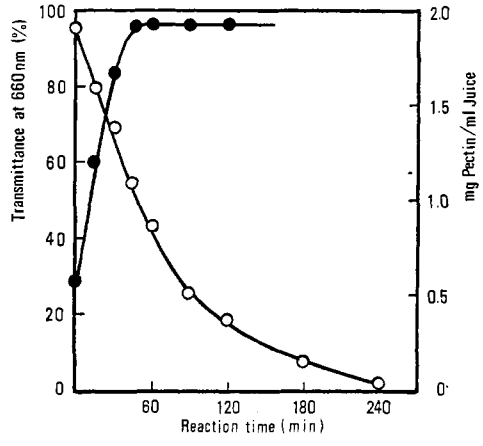

Figure 6. Relation between clarification and degradation of pectin. —●— clarification; —○— degradation of pectin

Optimum temperature of clarifying fruit juice by PTE was 45–50°C (Figure 5). The enzyme was found to be inactivated at temperatures higher than 60°C in fruit juice.

Physical and Chemical Changes during Clarification by PTE. Apple juice of Golden Delicious was used for the test of physical and chemical changes during clarification by PTE because of its higher viscosity and pectin content than found in other varieties.

To 1000 ml of apple juice of Golden Delicious 1 unit of PTE was added, and the mixture was incubated at 40°C. At intervals, transmittance at 660 nm of the juice and residual pectin in the juice were determined. The relation between clarification and degradation of pectin is shown in Figure 6. The apple juice was completely clarified after 60 min, showing transmittance of 96.25% at 660 nm, but about 50% of pectin still remained insoluble in 75% ethanol. The amount of pectin in the apple juice decreased linearly with incubation time until 60 min, but more slowly afterwards. Most of the pectin was converted to 75% ethanol-soluble forms after 4 hr.

The reduction of viscosity and the increase of reducing sugars and carboxyl groups in the apple juice during clarification by PTE are shown in Figure 7. More enzyme (0.05 units of PTE/10 ml of apple juice) than in other cases was used so that complete clarification was accomplished within 10 min. About 25% of viscosity was reduced at the time of clarification, but only little change was observed in the reducing sugars. It was previously found that PTE of *Aspergillus sojae* reduce the viscosity of pectin solutions by 50% at the

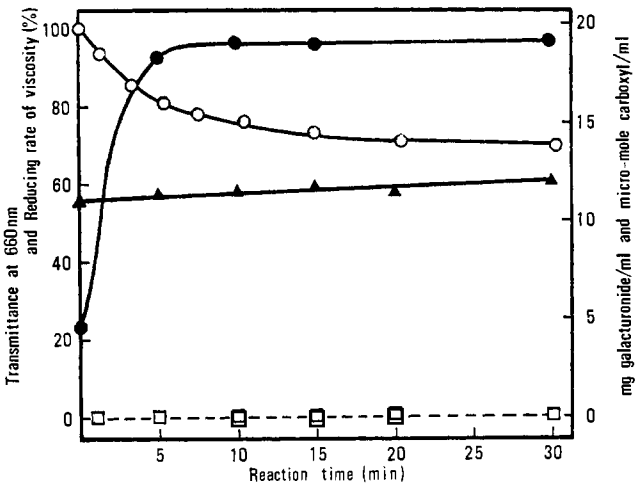

Figure 7. Changes during course of clarification of apple juice by PTE. —●— transmittance at 660 nm; —○— reduction of viscosity; —▲— reducing sugars; - -□- - - newly appeared carboxyl group

Table III. Formation of Methanol in Apple Juice Clarification

Variety of apple	Methanol (mg/ml of apple juice) Control	Clarified by PTE	Clarified by Ordinary hydrolytic pectinase	Pectin content, mg/ml of original juice
Kokko	0.001	0.001	0.010	0.246
Jonathan	0.002	0.001	0.008	0.160
Golden Delicious	0.001	0.002	0.136	1.126
Starking Delicious	0.001	0.002	0.086	0.525

time when the extent degradation of α-1,4-linkage was only 0.36% and degraded pectin in a random manner (Ishii *et al.*, 1970). These results strongly suggest that the clarification of fruit juice by PTE as well as hydrolytic pectinases occurs in the early stage of the degradation of pectin.

Clarification by PTE differed from that by hydrolytic pectinase in terms of formation of carboxyl groups. As shown in Figure 7, PTE does not produce carboxyl groups during clarification. However, Endo (1965a) and Yamasaki *et al.* (1967), in their investigation of clarifying apple juice by both purified PE and *endo*-PG preparations, showed an increase of carboxyl groups during clarification. And in our assay of a commercial hydrolytic pectinase, Sclase (Sankyo Co. Ltd.), used for clarifying fruit juices, produced carboxyl groups during clarification; in 5 ml of Golden Delicious juice 2.5 mg of Sclase produced 4.50 and 6.35 μmol carboxyl groups in 10 and 30 min, respectively. The amount of carboxyl groups produced by hydrolytic pectinase during clarification was found to vary with the varieties of apple.

Formation of Methanol in Apple Juice Clarification. The splitting of methyl ester bonds in the pectin molecule results in the formation of methanol during clarification.

As shown in Table III, only a negligible amount of methanol could be detected in original cloudy apple juice (control) or juice clarified by PTE. In contrast, methanol was clearly detected in every apple juice clarified by the ordinary hydrolytic pectinase. The amount of methanol produced by hydrolytic pectinase varied much according to the variety of apple; the amount of methanol in clarified Golden Delicious juice was 17 times larger than that in clarified Jonathan juice.

Table IV. Effect of Various Compounds on Clarification

Compound	Transmittance at 660 nm, % Units of PTE/10 ml of apple juice 0	0.010	0.025	PTE activity, %
No addition	24.75	83.00	98.25	100
NaCl	27.75	92.00	99.00	101.5
$CaCl_2$	25.00	77.25	99.00	103.0
$MgCl_2$	28.00	93.25	99.00	98.0
EDTA	23.75	87.75	98.25	100.5
Pectin	19.75	28.25	30.25	
Pectic acid	38.75	58.75	50.50	88.2
Alginic acid	18.25	25.25	27.75	101.5
Carboxymethyl-cellulose	20.50	33.00	42.25	102.6
Starch	23.00	89.00	98.50	104.6
Casein	7.50	26.00	93.00	103.1
Gelatin	15.50	57.00	98.50	100.0

These variations in methanol formation seem to be due to differences of pectin content in the juices, as given in Table III.

Effect of Various Compounds on Clarification by PTE. The effect of various compounds at final concentration of 0.1% on clarification by PTE was examined with Golden Delicious juice as substrate (Table IV). PTE activity was not affected by the tested compounds, except that the slight inhibition was shown by pectic acid. But alginic acid, pectic acid, and carboxymethylcellulose strongly inhibited the clarification. NaCl and $MgCl_2$ slightly stimulated the clarification but $CaCl_2$, EDTA, and gelatin had no effect.

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