Susceptibility of Fruit Juice to Enzymatic Clarification by Pectin Lyase and Its Relation to Pectin in Fruit Juice

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Differences in the enzymatic clarification of fruit juice from apples and grapes were revealed by adding purified pectin lyase and *endo*-polygalacturonase from *Aspergillus japonicus* individually and in combination. Pectin lyase showed almost the same clarifying activity as the crude enzyme but *endo*-polygalacturonase had no activity on apple juice clarification. In the case of grape juice, both enzymes showed clarifying activity, while the susceptibility of the juice to the enzyme varied according to the varieties and ripening. This variation was found to be mainly related to the degree of esterification of pectin in the juice, which may be converted by the pectinesterase occurring in grape tissues. A mixture of pectin lyase and *endo*-polygalacturonase brought about more rapid clarification of grape juice than did either of the two enzymes alone, indicating a synergistic effect. However, the clarification of grape juice by the crude enzyme could not be explained only by the combined action of the two enzymes.

Pectic substances occur in all higher plant tissues as cell wall constituents. The juice of ripe fruit is rich in dissolved pectin, which must be considered as solubilized protopectin. The pectin in fruit juice may suspend other materials in a stable colloidal system. For the clarification of fruit juice therefore, it is necessary to depolymerize the pectin.

Endo (1965) and Yamasaki *et al.* (1967) showed that cloudy apple juice was completely clarified by adding purified pectinesterase (EC 3.1.1.11) and *endo*-polygalacturonase (EC 3.2.1.15), although each of them had no effect on the clarification when added alone. Ishii and Yokotsuka (1971) found that purified pectin lyase (EC 4.2.2.3) from *Aspergillus sojae* was independently capable of clarifying not only apple juice but also grape juice. From these studies of the enzymatic clarification of fruit juice, it was concluded that only the depolymerization of pectin is enough for the clarification of apple and grape juices. However, grape juice was more resistant to the clarification by pectin lyase than apple juice (Ishii and Yokotsuka, 1972b,c). This may be due to the lower degree of esterification of grape pectin than that of apple pectin, since pectin lyase is specific for methyl-esterified galacturonide linkages in pectin molecules.

It is well known that pectolytic enzymes occur in various fruit tissues (Pilnik and Voragen, 1970) and are concerned with pectic changes during ripening and maturation (Arakji and Yang, 1969; Ben-Arie and Lavee, 1971; Hasegawa *et al.*, 1969; Pressey *et al.*, 1971; Shewfelt *et al.*, 1971). One of these enzymes, pectinesterase, catalyzes the hydrolysis of methyl ester bonds in pectin and results in a decrease in the degree of esterification.

In this paper the susceptibility of fruit juice to enzymatic clarification and its relation to pectin in the juice are reported using purified pectin lyase and *endo*-polygalacturonase from *Aspergillus japonicus*.

MATERIALS AND METHODS

Fruit Juice. Two varieties of apples (Jonathan and Golden Delicious) and three varieties of grapes (Delaware, Campbell Early, and Bailey A) 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: Jonathan, 3.33; Golden Delicious, 3.60; Delaware (I), 3.30; Delaware (II) (very ripe, obtained in late season), 3.52; Campbell Early, 3.30; and Bailey A, 3.20.

Enzyme Preparations. Pectolytic enzymes of *Aspergillus japonicus* 1744 were used in this study, since this mold produced a great amount of pectolytic enzymes, and the crude enzyme showed a higher fruit juice clarifying activity than that of *Aspergillus sojae* No. 48. *Aspergillus japonicus* 1744 was grown on moistened wheat bran at 25° for 65 hr. The culture medium was incubated with 5 vol of water, and the pH of the mixture was adjusted to 4.5 with 1 *N* HCl. After standing at room temperature for 2 hr, the suspension was squeezed through cotton cloth, followed by filtration with Celite. Three volumes of cold ethanol were added to the clear extract with continuous stirring. After standing overnight at 4°, the resultant precipitate was collected by centrifugation and dried *in vacuo*. This was used as a crude enzyme which contained 0.3 units of pectin lyase and 7.0 units of *endo*-polygalacturonase per milligram.

Purified pectin lyase and *endo*-polygalacturonase from the crude enzyme of *Aspergillus japonicus* were obtained by the following procedures, as shown in Figure 1. In this way the specific activity of pectin lyase and *endo*-polygalacturonase increased approximately 79- and 25-fold, respectively, and no other pectolytic enzyme activity could be detected in each final preparation. The details of the purification and properties of pectolytic enzymes from *Aspergillus japonicus* will be presented elsewhere (Ishii and Yokotsuka, 1972a).

Pectic Substances. Pectin N.F. (citrus pectin, No. 3442) and sodium polypectate (No. 6024) were purchased from Sunkist Growers, Inc. They were washed three times with 70% ethanol to remove soluble sugars. The anhydrogalacturonic acid content of pectin and sodium polypectate was 78 and 71%, respectively. The degree of esterification of pectin was 68%.

Assay of Pectolytic Enzyme Activity. Pectin lyase was determined by spectrophotometry (Albersheim and Killias, 1962). 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° for a definite period. The increase in absorbance at 235 nm was measured using a Hitachi model 101 spectrophotometer. One unit of pectin lyase was defined as an increase in absorbance at 235 nm by 1.0 in the reaction mixture per min.

endo-Polygalacturonase was determined by the viscosity-reducing method of Roboz *et al.* (1952). Reaction mixtures containing 1.0 ml of enzyme solution, 3.0 ml of

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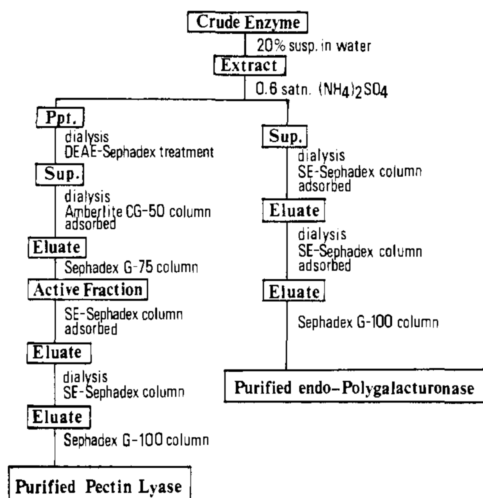

Figure 1. Purification procedure of pectin lyase and *endo*-polygalacturonase from *Aspergillus japonicus*.

1% sodium polypectate, and 3.0 ml of McIlvaine buffer, pH 4.5, were placed in an Ostwald viscosimeter and incubated at 40°. The readings of flow time were made at intervals and the rate of viscosity reduction was calculated. One unit of *endo*-polygalacturonase was defined as the amount of enzyme which reduces the viscosity of the reaction mixture by 50% per min at 40°.

Test for the Enzymatic Clarification of Fruit Juice. Each cloudy fruit juice was divided into four groups and incubated by adding the following enzymes: crude enzyme; a mixture of purified pectin lyase and *endo*-polygalacturonase; and either of the two enzymes alone. To 100 ml of apple juice was added 0.4 mg of crude enzyme, while 4 mg was used for grape juice clarification. Purified enzymes were added to fruit juice, making the amount of activity equal to the crude enzyme added. These fruit juices were incubated at 40° and, at intervals, clarification of fruit juice was measured as follows. Samples (5 ml) were withdrawn from the reaction mixtures and immediately heated in a boiling water bath for 5 min. After cooling they were centrifuged at 2500 × *g* for 5 min and the transmittance at 660 nm of the supernatant was measured.

Analysis of Pectic Substances in Fruit Juice. Cloudy fruit juice was ultracentrifuged at 100,000 × *g* for 30 min to completely remove suspended materials in the juices (Yamasaki *et al.*, 1964). To 25 ml of the clear supernatant was added 75 ml of 99.5% ethanol, and the mixture was stirred for 10 min. The resultant precipitate was collected by centrifugation at 8000 × *g* for 10 min, washed three times with 75% ethanol, and then dissolved in 25 ml of distilled water. After saponification of the sample obtained above at pH 12 and room temperature for 40 min, the pectin content was estimated by determining anhydrogalacturonic acid by the carbazole method (McComb and McCready, 1952). Neutral sugar was calculated as the difference between the total sugar estimated by the phenol–sulfate method (Dubois *et al.*, 1956) and galacturonic acid estimated by the carbazole method, and was expressed as milligrams of arabinose. The determination of esterification of the pectin was carried out by the saponification and methanol estimation method of Hatanaka and Ozawa (1966).

Determination of Methanol. The measurement of methanol in fruit juice was carried out according to the procedure described previously (Ishii and Yokotsuka, 1972b). Methanol in the pectin solution after saponification was determined by the chromotropic acid method of Yamamura and Matsuoka (1954).

Measurement of Pectinesterase Activity in Fruit Tissue. Pectinesterase activity of fruit tissue was determined by measuring an increase of methanol in fruit juice during incubation. Each mashed fruit was divided into two groups; one was incubated at 30° and the other was heated at 90° for 5 min before incubation. Toluene (1%, v/v) was added to the mashed fruit as preservative. At intervals the sample was withdrawn, squeezed through cotton cloth, and ultracentrifuged at 100,000 × *g* for 30 min. The supernatant was steam distilled and the distillant was subjected to methanol estimation.

RESULTS AND DISCUSSION

Purified *endo*-polygalacturonase, which may be preferable to pectin lyase in the depolymerization of lowly esterified pectin in fruit juice, was also used in this study.

Figure 2 shows the enzymatic clarification of apple juice, Jonathan and Golden Delicious. In both cases, the addition of 0.4 mg of the crude enzyme containing 0.12 unit of pectin lyase and 2.8 units of *endo*-polygalacturonase to 100 ml of apple juice was enough for complete clarification within 1 hr at 40°. As shown in Figure 2, *endo*-polygalacturonase had no effect on apple juice clarification by itself. The results agreed with those obtained by Endo (1965) and Yamasaki *et al.* (1967). The addition of 0.12 unit of purified pectin lyase to 100 ml of apple juice showed almost similar clarifying activity to the crude enzyme. This confirmed that apple juice clarification by the crude enzyme of *Aspergillus japonicus*, as well as that of *Aspergillus sojae* No. 48 (Ishii and Yokotsuka, 1971), was principally contributed by pectin lyase.

In the case of clarifying grape juice, the addition of 4 mg of the crude enzyme to 100 ml of the juice was necessary for the complete clarification within 2 hr at 40°. Although almost similar clarification rates were accomplished by adding the crude enzyme, the mode of enzymatic clarifications differed from each other (Figure 3). Both pectin lyase and *endo*-polygalacturonase showed clarifying activity to Delaware (I) juice, although the former was more active than the latter. Unlike apple juice, clarification of the juice by the crude enzyme could not be explained by the individual or combined actions of the two pectolytic enzymes, because an obvious difference in the rate of clarification between the crude enzyme and the mixture of the two enzymes was found. In the juice of Delaware (II), which was the same variety but was obtained in a later season than Delaware (I), *endo*-polygalacturonase was more effective than pectin lyase on the clarification. The fact that the crude enzyme was also more active than the mixture of pectin lyase and *endo*-polygalacturonase on the clarification of Delaware (II) juice suggests the existence of some factor in the crude enzyme capable of promoting the clarification of the grape juice together with pectolytic enzymes.

It is noteworthy, however, that the mixture of pectin

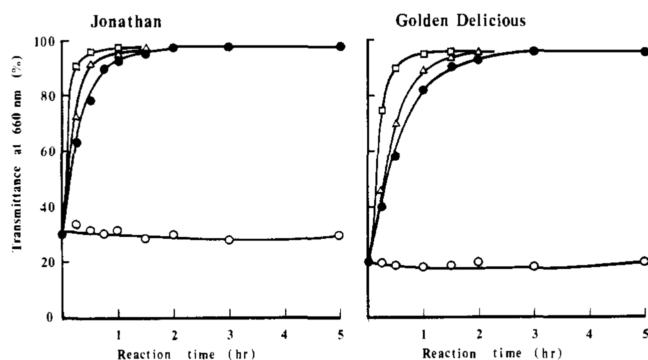

Figure 2. Enzymatic clarification of apple juice. ●, purified pectin lyase; ○, purified *endo*-polygalacturonase; △, mixture of the purified two enzymes; □, crude enzyme.

Table I. Pectin Content and Degree of Esterification of Pectic Substances Isolated from Fruit Juices

		Yield, mg/100 ml of juice			
		Pectin (anhydrogalacturonic acid)	Neutral sugar (arabinose)	Pectin content, %	Degree of esterification, %
Apple	Jonathan	13.0	9.8	57.0	92.0
	Golden Delicious	52.2	7.9	86.9	90.7
Grape	Delaware (I)	51.2	20.3	71.6	65.3
	Delaware (II)	57.4	23.2	71.2	43.9
	Campbell Early	49.5	15.3	76.4	44.8
	Bailey A	34.6	14.6	70.3	57.1

lyase and *endo*-polygalacturonase showed a higher activity than the one expected from individuals, indicating the synergistic effect. The effect is remarkable in an initial reaction time, when neither of the two enzymes alone showed little clarifying activity. This synergistic phenomenon was also observed in the other grape juices.

Campbell Early was similar in the mode of enzymatic clarification to Delaware (II). But Bailey A juice was somewhat different from other grape juices; pectin lyase and *endo*-polygalacturonase showed a similar clarifying activity, and the clarifying activity by the crude enzyme could be represented by the combined action of the two enzymes.

These results suggest that the differences in enzymatic clarification between apple juice and grape juice and among grape juices will be related to the characteristics of pectic substances occurring in these fruit juices. Table I shows the analysis of the pectic substances isolated as 75% ethanol-insoluble materials. The fact that the pectin isolated from apple juice, in either case, had a high degree of esterification may be a main reason that *endo*-polygalacturonase had no effect when added alone, but pectin lyase was very effective on clarifying apple juice. The degree of esterification of grape pectin was lower than that of apple pectin, and varied with the varieties and ripening. The degree of esterification of pectin was closely associated with the susceptibility of grape juice to enzymatic clarification by pectin lyase.

The result that the degree of esterification of grape pectin was lower than that of apple pectin and decreased with ripening suggests the occurrence of pectinesterase in grape tissues. Although an optimum pH of fruit pectinesterase has been found to be between 7 and 8 (Arakji and Yang, 1969; MacDowell *et al.*, 1950; Pithawala *et al.*, 1948), the activity of apple and grape was measured at the pH of fruit itself in order to detect whether or not the actual demethylation can occur in the fruit tissue. The results are given in Table II. No or only a negligible amount of pectinesterase activity was found in the apple tissues. McColloch and Kertesz (1947) and Pollard and Kieser (1951) demonstrated that pectinesterase activity in apple tissue was much lower than in orange or tomato tissues. On the other hand, a great increase of methanol during incubation, particularly in Delaware (I), was found in the grape tissues. The pectinesterase activity of grape is so strong, even at the pH of fruit itself, that the enzyme may play an important part in depolymerization of esterified pectin in grape juice together with *endo*-polygalacturonase. A considerable amount of methanol was already detected in the original grape juice, suggesting that demethylation of pectin may occur during ripening. Each control, which heated the mashed fruit at 90° for 5 min before incubation, did not increase methanol at all.

From the experiment described above we conclude as follows. Apple pectinesterase showed little activity in fruit tissue, and the pectin solubilized in the apple juice, therefore, has a high degree of esterification. On the other hand, grape tissue contained a high pectinesterase activity, which could cause a gradual decrease in the degree of esterification at the pH of the fruit itself. Therefore the pectin in grape juice may exist with various degrees of esterification, depending upon the variety and ripening.

Table II. Pectinesterase Activity Determined as Increase of Methanol in Mashed Fruit Tissues

		Methanol, µmol/ml of juice				
		Incubation time (day) at 30°				
	Variety	0	1	3	5	7
Apple	Jonathan	0.03	0.03		0.04	0.04
	Golden Delicious	0.06		0.07		0.09
Grape	Delaware (I)	0.49	3.35	3.69		4.11
	Delaware (II)	0.69	1.18	1.34		2.19
	Campbell Early	1.08	1.37	1.78	1.84	1.89
	Bailey A	0.39	0.55	0.91	1.02	1.43

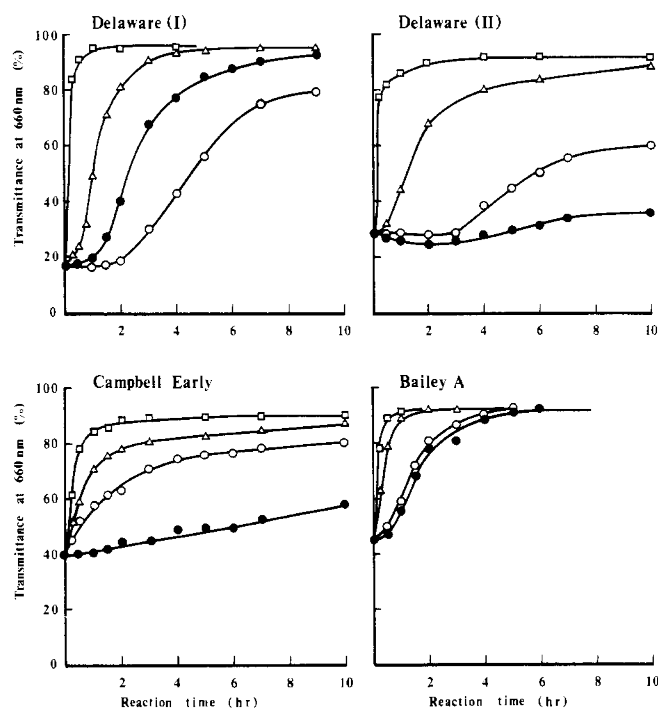

Figure 3. Enzymatic clarification of grape juice. ●, purified pectin lyase; ○, purified *endo*-polygalacturonase; △, mixture of the purified two enzymes; □, crude enzyme.

Table III. General Composition of Pectic Substances Isolated from Golden Delicious and Delaware Juices

Yield, mg/100 ml of juice	Golden Delicious 89.2	Delaware 675
Polysaccharide, %	67.4	11.9
Pectin, %	58.5	8.5
Neutral sugar, %	8.9	3.4
Protein, %	4.4	3.2
Ash, %	2.7	28.7
Unknown components, %	25.5	56.2

Such variation is related to the susceptibility of grape juice to enzymatic clarification. It seems likely that the boundary of susceptibility of fruit juice to pectin lyase and *endo*-polygalacturonase may exhibit about 55% of esterification of pectin in the juice.

The present study could not make it clear what kind of component in the crude enzyme may be a promoting factor and why it is necessary for clarifying only grape juice. Great difference was not found, as shown in Table II, in the pectin content of pectic substances isolated from apple and grape juice. However, it is not certain whether neutral sugar detected in these substances is bounded to pectin as a constituent or not, since these pectic substances were isolated as only 75% ethanol-insoluble materials and were not purified.

In general composition, these materials isolated from grape juice (Delaware), as compared with those from apple juice, were low in polysaccharide content but contained a very high percentage of ash and unknown components (Table III). We suggest that the elucidation of the nature and the functional role of the promoting factor in clarifying grape juice is not likely to be a simple matter.

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Use of Furfural Content as an Index of Storage Temperature Abuse in Commercially Processed Orange Juice

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Furfural was measured over a 16-week period in canned orange juice and orange juice packed in glass at 5, 10, 16, 21, and 30°. Examination of canned juice showed that for every 5° temperature rise in storage there was an approximate doubling of the furfural content. Organoleptic evaluation showed that when the level of furfural exceeded 55 μg/l. of juice, a taste panel observed a difference in flavor in comparison to controls at a significance of $p < 0.001$. Furfural, *per se*, was not the component responsible for the flavor change, but its levels appeared to parallel closely the extent of comparative flavor differences.

Commercially processed orange juice is highly susceptible to flavor change when stored at warm temperatures and prolonged storage periods. Over the past 30 years, many chemical changes occurring in adversely-stored citrus juices have been reported by scientists working in the citrus industry. Nolte and von Loesecke (1940) showed that temperature-aged Valencia orange juice differed from fresh juice by increased acidity, increased saponification and peroxide values, and by the presence of carbonyl compounds. Huskins *et al.* (1952) investigated the change in lipid composition of orange juice at 22° for 2 years and found that phospholipid phosphorus content decreased to one-tenth its original value, lipid nitrogen decreased to one-fifth its original value, and lipid choline had completely disappeared. The relationship of lipid degradation to flavor change has been further substantiated by the works of Curl and Veldhuis (1947), Swift (1951), and Nagy and Nordby (1970).

Although many chemical tests (Vandercook, 1970) are available to indicate temperature abuse of citrus juice, there has remained a need for a simple analysis which

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