Studies on Pectolytic Enzymes of Molds

Part XIII. Clarification of Apple Juice by the Joint Action of Purified Pectolytic Enzymes

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The clarification of apple juice has been studied using six pectolytic enzymes produced by *Coniothyrium diplodiella*, endo-PG (polygalacturonase) I, II and III, exo-PG and PE (pectinesterase) I and II. Each of these six enzymes had no effect on the clarification of apple juice when acted alone, whereas mixtures of any one of endo-PGs and PEs were all able to clarify the juice. Mixtures of exo-PG and either of PEs has no effect on the clarification. Clarifying activities of PG-PE mixtures were varied with the kind of endo-PG used in each mixture and not with the kind of PE. Clarifying activity of PG-PE mixture depended on either endo-PG or PE activities when the other was kept constant.

Crude enzyme from the mold and a mixture of the four PGs and PE in the ratio of the crude enzyme had essentially identical effect on apple juice as well as on artificial pectin and pectic acid.

INTRODUCTION

Clarification of apple juice has been carried out for many years with commercially available pectolytic enzymes.¹⁾ A large number of investigators have attempted to determine what enzymes are required for the clarification.

Joslyn et al.,²⁾ Neubeck,³⁾ Endo⁴⁾ and Arima et al.,⁵⁾ in their investigations of apple juice clarification by a series of pectolytic enzymes, found no correlation between the clarifying activity and the pectolytic activities on artificial pectin and pectic acid. Schubert⁶⁾ reported that pectolytic enzyme preparation from *Aspergillus niger* contained four PGs, only

one of which acted on native apple juice pectin at an optimum pH of 5.5. He differentiated the four PGs but did not purify them. Therefore, the presence of other enzymes, especially PE, in Schubert's preparations might have contributed to the clarification.

Previous works in this series dealing with fractionation of pectolytic enzymes produced by *Coniothyrium diplodiella* have shown that the enzymes consist of at least PE (pectinesterase) and four PGs (polygalacturonase).⁷⁾ All of these pectolytic enzymes have been separately purified. Furthermore, some of their properties have been studied.⁸⁾ It appeared of importance and of interest to study the relation between pectolytic enzyme and apple juice clarification using purified enzymes.

The present paper reports some results with these problem.

1) A. L. Demain and H. J. Phaff, *Wallerstein Lab. Commn.*, **20**, 119 (1957).

2) M. A. Joslyn, S. Mist and E. Lambert, *Food Technol.*, **6**, 133 (1952).

3) C. E. Neubeck, *J. Assoc. Off. Agr. Chemists*, **42**, 374 (1959).

4) A. Endo, *This Journal* **25**, 389 (1961).

5) K. Arima, M. Yamasaki and T. Yasui, *This Journal*, **28**, 248 (1964).

6) E. Schubert, *Nature* **169**, 931 (1952); *Schweiz. Brauereirundschau*, **62**, 39 (1951).

7) A. Endo, *This Journal*, **27**, 741, 751 (1963).

8) A. Endo, *This Journal*, **28**, 535, 543, 551, 639, 757 (1964).

TABLE I. SOME PROPERTIES OF SIX PECTOLYTIC ENZYME FRACTIONS FROM
Coniothyrium diplodiella

	Endo-PG I	Endo-PG II	Endo-PG III	Exo-PG	PE-I	PE-II
Content in crude enzyme as PGU (%)	18~20	7~10	25~30 ¹⁾	3~5 ¹⁾		
Specific activity (PGU)mgN or (PEU)mgN ²⁾	7,882	3,140	2,560	538.5	1,840	1,870
Sedimentation coefficients (S)	2.7	3.3	3.6			
Optimum pH range	4.0~4.5 ³⁾	4.0~4.5 ³⁾	4.0~4.5 ³⁾	4.0~4.5 ³⁾	4.5~5.0 ⁴⁾	4.5~5.0 ⁴⁾
Stable pH range	4.0~4.5	4.0~6.0	5.0~6.5	3.5~6.0	4.0~5.5	4.0~4.5
Stable temperature range (°C)	40>	45>	40>	50>	40>	40>
VRu/PGU ⁵⁾	2.30	1.32	3.00	0.168		
H ₂ SO ₄ precipitation test ⁶⁾	15	45	15>	80		
Hydrolysis of pectic acid {	Extent of hydrolysis (%)	73.8	68.8	54.0	100	
	End product	mono-, di-,	mono-, di-, tri-,	mono-,		
Decrease in viscosity of citrus pectin solution	+	+	+	-		

1) These figures are slightly different from previous data.⁸⁾

2) PG or PE unit per mg of total nitrogen.

3) Activity was measured by hypoiodite method with pectic acid as substrate.

4) Activity was measured by alkali-titration method with citrus pectin of esterification degree 64%.

5) The ratio of liquefying activity to saccharifying activity.

6) Per cent hydrolysis when no further precipitate is observed by adding 2ml of 2M H₂SO₄ solution to 2ml of reaction mixture.

MATERIALS AND METHODS

(1) Purified Pectolytic Enzymes

Endo-PG I, II and III, exo-PG, PE I and II used here were isolated from crude enzyme preparation from *Con. diplodiella* according to the procedures described before.⁸⁾ A summary of some properties of these enzyme fractions is shown in Table I.

(2) Crude Enzyme Preparation

Crude enzyme preparation *Con. diplodiella* was prepared as described previously.⁷⁾

(3) Apple Juice

Apple juice was obtained by crushing commercially available apples (Ralls apples) and squeezing the crushed apples with several pieces of gauze. Freshly pressed apple juice which had no detectable PG and PE activity was adjusted to pH 3.5 with citric acid or NaOH prior to clarification test. Unless otherwise stated, freshly pressed apple juice of Ralls apples was used.

(4) Pectin and Pectic Acid

Pectin (esterification degree 64%) and pectic acid were obtained commercially as described previously.⁷⁾

(5) Determination of Enzyme Activities

Determinations of polygalacturonase (PGU), viscosity-reducing (VRu) and PE (PEU) activities were the same as those described before.^{7,8)} Unless other-

wise stated, these enzyme activities were determined at pH 4.4

(6) Clarification of Apple Juice

Clarification of apple juice was carried out by the method described previously.⁹⁾ Into a test tube, 1.6 cm in diameter, were measured 10 ml of apple juice, and then an enzyme solution and water were added to make the total volume 11 ml. After incubating at 30°C for a suitable period, the reaction was stopped by heating for 5 min. in boiling water. After cooling with running tap water, the mixture was centrifuged at 3,000 r.p.m. for 10 min., and the resultant supernatant was collected. Transmittance (T %) of the supernatant at 660 mμ was determined in a Hitachi spectrophotometer (Hitachi Ltd., Japan) with 1-cm light path.

Centrifugation of the reaction mixture was carried out with the commercially available centrifuge which was capable of treating 24 samples at a time. In order to avoid the effect of centrifugation on transmittance of the supernatant, a series of reaction mixtures were centrifuged with the same centrifuge and at the same time.

The relative clarification values of a series of enzyme samples were calculated from the following equation, in which the clarification value of any one of the

9) A. Endo, This Journal, 28, 234 (1964).

FIG. 1. Relation between Enzyme Concentration and Transmittance.

Apple juice, freshly pressed juice of pH 3.5; incubation period, 20 min.; enzyme solution, 0.5% water extract of the crude enzyme preparation.

samples tested was compared with that of standard assigned to 100. The enzyme concentration required to reach 50% transmittance at 660 mμ after 20-min. incubation was determined from the relation between the enzyme concentration and the transmittance (Fig. 1).

$$A_{sa} = \frac{D_{st}}{D_{sa}} \times A_{st}(100)$$

where A_{sa} is the relative clarification value of the sample; A_{st} , the clarification value of the standard (100); D_{sa} , the enzyme concentration of the sample required to reach 50% transmittance in 20 min.; D_{st} , the enzyme concentration of the standard required to reach 50% transmittance in 20 min.

RESULTS

(1) Action of Endo-PG I, II and III, Exo-PG, and PE I and II on Apple Juice

The enzyme fractions described in Table I were applied to clarification of freshly pressed apple juice to determine whether these fractions were able to clarify apple juice. As shown in Fig. 2, every one of these fractions by itself could not clarify apple juice except for a slight action of endo-PG III.

Fig. 3 shows the results of the joint action of PG and PE on apple juice. It was observed that the clarification of apple juice

was accomplished by the joint action of one of endo-PGs and one of PEs, and that a mixture of oxo-PG and PE had no activity for apple juice. It is of interest that there was a lag in the clarification curve during initial stage of incubation (Fig. 3). There was no difference between the use of PE I and II. Endo-PG III was the most effective of the three endo-PGs on the clarification of apple juice when equal polygalacturonase unit at pH 3.5 and 4.4 was used as in Fig. 3. Moreover, endo-PG III was the most dominant polygalacturonase in the crude enzyme from *Con. diplodiella* (Table I).

Heat-treated juice of Ralls apples and fresh juice of Jonathan apples were similarly clarified by the joint action of one of endo-PGs and one of PEs. These results indicate that clarification of apple juice was done by the joint action of endo-PG and PE, and other hydrolases such as protease, amylase, cellulase and hemicellulase are not indispensable for the clarification of apple juice.

When the floc formation of apple juice occurred by the action of these enzymes, transmittance of the supernatant at 660 mμ reached 95~98%.

In Table II are given the results of relative clarification values of a series of the enzyme mixtures. Of the three endo-PGs, endo-PG III was the most effective on apple juice clarification in terms of PGU at pH 4.4. Exo-PG added to a mixture of endo-PG and PE had no stimulating effect on the clarification.

(2) Effect of the Ratio of PG to PE on the Clarification

With endo-PG I and PE I, the effect of the ratio of PG to PE on the clarification was determined as shown in Fig. 4 and Table III. The results in Table III are shown as relative clarification value. As shown in Table III, clarification value varied with either endo-PG I or PE I activities when the other was kept constant. These results indicate that the ratio of endo-PG to PE as well as the absolute endo-PG and PE activities

FIG. 2. Action of Each Enzyme Fraction on Apple Juice.

To 10ml of freshly pressed apple juice of pH 3.5 was added 0.15 PGU of PG or 0.15 PEU of PE. Activities of PG and PE were determined at pH 4.4(A) and at pH 3.5(B) respectively.

—○— endo-PG I, —●— endo-PG II,
—▲— endo-PG III, —△— exo-PG,
—■— PE I and II.

was important to the clarification of apple juice. About the same effect of the ratio of PG to PE on the clarification was obtained also with PE I and endo-PG II, and with PE I and endo-PG III.

In a previous paper dealing with the clarification of apple juice by two crude enzymes from *Con. diplodiella* and *Sclerotinia libertiana*, it was found that the clarification curve of apple juice varied with enzymes even when clarifying activity of these two enzymes was adjusted to nearly the same values.⁹⁾ Accordingly, the effect of the ratio of endo-PG to PE on the clarification curve was studied. Enzyme solutions varying in the ratio of endo-PG to PE were appropriately diluted so that clarification activities were approximately the same value.

Of the three endo-PGs, endo-PG I and endo-PG II exhibited nearly the same clarification curve respectively when the ratio of PG to PE varied (Fig. 5 and 6). In the case of endo-PG III, however, the clarification curve varied with the ratio of the PG to PE (Fig. 7). As the ratio of endo-PG III to PE was increased, the lag in the clarification was shortened but longer time was required for the final clarification. Therefore, crossing of the clarification curves was observed as shown in Fig. 7.

(3) Reconstruction of Crude Enzyme Action by Mixing Purified Pectolytic Enzymes

As stated in previous papers,⁸⁾ crude enzyme from *Con. diplodiella* contains four PG and two PE fractions. The endo-PG I, endo-PG II, endo-PG III and exo-PG contents of the

FIG. 3. Joint Action of PG and PE on Apple Juice.

To 10ml of fresh apple juice of pH 3.5 were added 0.15 PGU of one of PGs and 0.15 PEU of one of PEs. Activities of PG and PE were determined at pH 4.4(A) and at 3.5(B) respectively.

○--- endo-PG I +PE I, ●--- endo-PG II+PE I,
 ▲--- endo-PG III+PE I, △--- exo-PG +PE I,
 ○--- endo-PG I +PE II, ●--- endo-PG II+PE II,
 ▲--- endo-PG III+PE II, △--- exo-PG +PE II.

crude enzyme as PG-activity at pH 4.4 were 18~20, 7~10, 25~30 and 3~5%, respectively (Table I). The PE content of the crude enzyme could not be determined exactly since it was difficult to separate PE from other PGs quantitatively and, moreover, PE activity was strongly affected by the presence of PG. Therefore, to the mixture of the four PG in the ratio mentioned above was added appropriate unit of PE as shown in Table IV-(1). Composition of the mixtures of PG and PE, and some pectolytic enzyme activities of the mixtures and the crude enzyme are shown in Table IV-(1) and (2). Of the three mixtures a, b and c in Table IV, the latter two were nearly the same with crude enzyme in both several pectolytic enzyme activities tested and the clarification of apple juice (Fig. 8).

Precisely speaking, one might conclude from these results that pectolytic enzyme system of crude enzyme from *Con. diplodiella* consists essentially of four PGs and two PEs in the ratio between the mixtures b and c in Table IV, and moreover that crude enzyme from the mold contains no other factor having a pronounced effect on the hydrolysis of pectin and pectic acid, and on the clarification of apple juice than those six enzymes.

DISCUSSION

It has been shown that apple juice was clarified by the joint action of endo-PG and PE, and that exo-PG had no effect on the clarification. On the basis of these data, it seemed reasonable to assume that hydrolysis of pectic materials in apple juice was in-

FIG. 4. Effects of the Ratio of PG to PE on the Clarification of Apple Juice.

To 10ml of fresh apple juice were added 0.113 PEu of PE I, and 0.024(—Δ—), 0.121(—○—), 0.605(—■—), 3.03(—▲—) or 15.13(—●—) PGu of endo-PG I, respectively.

TABLE II. RELATIVE CLARIFICATION VALUES OF A SERIES OF PECTOLYTIC ENZYME MIXTURES

(PGu)ml in enzyme mixture					Clarification value
Endo-PG I	Endo-PG II	Endo-PG III	Exo-PG	Total (PGu)ml	
0.15				0.15	0.15 **100
	0.15			0.15	0.15 120
		0.15		0.15	0.15 180
			0.15	0.15	0.15 0
0.15	0.15			0.3	0.15 185
0.15		0.15		0.3	0.15 240
0.15			0.15	0.3	0.15 100
	0.15	0.15		0.3	0.15 250
	0.15		0.15	0.3	0.15 120
		0.15	0.15	0.3	0.15 180
0.15	0.15	0.15		0.45	0.15 270
0.15	0.15		0.15	0.45	0.15 185
	0.15	0.15	0.15	0.45	0.15 250
0.15	0.15	0.15	0.15	0.6	0.15 265

* PE I was used.

** A value of 100 was assigned to a enzyme mixture containing 0.15 PGu of endo-PG I and 0.15 PEu of PE I.

TABLE III. RELATION BETWEEN THE RATIO OF PG TO PE AND CLARIFICATION VALUES

	Enzyme activity, unit/ml		Relative clarification values
	PE-I	Endo-PG-I	
PE constant	0.113	0.024	62
	*0.113	0.121	100
	0.113	0.605	203
	0.113	3.03	420
	0.113	15.13	840
PG const.	0.022	0.121	42
	0.113	0.121	100
	0.55	0.121	235
	2.75	0.121	950

* A value of 100 was assigned to a enzyme mixture containing 0.113 PEu of PE I and 0.121 PGu of endo-PG I.

dispensable for clarification of apple juice. Every endo-PG could partially hydrolyze purified citrus pectin of esterification degree 64% without PE and decreased its viscosity

FIG. 5. Effects of the Ratio of PG to PE on the Clarification Curves of Apple Juice(1).

One ml of the mixtures of endo-PG I and PE I was added to 10 ml of fresh apple juice. The ratio of the PG to PE and other experimental conditions were as follows:

Curve	PG/PE	(PGu)ml	(PEu)ml
—○—	0.2	0.03	0.15
—●—	1.0	0.15	0.15
—△—	5.0	0.375	0.075
—▲—	25.0	0.938	0.0375

as described in previous papers.⁶⁾ However, these endo-PGs had no ability to clarify apple juice as described here. These results indicate that native pectin in apple juice behaved very differently from artificial pectin solution made from purified pectin.

Clarifying activity of mixtures of endo-PG and PE depended on either endo-PG or PE activities when the other was kept constant as described above. Joslyn et al.²⁾ reported that the rate of decrease in viscosity and the time for clarification of apple juice did not depend on the ratio of PE to PG or on the absolute PE or PG activities. However, since their method of estimating PG activity was based solely on its hydrolytic activity of pectic

FIG. 6. Effects of the Ratio of PG to PE on the Clarification Curves of Apple Juice(2).

One ml of the mixtures of endo-PG II and PE I was added to 10 ml of fresh apple juice. Other experimental conditions were as follows:

Curve	PG/PE	(PGu)ml	(PEu)ml
—○—	0.2	0.03	0.15
—△—	1.0	0.15	0.15
—▲—	5	0.375	0.075
—●—	25	0.938	0.0375
—□—	125	2.34	0.0188

acid by the hypoiodite method,¹⁰⁾ it may be possible to assume that their preparations were contaminated with exo-PG, which had no effect on the clarification as described above. With a preparation from *Aspergillus niger*,⁶⁾ Schubert found that PE content was the rate-limiting factor for clarification of apple juice. In later work he reported that only one of PGs acted on native apple pectin without PE.¹¹⁾ However, it would seem possible that the presence of other factor, especially PE, in Schubert's PG or in apple juice used might have contributed to the

10) E. F. Jansen and L. R. MacDonnell, *Arch. Biochem.*, **8**, 97 (1945).

11) E. Schubert, *Schweiz. Brauereirundschau*, **63**, 95 (1952).

FIG. 7. Effects of the Ratio of PG to PE on the Clarification Curves of Apple Juice(3).

One ml of the mixtures of endo-PG III and PE I was added to 10 ml of fresh apple juice. Other experimental conditions were as follows:

curve	PG/PE	(PGu)ml	(PEu)ml
—○—	0.2	0.03	0.15
—●—	1.0	0.15	0.15
—△—	5.0	0.375	0.075
—▲—	25	0.938	0.0375
—□—	125	1.88	0.015

clarification.

Crude enzyme from *Con. diplodiella* was essentially the same with a mixture made from the four PGs and PE in the activity for pectic acid, pectin and apple juice. These results indicate that the effect of other enzymes such as cellulase, amylase, protease, etc. which might be present in crude enzyme was not indispensable for clarification of freshly pressed apple juice.

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TABLE IV. COMPARISON BETWEEN THE MIXTURES OF PURIFIED PGs AND PE, AND A CRUDE ENZYME FROM *Con. Diplodiella*

(1) Composition of enzyme mixtures.

En- zyme	Enzyme added, units/ml of mixture					PE*	PG/PE
	Endo- PG I	Endo- PG II	Endo- PG III	Exo- PG	Total PG		
a	0.8	0.4	1.2	0.2	2.6	0.26	10
b	0.8	0.4	1.2	0.2	2.6	0.13	20
c	0.8	0.4	1.2	0.2	2.6	0.065	40

(2) Pectolytic activities of the above mixtures and a crude enzyme from *Con. diplodiella*.

(i) Determined at pH 4.4.

En- zyme	(PGu) ml	(PMGu) ml	(VRu) mlPA**	(VRu) mlP***	(PEu) ml	PG/PE
a	2.4	0.63	3.08	9.10	0.39	6.2
b	2.4	0.51	3.08	8.77	0.24	10.0
c	2.4	0.47	3.08	8.55	0.14	17.1
crude****	2.4	0.48	3.14	8.81	0.19	12.6

(ii) Determined at pH 3.5.

a	b	c	crude
0.90	0.51	0.63	0.89
0.90	0.45	0.63	0.43
0.90	0.43	0.63	0.43
2.78	2.71	2.65	2.71
0.24	0.13	0.08	0.10
0.41	0.36	0.30	0.27

* PE I was used. Enzyme activities were determined at pH 4.4.

** Pectic acid was used as substrate.

*** Pectin was used as substrate.

**** Crude enzyme was moderately diluted, and both mixtures, a, b and c, and crude enzyme had 0.24 (PGu)ml at pH 4.4.

FIG. 8. Clarification of Apple Juice by the Mixtures of Purified PGs and PE, and a Crude Enzyme from *Con. diplodiella*.

One ml of 1:5 dilution of the enzyme mixtures or a crude enzyme solution in Table IV was added to 10 ml of fresh apple juice of pH 3.5.

—○—	enzyme mixture a,
—●—	enzyme mixture b,
—□—	enzyme mixture c,
—△—	crude enzyme.