Studies on Pectolytic Enzymes of Molds

Part VII. Turbidimetry of Apple Juice Clarification and its Application to Determination of Enzyme Activity

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The process of apple juice clarification by pectolytic enzymes has been successfully observed turbidimetrically and macroscopically by heating of reaction mixtures. It has been shown that the process of apple juice clarification varies with the varieties and conditions of apple juices as well as with the sources of enzyme preparations. From a study of the turbidimetry of apple juice clarification, a method for determination of clarification values been described.

INTRODUCTION

Clarification of apple juice has been carried out for many years with commercially available pectic enzymes¹⁾. A large number of investigators have attempted to determine what enzymes are required for the clarification. It has been shown that the clarification occurs in the early stage of the hydrolysis of pectin. It is generally observed that the viscosity decrease takes place, followed by a stage in which juice becomes cloudy because of the formation of floc. The size of the floc particles increases, and then sedimentation occurs. Newbeck²⁾, Joslyn et al.³⁾, Endo⁴⁾ and Arima et al.⁵⁾, in their investigations of apple juice clarification, using a series of enzymes, found that similar clarification results were obtained, even though the ratio between PG (polygalacturonase) to PE (pectinesterase) varied about 100-fold. Schu-

bert⁶⁾ described that standardization of enzymes by using purified pectin could not show the correlation with fruit juice clarification, since native pectin in fruit juice behaves very differently from purified pectin. He advised that the use of a solution of fresh apple juice was necessary for standardizing clarification enzymes. Clarification values have generally been determined by adding pectic enzymes to apple juice and measuring the time for floc formation or sedimentation of floc particles^{2,4)}.

These methods are not only somewhat cumbersome, but also give poor reproducibility. Moreover, by these methods, it is impossible to observe the intermediate stages before the floc formation.

During the course of the author's investigation on the clarification of apple juice, the intermediate steps could be observed both macroscopically and turbidimetrically by heating the reaction mixture from each step in boiling water. In the present paper, this

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5) K. Arima, M. Yamazaki, S.Y. Chu, T. Yasui and G. Tamura, presented at the Annual Meeting of the Agricultural Chemical Society of Japan, Sendai, April 29, 1962.

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

heating method is applied to a study on the clarification of apple juice and the determination of clarification values with a spectrophotometer.

MATERIALS AND METHODS

(1) **Enzyme Preparations.** Defatted rice bran was used as solid medium for *Coniothyrium diplodiella* and *Sclerotinia libertiana*. The mass was incubated at 30°C for 2 to 4 days and then extracted with about 3 volumes of water. Three volumes of ethanol were added to the water extract in the cold.

The resultant precipitate was separated by centrifuging and dried in vacuum. The dried materials—A (*Con. diplodiella*) and B (*Scl. libertiana*)—prepared in this manner, were used as enzyme preparations⁽²⁾.

(2) **Apple juice.** Apple juice obtained by crushing commercially available apples (Ralls and Jonathan) and squeezing the crushed apples with several pieces of gauze was used.

(3) **Centrifuge.** A commercial centrifuge which is capable of treating 24 samples at a time was used.

(4) **Transmittance Measurement.** The transmittance of apple juice was measured at 660 m μ (T%) in a Hitachi spectrophotometer (Hitachi Corp., Japan) with 1-cm light path.

(5) **Clarification of Apple Juice.** Into a test tube, 1.6 cm in diameter, were put 10 ml of apple juice, and an enzyme solution and water were added to make the total volume 11 ml. After being incubated at 30°C for a suitable period, the reaction was stopped by heating for 5 min. in boiling water. The mixture was cooled with running tap water, and centrifuged at 3,000 to 4,000 r.p.m. for 10 to 20 min. and the supernatant collected.

Transmittance (T%) of the supernatant at 660 m μ was determined with the spectrophotometer.

RESULTS

(1) Change in Transmittance of Apple Juice.

To fresh apple juice was added 1% water extract of preparation B, and the reaction mixture was allowed to stand for a suitable period at 30°C. The reaction mixture was centrifuged at 3,000 r.p.m. for 15 min. and transmittance (T%) at 660 m μ of the supernatant was measured. As shown in Fig. 1,

FIG. 1. Time Course of Clarification.

To 10 ml of fresh apple juice of pH 3.6 was added 1 ml of 1% water extract of preparation B (*Scl. libertiana*) and then the reaction mixture was kept at 30°C. After suitable period the reaction was stopped by heating in boiling water for 5 min. and then the mixture was centrifuged at 3,000 r.p.m. for 15 min.

—○— Ralls —●— Jonathan

increase in transmittance was proportional to the period of incubation. Difference in clarification curves was observed between the two juices.

(2) Centrifugation and Transmittance.

The transmittance of the supernatant varied with condition of the centrifugation. As shown in Fig. 2, increase in transmittance was proportional to either force or time of centrifugation when the other factors were kept constant. In order to avoid the effect of centrifugation on transmittance, a series of reaction mixtures were centrifuged with the same centrifuge and at the same time in the following experiments.

(3) Apple Juice and Clarification.

Apple juice stored at 4°C was found to be different from the fresh one in the clarification curve. Fig. 3 shows relations between the storage of fresh apple juice and the clarification. It was shown that some changes in apple juice occur during storage. Such differences were observed with both of the juices of Ralls and Jonathan.

(4) Enzyme Preparation and Clarification.

As stated in an earlier paper⁽⁴⁾, preparation

FIG. 2. Centrifugation and Clarification.

(1) Fresh apple juice (Ralls) and 1% water extract of preparation B were used and the reaction mixture was incubated for 20 min. Centrifuge: 4,000 r.p.m.

—×— 10 min., —●— 15 min., —○— 20 min.

(2) Fresh apple juice and 0.2% water extract of preparation A were used. Ralls was treated for 20 min. and Jonathan, 30 min.

—○— Ralls, 3,000 r.p.m.,

---○--- Jonathan, 3,000 r.p.m.,

—●— Ralls, 3,500 r.p.m.

---●--- Jonathan, 3,500 r.p.m.

FIG. 3. Apple Juice and Clarification.

(1) Enzyme: preparation A (0.2% water extract); reaction time: 30 min. (Rall) or 40 min. (Jonathan); centrifugation: 4,000 r.p.m. for 10 min. —×— Rall, fresh juice, —●— Ralls, juice stored overnight at 4°C, —○— Ralls, juice stored at 4°C for 3 days, ---×--- Jonathan, fresh juice, ---●--- Jonathan, juice stored overnight at 4°C, ---○--- Jonathan, stored at 4°C for 3 days.

(2) Enzyme: 1% water extract of preparation B; reaction time: 20 min. (Ralls) or 30 min. (Jonathan); centrifugation: 3,000 r.p.m. for 15 min. —●— Ralls, fresh juice, —○— Ralls, juice stored overnight at 4°C, ---●--- Jonathan, fresh juice, ---○--- Jonathan, juice stored overnight at 4°C.

B has a higher ratio of the PE to the PG than that of preparation A. Fig. 4 shows a comparison of clarifications by the two preparations. As shown in the figure, the difference

between properties of the two preparations was observed.

FIG. 4. Enzyme Preparation and Clarification.

Experimental conditions are the same as in Fig. 3.

—○— preparation A and fresh juice, ---○--- preparation A and overnight-stored juice, —●— preparation B and fresh juice, ---●--- preparation B and overnight-stored juice.

(1) Ralls,

(2) Jonathan

(5) Possibility of the Determination of Clarification Values.

The experiments described above showed that clarification values of the preparations can be measured from the relations between transmittance and the concentration of enzymes. The clarification value of a preparation can be calculated as follows:

$$Asa = \frac{Dst}{Dsa} \times Ast$$

where Asa is the clarification value of the preparation; Ast, the clarification value of

the standard; Dsa, the amount of the preparation; Dst, the amount of the standard.

In Table I are given the results of clarification values of several samples at a transmittance of 50%. Although the results more or less varied with the variety of apple fruits, essentially identical results were obtained with the same apple juice.

DISCUSSION

When the floc formation of apple juice occurred, transmittance of the supernatant at 660 mμ reached 90~98%. Previous investigations of the clarification of apple juice have dealt almost exclusively with the observation of floc formation. This method is not only somewhat cumbersome, but also is poor in reproducibility.

By means of the method presented here, the intermediate steps before the floc formation could be followed not only macroscopically but also turbidimetrically. Thus, the method can be widely used in the determination of clarification values. Moreover, the method will give a new approach in understanding the mechanism of apple juice clarification.

TABLE I. CLARIFICATION VALUES OF SOME PREPARATIONS

(1) Fresh apple juice

Sample	Ralls		Jonathan	
	Juice A	Juice B	Juice A	Juice B
1	526	520	440	443
2	519	513	420	426
3	880	870	750	760
4	708	720	578	597
5	445	453	370	375

(2) Apple juice stored overnight at 4°C

Sample	Juice A	Juice B	Juice A	Juice B
1	612	612	452	446
2	607	600	440	440
3	1050	1080	770	755
4	809	858	609	617
5	503	503	365	345

Pectic substances in apple fruit can be classified into insoluble protopectin and soluble pectin. The ratio between the two varies with such factors as maturity, storage and variety of apples. The $-\text{OCH}_3$ content of pectin also varies with these factors⁸⁾. It was thought that such variations of pectic substances in apple fruits influenced the clarification of apple juice.

In previous works in this series^{4,9)} dealing with the fractionation of pectic enzymes of *Con. diplodiella*, it had been shown that pectic enzymes of the mold consisted of at least one PE and four PGs. As will be stated in a forthcoming paper, clarification of ap-

ple juice can only be effected by the joint action of PE and PG. Differences in the ratio of PE to PG and the absolute PE and PG activities were observed between the two enzyme preparations from *Con. diplodiella* and *Scl. libertiana*^{4,10)}. These results indicate that clarification of apple juice depends on PE and PG activities of enzyme preparation as well as the nature of apple juice itself. Further studies on the mechanism of clarification are now being carried out using the method presented here.

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