Pectic Enzymes in the Clarification of Apple Juice

Part I. Study on the Clarification Reaction in a Simplified Model*

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To know the role of pectic enzymes in the clarification reaction of apple juice, a simplified model for apple juice, that is, aqueous re-suspension of ultracentrifugal precipitates of apple juice, was employed. It was found that the precipitates (i.e., suspended materials) contained 36% of protein and that the surface of the suspended materials was negatively charged at pH 3.5. Positively charged colloids at pH 3.5 such as gelatin enhanced the clarification reaction or mutually coagulated with the suspended materials. While negatively charged colloids at pH 3.5, such as sodium alginate completely inhibited the clarification reaction. The direct participation of pectic enzymes in the clarification of apple juice was shown, and a supposed mechanism of the enzymic clarification was presented.

INTRODUCTION

It has long been known that pectic enzymes were responsible for the clarification of cloudy fruits juices.

And several investigators¹⁻⁵⁾ studied the clarification phenomenon, though the mechanism of it has long been unelucidated.

Of course, it has not been known too that how many kinds of pectic enzymes or what kind of other enzymes are responsible for the clarification. Recently Endô⁶⁾, however, discovered that only two kinds of pectic enzymes, i.e., purified endo-polygalacturonase I and pectinesterase, could cooperatively cause the clarification reaction.

tion reaction.

To know the role of the pectic enzymes in the clarification reaction, it seems necessary for the authors to adopt more simplified system than apple juice which is very complicated system.

The authors happened to know that the suspended materials in apple juice could be precipitated by ultracentrifugation, e.g., 75,000 g for thirty minutes without using pectic enzymes, and that the ultracentrifugal precipitates were readily re-suspended in water or other aqueous solutions, resulting in stable suspensions.

These suspensions may be regarded as simplified models for apple juice. The present paper deals with several observations on the clarification phenomenon in the re-suspension systems, and the colloidal properties of the suspended materials in apple juice.

MATERIALS AND METHODS

Apple Juice

The variety of apple: Kôgyoku, pasteurized at 60°C for one hour, pH: 3.50, sp. gr. 1.037 (at 25°C), total acid: 0.434% (as malic acid), pectin: 0.13% (as anhydro-

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1) Z.I. Kertesz, *N.Y. State Agr. Expt., Bull.*, **589**, 3 (1930).

2) J.J. Willaman and Z.I. Kertesz, *N.Y. State Agr. Expt. Sta. Tech. Bull.*, No. 178, 3 (1931).

3) A. Mehlitz and M. Scheuer, *Biochem. Z.*, **268**, 345, 355 (1934).

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

5) C.E. Neubeck, *J. Assoc. Offic. Agr. Chemists*, **42**, 374 (1959).

6) A. Endô, Proceeding of the annual meeting of the Agricultural Chemical Society of Japan, Tokyo, 1963, p. 4.; A. Endô, Proceeding of 15th Symposium on Enzyme Chemistry, Ôsaka, 1963, p. 126.

galacturonic acid). Before use, lees were filtered off with cotton.

Chitosan Acetate

Chitosan acetate was prepared from chitin after Tracey's method.⁷⁾

Infrared spectra revealed that the absorption bands around 1570 cm^{-1} and 1650 cm^{-1} characteristic of amide bond in the N-acetyl group were almost disappeared.

Alginase

Crude alginase preparation was obtained from whole liver of abalone (*Haliotis discus hamai*) after Tsujino and Saitô's method.⁸⁾

Preparation of Enzymic Precipitates

Apple juice was treated with a pectinase preparation (Miles Lab., Clifton, New Jersey, U.S.A.) at the concentration of 0.5 mg/ml of juice. Reacted three hrs. at 40°C , precipitates were collected by centrifugation at $3,500\text{ r.p.m.}$ for 20 min. The precipitates were re-suspended in a dilute HCl solution (pH 3.50) and centrifuged.

The washing was repeated three times. The final precipitates were re-suspended in the dilute HCl solution and lyophilized.

Preparation of Ultracentrifugal Precipitates

The apple juice was ultracentrifuged at $75,000\text{ g}$ for 30 minutes.

The precipitates were re-suspended in the dilute HCl solution and ultracentrifuged again. This washing was repeated once again.

The final precipitates were re-suspended in the dilute HCl solution and lyophilized.

Bioassay of Amino Acids

Both enzymic and ultracentrifugal precipitates were hydrolyzed and routinely bioassayed for 18 amino acids. Samples were hydrolyzed as follows: (1) for methionine and histidine, in 10 times volume of 5N HCl at 120°C for 2 hrs., (2) for tryptophan, in 10 times volume of 3.5N NaOH with cysteine (40%) at 120°C for 6 hrs., (3) for other amino acids, in 10 times volume of 6N HCl at 120°C for 6 hrs.

Determination of Sugar Components

The sugar components of both enzymic and ultracentrifugal precipitates were examined paper-chromatographically by using pyridine, ethyl acetate, water (5:12:4) as the solvent and ammoniacal silver nitrate or aniline-phthalate as the color reagents.

Nearly 20 mg of each precipitate was suspended in 4 ml of 1N HCl and heated at 100°C for eight hrs. in a sealed tube. After HCl was removed by the concentration repeated under reduced pressure, the hydrolyzate was dissolved in 0.5 ml of distilled water and the solution resulted was spotted 10~15 times on a Tôyô filter paper No. 51 ($40\times 40\text{ cm}$). This paper was developed twice in the solvent aforementioned.

Clarification Reaction

Apple juice was ultracentrifuged at $75,000\text{ g}$ for 30 minutes.

Under this condition, almost all pectin in the juice remained in the supernatant. The precipitates were usually re-suspended in a final 0.05 M acetate buffer (pH 3.50) with or without an aid of mortar. The re-suspensions were always at the same concentration as that found in original juice in relation to suspended materials.

These suspensions could be clarified by pectinase (Miles Lab.) faster than in the case of the original apple juice. The clarification reaction was always carried out at pH 3.50 and 40°C , and the reaction mixture was 5 ml in total. The time required for the first appearance of the flocculation of the suspended materials was recorded. Other detail of experimental conditions will be shown in individual experiments.

Electrophoresis of the Suspended Materials

The twice concentrated re-suspension in 0.01 M acetate buffer (pH 3.50) was electrophoretically migrated in a small U-tube under the condition of 26.5 mA/cm^2 and 11 V/cm . The section area and the mean current path of the U-tube were 0.94 cm^2 and 18 cm respectively. The electrode vessels and the U-tube, which was being dipped in ice bath during electrophoresis, were connected with agar-agar bridges.

Determination of the Amount of Galacturonide Liberated from the Suspended Materials by the Action of Pectinase.

The twice concentrated re-suspension in the dilute HCl solution (pH 3.50) was treated by pectinase (Miles Lab.) at the concentration of 0.1 mg per ml of the suspension at 40°C . At a given time, 10 ml of aliquot was withdrawn and immediately ultracentrifuged at $75,000\text{ g}$ for 7 min. Pectic substances in 9.5 ml of supernatant were demethylated with 0.5 ml of 1N NaOH for 30 min. at room temperature. Exactly 2 ml of the reaction mixture was applied to the quantitative analysis of pectic substances by the colorimetric method with carbazole.⁹⁾

7) M.V. Tracey, *Biochem. J.*, **61**, 579 (1955).

8) I. Tsujino and T. Saitô, *This journal*, **26**, 115 (1962).

9) E.A. McComb and R.M. McCready, *Anal. Chem.*, **24**, 1630 (1952).

RESULTS

Comparison between Enzymic and Ultracentrifugal Precipitates

The dry matter weight and the elementary compositions of the enzymic and ultracentrifugal precipitates are shown in Table I.

The compositions of amino acid of both precipitates are compared in Table II. As for the sugar components, both precipitates were found to contain at least four kinds of sugar, i.e., arabinose, galactose, glucose, and galacturonic acid. (Fig. 1)

From these data, any notable difference between both precipitates could not be observed.

Liberation of Galacturonides from the Suspended Materials by the Action of Pectic Enzymes

As the suspended materials in apple juice contained galacturonic acid as their component as shown in Fig. 1, pectin or hemicellulose contain-

ing galacturonic acid residue may be the components or protective colloids of the suspended materials. Then it is expected that galacturonic acid or galacturonides will be liberated from the suspended materials by the action of pectic enzymes.

As expected, galacturonic acid or galacturonides were liberated by pectic enzymes as shown

TABLE II. THE COMPARISON OF AMINO ACID COMPOSITIONS

	Enzymic	Ultracentrifugal
Asp.	3.70%	3.48%
Glu.	4.59	4.31
Gly.	2.37	2.14
Ala.	2.72	2.67
Val.	2.47	2.18
Leu.	3.01	2.69
Ileu.	2.06	1.81
Pro.	1.81	1.70
Phe.	1.86	1.72
Tyr.	1.31	1.13
Try.	0.47	0.28
Ser.	2.18	1.91
Thr.	1.93	1.69
Met.	0.68	0.50
Cys.	0.43	0.27
Arg.	2.34	1.97
His.	0.81	0.68
Lys.	2.54	2.38
Total	37.28	33.31
N% $\times 6.25$	37.6	36.3

TABLE I. THE COMPARISON OF DRY MATTER WEIGHT, ELEMENTARY COMPOSITION AND ASH BETWEEN THE ENZYMIC AND ULTRACENTRIFUGAL PRECIPITATES.

	Enzymic	Ultracentrifugal
Dry matter*	0.051%	0.046%
Nitrogen	6.02%	5.80%
Carbon	55.13%	54.35%
Hydrogen	7.11%	6.58%
Ash	$\approx 2\%$	$< 2\%$

* Expressed in weight percentage in original apple juice.

FIG. 1. Paper Chromatogram of the Hydrolyzates of Enzymic and Ultracentrifugal Precipitates.

Alg.*: Enzymic hydrolyzate of alginic acid as a standard for mannuronic acid.

FIG. 2. The Liberation of Galacturonides by the Action of Pectinase.

in Fig. 2. For purposes of qualitative analysis, the reaction mixture in the colorimetric analysis was diluted with distilled water. Pink color turned to colorless in a few minutes. This means that there was no detectable amount of concomitants of hexoses.¹⁰⁾ As the suspended materials contained galacturonic acid as the sole uronic acid component, the liberated substances were determined as galacturonic acid or galacturonides.

The liberated amount was as much as endogenous amount of galacturonides in the supernatant.

Effect of the Concentration of Pectin on the Clarification Reaction.

The viscosity of apple juice is mainly ascribed to pectin in the juice. The effect of the con-

TABLE III. THE EFFECT OF THE CONCENTRATION OF PECTIN ON THE CLARIFICATION REACTION

Re-suspension system*	pH after the reaction	Time required for the clarification
water	3.55	6 min.
0.05% pectin soln.	3.49	12
0.1% pectin soln.	3.49	16
0.2% pectin soln.	3.45	22
0.4% pectin soln.	3.45	62
apple juice	3.50	16

* 0.5 mg of pectinase per 5 ml of the re-suspension or apple juice, and buffer free.

10) G. Ashwell, "Methods in Enzymology Vol. 3", Academic Press, 1957, p. 94.

centration of pectin on the clarification reaction was studied by applying re-suspension systems.

From the data shown in Table III, following facts could be pointed out: (1) The water re-suspension was clarified much faster than original apple juice. (2) The clarification reaction was apparently retarded with the increase of the amount of pectin in the resuspensions.

Effect of pH on the Clarification.

It was incidentally found that the enzymic clarification reaction did not occur at pH 6.0 at least within several hours. But the clarification reaction occurred after some lag time (about three minutes), if the apparently unchanged reaction mixture was adjusted to pH 3.5 with an appropriate buffer. On the other hand, the clarification reaction under the similar condition was observed after six minutes, if the reaction was carried out at pH 3.5 from the beginning. This means that enzymic reactions proceeded even at pH 6.0 without resulting in the clarification and that the clarification reaction was dependent on pH of the re-suspensions.

Then the effect of pH on the clarification reaction was studied.

FIG. 3. The Effect of pH on the Clarification Reaction.

The clarifying activity at pH 3.5 was referred to as 1.0.

---○--- Re-suspension
—●— Apple juice

As shown in Fig. 3, the reaction could never be observed above pH 4.75 either with apple juice or the re-suspensions. A great difference, however, of the behavior to pH was observed below pH 3.5 between apple juice and the re-suspensions. The enzymic clarifying activity was retarded with the decrease of pH value in apple juice, while the flocculation of the suspended materials in the re-suspension was markedly accelerated along with increase of acidity. Below pH 3.0 in the re-suspension systems, it will be more accurate to say that the mutual coagulation between the suspended materials and enzyme proteins occurred in place of enzymic clarification reaction.

Interaction between the Suspended Materials and Molecular Colloids.

The amount of pectin markedly affected the time required for the clarification. (Table III) Then the effect of the presence of various kinds of molecular colloids on the clarification reaction was studied. The suspended materials seemed to be negatively charged, because the non-

enzymic mutual coagulation occurred when positively charged colloids were added. (Table IV) In apple juice system, however, this mutual coagulation could not be observed in spite of adding a large amount of positive colloids.

Negatively charged colloids such as sodium alginate, CMC, and gum arabic completely inhibited the clarification reaction even at the final concentration as low as 0.05%.

Electrostatically neutral colloids, such as dextran or inulin, had little effect on the clarification, but the reaction time was a little prolonged by the addition of soluble starch.

Surface Electrostatic Charge of the Suspended Materials.

To know more exactly the electrostatic charge of the surface of the suspended materials, the electrophoresis was applied. At first Tiselius type apparatus was used with samples of apple juice or the re-suspensions dialyzed against an appropriate buffer as stated by Ôki.¹¹⁾ But this method did never give good results.

Then the classical method using a U-tube as reported by Endô⁶⁾ was employed with a slight modification. All the suspended materials migrated to anode within 60 minutes. This result is consistent with the observations reported by Ôki¹¹⁾ and Endô.⁶⁾

Effect of a Small Amount of Protein.

It has long been known that the stability of a hydrophobic colloid having either positive or negative charge is reduced by adding a small amount of a hydrophilic colloid having opposite

TABLE IV. THE EFFECT OF THE PRESENCE OF VARIOUS KINDS OF COLLOIDS ON THE CLARIFICATION REACTION

Sorts of colloid	Concn. tested	Clarification reaction*	Mutual coagn.**	Charge at pH 3.5
Pectin	0.05~0.2%	+	—	—
Na-pectate	//	+	—	—
Na-alginate	//	—	—	—
CMC	//	—	—	—
Gum arabic	//	—	—	—
Soluble starch	0.17~0.67%	+	—	0
Inulin	0.05~0.2	+	—	0
Dextran	//	+	—	0
Chitosan acetate	0.025%		+	+
Albumin	0.05%		+	+
Gelatin	//		+	+
Casein	//		+	+
Pepsin***	0.2%	+	—	—

* Re-suspension systems contained 0.75 mg of pectinase per 5 ml.

** Mutual coagn.: Non-enzymic mutual coagulation.

*** Pepsin (Crys.): Purchased from Nutritional Biochemicals Corporation, Ohio, U.S.A.

TABLE V. THE EFFECT OF THE PRESENCE OF A SMALL AMOUNT OF PROTEINS

Treatment	Time required for the clarification
pectinase ¹⁾	21.5 min.
pectinase ¹⁾ + protease ^{2)*}	13.5
pectinase ¹⁾ + heated protease ²⁾	13.5
pectinase ¹⁾ + heated pectinase ²⁾	15
pectinase ¹⁾ + casein ²⁾	16

1) 0.06 mg of pectinase per 5 ml of the re-suspension.

2) 0.12 mg of protein per 5 ml of the re-suspension.

* protease: Partially purified protease of *Streptomyces griseus* kindly given by Dr. M. Nomoto.

11) T. Ôki, *J. Agr. Chem. Soc., Japan*, **33**, 1005 (1959).

charge. This phenomenon called "sensitization" was also found in the re-suspension systems. (Table V) It is clear that the time required for the clarification was markedly shortened by the addition of a small amount of proteins. But the sensitization phenomenon could not be observed in the case of apple juice.

As far as examined, proteases had no accelerating effect on the clarification reaction.

Effect of Negatively Charged Colloids.

The fact that sodium alginate or carboxymethyl cellulose (CMC) completely inhibited the clarification reaction can be understood by the alternative explanation either that the added pectinase and these colloids reacted to form complex coacervates resulting in the loss of the enzymic activity, or that these colloids acted as protective colloids for the suspended materials. The formation of complex coacervates, however, could never be detected even microscopically. Then sodium alginate or CMC which pectic enzymes can not attack seems to act as protective colloids.

If so, inhibitory effect of sodium alginate or CMC will be deservedly lost by pre-treating with alginase or cellulase respectively.

The data shown in Table VI were obtained under the condition that 100 ml of 0.4% CMC

TABLE VI. THE EFFECTS OF THE PRESENCE OF NEGATIVELY CHARGED COLLOIDS AND THE PRE-TREATMENT OF THE COLLOIDS ON THE CLARIFICATION REACTION

Re-suspension system*	Final conc. of colloid	Pretreatment	Time required for the clarification
Na-pectate soln.	0.05%	+(pectinase)	6.5 min.
Na-pectate soln.	0.10	+(pectinase)	6.5
Na-pectate soln.	0.05	—	11.5
CMC soln.	0.05	+(cellulase)	37
CMC soln.	0.10	+(cellulase)	210
CMC soln.	0.05	—	—
Na-alginate soln.	0.05	+(alginase)	20
Na-alginate soln.	0.10	+(alginase)	27.5
Na-alginate soln.	0.05	—	—

* Reaction mixture contained: 0.5 ml of 0.5 M acetate buffer pH 3.5, 2.0 ml of the twice concentrated re-suspension of the suspended materials, 2.0 ml of pre-treated or non-treated colloid solution, and 0.5 ml of pectinase soln. (0.5 mg/ml).

solution (pH 5.0) was pretreated with 15 mg cellulase (of *Trichoderma coningi*, Ueda Kagaku Co.) at 37°C for 19 hrs, and that 100 ml of 0.4% sodium alginate solution (pH 6.0) was pretreated with 10 ml of crude alginase solution at 37°C for 19 hrs.

As is shown in Table VI, pre-treated negatively charged colloids had no more inhibitory effect on the clarification.

If pectic enzymes and cellulase were added simultaneously to the system containing CMC, the clarification reaction was expected to occur as well. The clarification reaction was observed only when both pectinase (Miles Lab.) and cellulase (Ueda Kagaku Co.) existed as shown in Table VII. As the pectinase used in this experiment contained rather high CMCase activity, it partly caused the clarification, only if the reaction period was prolonged to 24 hrs.

TABLE VII. THE ROLE OF PECTIC ENZYMES IN THE CLARIFICATION

Re-suspension system	Enzymes added*	Time required for the clarification
Buffer	pectinase	10 min.
Buffer	cellulase	—
CMC 0.05% soln.	pectinase	—
CMC 0.05% soln.	cellulase	—
CMC 0.05% soln.	pectinase+heated cellulase	—
CMC 0.05% soln.	pectinase+cellulase	5.5 hrs

* pectinase: 0.5 mg per 5 ml of the re-suspension.
cellulase: 1.0 mg per 5 ml of the re-suspension.

DISCUSSION

The suspended materials were supposed to contain about 36% of protein on the basis of good accordance between calculated protein content (nitrogen content $\times 6.25$) and total amino acid content, and the presence of 18 natural amino acids. (Table II)

This protein would be positively charged at pH 3.50, provided that the isoelectric point of the protein was between pH 4.0 and 5.0. And this protein was supposed to exist as a protein-carbohydrate complex from the following facts: (1) The enzymic precipitates, which were thought to be partly or wholly deprived of their

protective colloid, pectin, by the action of pectic enzymes, could not be solubilized by partially purified pronase which had the wide substrate specificity.^{12)*} (2) Both enzymic and ultracentri-

fugal precipitates contained at least three kinds of sugar in addition to galacturonic acid.

On the other hand, judging from the interaction with various kinds of molecular colloids

(A) Below pH 4.0

(B) Above pH 5.0

(C) Blocking effect

FIG. 4. A Supposed Mechanism of the Flocculation.

Suspended materials.
(The protein-carbohydrate complex is surrounded by negatively charged protective colloids, such as pectin.)

Enzymes or proteins.

Pectin

Negatively charged protective colloids which pectic enzymes can not attack.

* pronase: protease of *Streptomyces griseus* kindly given by Dr. M. Nomoto.

12) M. Nomoto, Y. Narahashi, and M. Murakami, *J. Biochem.*, **48**, 593, 906 (1960)

and the result of the electrophoresis, the surface of the suspended materials was negatively charged.

Assumed that the protein-carbohydrate complex was surrounded by the negatively charged protective colloids, e.g., pectin, positive charge of the complex would be exposed by the whole or partial degradation of the protective colloid with pectic enzymes. Then appeared positive charge of the complex and the neighbouring negative charge of the protective colloid which was not yet degraded by the action of pectic enzymes would attract to each other, resulting in the flocculation. (Fig. 4A) If the pH of the re-suspension system was above 4.75, the charge of the complex would be converted to negative so that the flocculation could not be occurred. (Fig. 4B)

The sensitization by a small amount of proteins (supposed to be positively charged at pH 3.5) can be understood as the connecting effect of the proteins among negatively charged protective colloids. When the concentration of proteins was elevated, the mutual coagulation occurred in place of the sensitization. In apple juice system, the sensitization and the mutual coagulation could not be observed. This may be partly because of the pre-existence of soluble proteins in apple juice. Freshly prepared apple juice, however, showed mutual coagulation between large particles of the suspended materials and added gelatin (at the final concentration of 0.0005~0.05%). It was well-known that the addition of gelatin improved the enzymic clarification reaction of apple juice.¹³⁾ This effect of gelatin is explained as an agent causing mutual coagulation and/or sensitization.

The fact that negatively charged colloids which pectic enzymes could not attack, such as sodium alginate and carboxymethyl cellulose, completely inhibited the clarification reaction even at the low concentration was explained as the blocking effect in which exposed positive charge of the complex was blocked or neutralized by the added negative colloids. (Fig. 4C) In

apple juice system, pectin may act as the protective colloid for the suspended materials. For the complete clarification of cloudy apple juice, pectin should be degraded by the action of pectic enzymes and be lost its protective or blocking effect.

SUMMARY

(1) Cloudy apple juice could be clarified by ultracentrifugation, e.g., 75,000 g for 30 minutes, without using pectic enzymes. The ultracentrifugal precipitates were readily able to be re-suspended in water or in other aqueous solutions, resulting in stable suspensions. These suspensions were regarded as simplified models for apple juice.

(2) Any notable difference was not found between the enzymic and ultracentrifugal precipitates in relation to dry matter weight, elementary composition, ash, amino acid and sugar compositions. Both precipitates were found to contain about 36% of protein.

(3) The enzymic clarification reaction was observed below pH 4.75, but not above pH 4.75 in the cases of apple juice and the re-suspensions.

(4) Judging from the interactions with other colloids and the results of electrophoresis, the surface of the suspended materials in apple juice was thought to be negatively charged.

(5) The enzymic clarification reaction in the re-suspension systems was markedly accelerated by the addition of a small amount of protein.

(6) Negatively charged molecular colloids, such as sodium alginate or carboxymethyl cellulose even at the low concentration, completely inhibited the clarification reaction.

(7) From the electrostatical and colloidal points of view, a supposed mechanism of the flocculation of the suspended materials was presented.

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13) E. Schubert, *Schweizer Brauerei-Rundschau*, Nr. 3 und 4, 1 (1951).

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