Studies on Pectolytic Enzymes of Molds

Part XV. Effects of pH and Some Chemical Agents on the Clarification of Apple Juice

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Effects of pH and some chemical agents (1) on the solubilization of insoluble pectin bound to suspended particles, (2) on the viscosity decrease of native soluble pectin, (3) on the flocculation of suspended particles and (4) on the over-all clarification of apple juice have been studied. Optimum pH ranges were: (1), 3.2~4.0; (2), 3.6~4.1; (3), lower than 3.0; (4), 3.2~3.8. Gelatin had no effect on (1) and (2) but stimulated (3) and (4). NaCl had no detectable effect on (2) and (3) but slightly stimulated (1) and (4). CaCl₂ strongly inhibited (1), (2) and (3). SnCl₄ stimulated (3) but strongly inhibited (1), (2) and (4). EDTA (ethylenediaminetetraacetic acid) accelerated (1) and (4), and had no detectable effect on (2) and (3).

INTRODUCTION

Pectic substances in plant tissues are classified into two groups, soluble and insoluble pectin.¹⁾ Softening of fruits takes places relatively rapidly during ripening,²⁾ and the classical hypothetical mechanism of the softening assumes the solubilization of pectin and de-esterification with PE (pectinesterase) followed by its degradation with PG (polygalacturonase).³⁾ An explanation of the solubilization of pectin involving a movement of calcium in the cell walls was offered by Doesburg.⁴⁾ Kaji⁵⁾ and Letham⁶⁾ reported that EDTA (ethylenediaminetetraacetic acid) was effective in the extraction of pectin from plant tissues. McCready and McComb⁷⁾

proposed using EDTA and pectinase to extract pectic substances from ethanol-insoluble solids of fruit. Shioiri and Hagimura⁸⁾ reported that salts such as NaCl or Na₂SO₄ were just as effective as polyphosphates in extracting pectin from apple marc.

The commercial treatment of apple juice sometimes includes treatment with gelatin for clarification.⁹⁾ It was generally considered that gelatin reacts with the tannin present in the juice to give a gelatin-tannin complex precipitate which carries down much of the sediment. The commercial clarification of apple juice is sometimes inhibited probably by the contamination with tin salts from the vessel used.¹⁰⁾

In a previous paper¹¹⁾ it was observed that there were two types of pectin in cloudy

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11) A. Endo, *This Journal*, **29**, 137 (1965).

apple juice, soluble pectin and a small amount of insoluble pectin bound to suspended particles. Suspended particles, moreover, were successfully separated from the soluble fraction by ultracentrifugation.

In the present investigation, effects of pH, NaCl, CaCl₂, SnCl₄, EDTA, gelatin and some other chemical agents on the extraction of insoluble pectin, on the viscosity decrease of native soluble pectin, on the flocculation of suspended particles and on the over-all clarification of the juice were studied.

MATERIALS AND METHODS

1) **Purified Pectolytic Enzymes.** Endo-PG I, endo-PG II, endo-PG III, exo-PG, PE I and PE II used here were isolated from crude enzyme preparation from *Coniothyrium diploidiella* by the methods described before.¹²⁾

2) **Pectin and Pectic Acid.** Citrus pectin (esterification degree 64%) and pectic acid were obtained commercially as described previously.¹³⁾

3) **Apple Juice and its Clarification.** Apple juice was obtained by crushing commercially available apples (Ralls apples) as described before.¹⁴⁾ Fresh apple juice was adjusted to pH 3.5 with citric acid or NaOH prior to clarification test. Clarification test was carried out by the method described before.^{14 15)}

4) **Determination of Enzyme Activities.** Determinations of PG, viscosity-reducing (VRu) and PE activities were identical with those described before.^{12 13)}

5) **Determination of Pectic Substances.** Pectic substances were determined by the method of McCready and McComb as described before.^{7 11)}

6) **Native Soluble Pectin and Suspended Particles.** Apple juice was centrifuged at 100,000×g for 30 min. The resultant supernatant was used as native soluble pectin.¹¹⁾ The resultant sediment was washed twice with water and used as suspended particles.

About 250~290 mg of suspended particles was recovered from 100 ml of apple juice on dry weight basis. The particles contained 6~7% of total nitrogen, 1.4% of total ash and about 4% of pectic substances on dry weight basis.

7) Determination of Solubilizing Activity.

Suspended particles isolated were homogenized in a hand-operated mill and then suspended in 0.1M acetate buffer of pH 3.5. To 10 ml of the resultant mixture containing about 3.0 mg of pectin was added 1.0 ml of enzyme solution. After the incubation 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 filtered. Then total pectin content of the filtrate was determined as described above.

8) **Determination of Relative Viscosity.** A 10-ml aliquot of samples was placed in an Ostwald viscosimeter, and the relative viscosity was measured at 30°C.

RESULTS

[1] Solubilization of Insoluble Pectin.

Solubilization of insoluble pectin was accomplished by the joint action of endo-PG and PE, and exo-PG-PE mixture had no detectable effect on the solubilization as in the case of the clarification of apple juice¹⁵⁾ (Table I). Endo-PG I was the most effective of the three endo-PGs on the solubilization in terms

TABLE I. SOLUBILIZATION OF INSOLUBLE PECTIN BY PURIFIED PECTOLYTIC ENZYMES

Enzyme	Solubilized pectin, γ/ml	
	at pH 3.5	at pH 4.4
(1)		
endo-PG I	18	28
endo-PG II	21	12.5
endo-PG III	17	18.5
exo-PG	2	0
PE I	2	0
PE II	2	0
(2)		
endo-PG I + PE I	171	153
endo-PG II + PE I	122	111.5
endo-PG III + PE I	130	90.5
exo-PG + PE I	1	0
endo-PG I + PE II	185	149
endo-PG II + PE II	131	105
endo-PG III + PE II	130	100
exo-PG + PE II	2	0

To 10 ml of suspension of suspended particles in 0.1M acetate buffer was added 0.5 PGU of PG or 0.5 PEU of PE(1) or 0.5 PGU of PG and 0.5 PEU of PE(2), and the mixtures were incubated at 30°C for 40 min.

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

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

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

15) A. Endo, This Journal, 29, 129 (1965).

of PGu at pH 4.4.

1) Effect of pH.

Optimal pH range for enzymatic solubilization of insoluble pectin was observed to be 3.2~4.0 as shown in Fig. 1. At low pH values, especially at lower than 3.5, some non-enzymatic solubilization was observed.

FIG. 1. Effect of pH on the Solubilization of Insoluble Pectin.

Enzyme solution, endo-PG I-PE I mixture.
Reaction was carried out at 30°C.

2) Effect of Some Chemical Agents.

NaCl and EDTA slightly activated the solubilization, and gelatin had no detectable effect on it. SnCl_4 and CaCl_2 inhibited the enzymatic solubilization as shown in Table II.

TABLE II. EFFECT OF CHEMICAL AGENTS ON THE SOLUBILIZATION OF INSOLUBLE PECTIN

Reagent	Concentration	Relative activity (%)
Control		100
NaCl	0.1M	131
CaCl_2	0.05M	65
SnCl_4	0.002M	31
EDTA	0.01M	145
Gelatin	0.01%	98

Enzyme, endo-PG I-PE I mixture; incubation period, 40 min. at pH 3.5 and 30°C.

[2] Viscosity Decrease of Native Soluble Pectin.

Optimal pH for decrease in viscosity of

FIG. 2. Effect of pH on the Viscosity Decrease of Native Soluble Pectin.

Enzyme solution, endo-PG I-PE I mixture.
Reaction was carried out at 30°C.

FIG. 3. Effect of Chemical Agents on the Viscosity Decrease of Native Soluble Pectin.

To 10 ml of native soluble pectin solution containing about 0.1% of pectin was added 1 ml of enzyme solution containing 0.5 PGu of endo-PG I and 0.5 PEu of PE I, and the mixture was incubated at 30°C and pH 3.5.

—●— control, —○— 0.1M NaCl,
—●— 0.01M EDTA, —△— 0.05M CaCl_2 ,
—■— 0.01% of gelatin, —□— 0.002M SnCl_4 .

(A)

(B)

FIG. 4. Effect of Several Proteins and Salts on the Flocculation of Suspended Particles.

To 10 ml of suspension of suspended particles in 0.1M acetate buffer of pH 3.5 was added 0.1 ml of 0.5% protein solution (A) or 0.1 ml of 0.5M salts solution (B) with stirring. After standing at room temperature for 120 min., the mixtures were photographed.

(A): 1, control; 2, gelatin; 3, protamin sulfate; 4, bovine γ -globulin; 5, α -globulin; 6, casein; 7, lactalbumin; 8, egg albumin; 9, containing 0.1% of crude soluble pectin isolated from apple juice¹¹⁾ and 0.005% of gelatin.

(B): 1, control; 2, SnCl_4 ; 3, FeCl_3 ; 4, AlCl_3 ; 5, ZnSO_4 ; 6, CaCl_2 ; 7, MgSO_4 ; 8, KCl ; 9, NaCl ; 10, containing 0.1% of crude soluble pectin and 0.1 ml of 0.5M SnCl_4 .

native soluble pectin was found to be 3.6~4.1 as shown in Fig. 2.

Decrease in viscosity of the native soluble pectin was almost completely inhibited by 0.002M SnCl_4 and partially by 0.05M CaCl_2 . NaCl, EDTA and gelatin exhibited no noticeable effect on the viscosity decrease as shown in Fig. 3.

[3] Flocculation of Suspended Particles.

1) Effect of pH.

Stability of the colloidal system of apple juice was observed to vary with pH values. Effect of pH on the stability and flocculation of apple juice was determined by measuring the relative viscosity at which flocculation occurred during clarification. As the pH value was lowered, the relative viscosity at which the flocculation occurred was raised (Table III). In other words, suspended particles were more ready to flocculate at lower pH.

TABLE III. RELATION BETWEEN pH AND THE RELATIVE VISCOSITY AT WHICH FLOCCULATION OCCURRED DURING CLARIFICATION OF APPLE JUICE

		pH				
		2.5	3.0	3.5	4.0	4.5
Relative viscosity	Juice A	2.81	2.60	2.04	1.69	1.40
	Juice B	2.15	1.82	1.63	1.48	1.41

To 10 ml of fresh apple juice was added 1 ml of enzyme solution containing 0.15 PGU of endo-PG I and 0.15 PEU of PE I, and the mixture was incubated at 30°C. The relative viscosity at which flocculation occurred was determined in an Ostwald viscosimeter. The mixture was centrifuged at 10,000 r.p.m. for 10 min. prior to the determination of the relative viscosity.

2) Effects of Some Chemical Agents.

To 10 ml of suspension of suspended particles in 0.1M acetate buffer of pH 3.5 was added 0.005% of several proteins with stirring. After standing at room temperature for 120 min., nature of flocculation was observed. Gelatin, protamin sulfate and bovine- γ -globulin caused flocculation, whereas other proteins added had no visible effect on the flocculation as shown in Fig. 4-A. Gelatin

was the most effective of the proteins tested on the occurrence of flocculation. However, gelatin could not cause flocculation when 0.1% of pectin was added to the suspension as shown in Fig. 4-A.

Of the several salts tested, SnCl_4 , FeCl_3 and AlCl_3 caused flocculation of the suspended particles. Other salts had no detectable effect on the suspension as shown in Fig. 4-B. Added pectin inhibited floc formation caused by SnCl_4 .

The data described above indicate that suspended particles had a negative charge at pH 3.5.

[4] Over-all Clarification of Apple Juice.

1) Effect of pH.

Optimal pH for the over-all clarification of apple juice was found to be 3.2~3.8 with a sharp peak at about 3.5 as shown in Fig. 5.

FIG. 5. Effect of pH on the Clarification of Apple Juice.

Enzyme solution, endo-PG I-PE I mixture.
Reaction was carried out at 30°C.

2) Effect of Added Gelatin.

Table IV shows the effect of added gelatin on the clarification. A moderate amount of gelatin, lower than 0.025%, stimulated the clarification of both fresh and heat-treated

TABLE IV. EFFECT OF GELATION ON THE CLARIFICATION OF APPLE JUICE

(1) Fresh apple juice.

Gelatin (%)	*Control	Transmittance at 660 m μ	
		30-min. incubation	120-min. incubation
0	2.6	67.2	91.7
0.005	5.1	67.5	94.7
0.01	6.2	62.5	94.7
0.025	5.7	71.3	92.6
0.05	4.3	49.7	91.8
0.10	3.5	29.1	91.8

(2) Apple juice pre-treated at 70°C for 10 min.

Gelatin (%)	*Control	Transmittance at 660 m μ	
		30-min. incubation	120-min. incubation
0	10.6	70.6	93.8
0.005	13.2	80.6	98.7
0.01	12.2	85.7	98.7
0.025	11.0	75.8	97.8
0.05	10.0	27.8	86.7
0.10	9.3	19.6	26.6

Enzyme solution contained 0.1 PGU of endo-PG I and 0.1 PEU of PE I per ml.

* Mixtures contained no enzyme was incubated at 30°C for 120 min.

TABLE V. EFFECT OF SEVERAL SALTS ON THE CLARIFICATION OF APPLE JUICE

Salt	Molarity	Relative clarification value
Control		100
NaCl	0.0125	107
	0.042	113
	0.125	151
	0.208	171
	0.417	191
CaCl ₂	0.0042	80
	0.0125	73
	0.042	37
	0.104	27
	0.313	5
KCl	0.1	130
MgSO ₄	0.025	100
CoSO ₄	0.01	43
ZnSO ₄	0.01	35
AlCl ₃	0.01	50
FeCl ₃	0.01	5>
SnCl ₄	0.01	10>
	0.002	39
EDTA	0.01	155

apple juice. An excess of gelatin, however, rather inhibited the clarification. This indicates that gelatin, if added in excess, will protect and stabilize colloidal system.

3) Effect of Some Chemical Agents.

NaCl, KCl, EDTA, etc. stimulated the clarification, whereas CaCl₂, FeCl₃ and SnCl₄ strongly inhibited the clarification as shown in Table V

Even in the absence of enzyme, gelatin could sometimes cause flocculation and partial clarification, if the juice low in pectin content was used. SnCl₄ itself could cause flocculation and partial clarification. However, further clarification by enzyme could not proceed because of the inhibitive action of SnCl₄ on the enzyme.

DISCUSSION

Effects of pH and some chemical agents on the solubilization of insoluble pectin in suspended particles, on the viscosity decrease of native soluble pectin, on the flocculation of suspended particles and on the over-all clarification of apple juice have been studied here. A summary of the data obtained is shown in Table VI.

Both NaCl and EDTA slightly stimulated the solubilization and the over-all clarification but showed no detectable effect on the viscosity decrease and the flocculation. Their stimulating effect on the over-all clarification,

TABLE VI. A SUMMARY OF THE EFFECTS OF pH AND SOME CHEMICAL AGENTS ON THE CLARIFICATION OF APPLE JUICE

	Over-all clarification	Solubilization of insoluble pectin	Viscosity decrease	Flocculation of suspended particles
Optimum pH	3.2~3.8	3.2~4.0	3.6~4.1	3>
NaCl	+	+	no	no
CaCl ₂	—	—	—	no
SnCl ₄	—	—	—	+
EDTA	+	+	no	no
Gelatin	+	no	no	+
+, Stimulation				
—, Inhibition				
no, No detectable effect				

therefore, seems to be due largely to their stimulating effect on the solubilization. CaCl_2 had no detectable effect on the flocculation. However, it considerably inhibited the solubilization and the viscosity decrease, probably resulting in the inhibition of the over-all clarification. SnCl_4 strongly inhibited the solubilization and the viscosity decrease. These effects probably caused the inhibition of the over-all clarification in spite of its stimulating effect on the flocculation. Gelatin

stimulated the flocculation but had little effect on the solubilization and the viscosity decrease. This indicates that the stimulation of the over-all clarification by gelatin was owing to its stimulating effect on the flocculation.

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