

Effect of a Wheat Protein on pH-Dependent Water-Binding Capacity and Viscosity of Wheat Tailings Fractions

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ABSTRACT

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Wheat flour was fractionated with acetic acid using a mortar and pestle method or a blender method. Higher pH-dependent water-binding capacity (WBC) and viscosity were obtained only in the tailings fraction. The higher pH-dependent WBC was rather stable at 5–37°C, however it decreased with salt addition. Pepsin or bromelain treatment stopped the

pH-dependent changes in WBC and viscosity, which suggests that this characteristic of the tailings fraction is due to the presence of proteins. HPLC indicated that the M_r of the proteins associated with high WBC at low pH was >200 kDa.

It has been reported that acetic acid treatment of wheat flour improved its breadmaking performance (Seguchi et al 1997). Maximum bread heights and specific volumes were observed with the addition of acetic acid at low levels (2–3 mL/kg of wheat flour). Maximum gas generation velocities and dough expansion rates were observed at the same level of acetic acid addition as those giving maximum breadmaking performance (Seguchi et al 1997). Mixograms (Hoseney and Brown 1983) and farinograms (Tanaka et al 1967) of acetic acid-treated doughs indicated a weakening of the doughs. Acetic acid addition has also been shown to increase water-binding capacity (WBC) and viscosity of flour-water suspensions (Seguchi et al 1997). It was suggested that these rheological changes were related to the improved breadmaking performance of the treated doughs. WBC of wheat flour doughs has been studied by many investigators. Bushuk (1966), Jelaca and Hlynka (1971), Yeh et al (1980), Hashimoto et al (1987), and Shogren et al (1988) reported that wheat flour pentosans were responsible for the higher WBC and that they were related to the mixing characteristics of dough. The increased viscosity may result from oxidative gelation reactions involving polysaccharide-polysaccharide or polysaccharide-protein covalent cross-linking through diferulic acid, ferulic acid-tyrosine, or ferulic acid-cysteine bridges (Fincher and Stone 1986). The role of pentosans in bread and dough was reviewed by D'Appolonia (1971). Michniewicz et al (1991) examined the influence of pentosans on the rheological behavior of hydrated gluten as well as on the aggregation-disaggregation process of gluten proteins during mixing. However, there are no reports about pH-dependent changes in WBC of doughs and viscosity of flour-water suspensions and their subsequent relationships to breadmaking performance. Furthermore, it was not clear why WBC and viscosity increased when the pH of flour dough decreased from 5.0 to 3.5. In this study, we sought to determine the mechanism of increases in WBC and viscosity at low pH using an acetic acid fractionation technique in conjunction with protease digestion.

MATERIALS AND METHODS

Wheat Flour

A hard red spring wheat flour (Red Knight, Nitto Flour Milling Co., Japan) was used for this study.

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Fractionation of Wheat Flour by Acetic Acid (pH 3.5)

Fractionation of wheat flour was performed by the method of Sollars (1958) with slight modification. The general procedure for acetic acid fractionation is outlined in Fig. 1. Flour (50 g) was mixed with water (150 mL) and homogenized for 20 min using either a Waring blender (Excel Auto Homogenizer, Tokyo Nihon Seiki Seisakusho Co., Japan) or a mortar and pestle run at 120 and 7 rpm, respectively (ANM-1000 and ANM-150, Nittokagaku Co., Japan). Samples were cooled on ice homogenized in the blender. The mixture was then centrifuged at $1,700 \times g$ for 20 min at room temperature. Two fractions were taken: the supernatant (water solubles fraction) and the pellet. The water solubles fraction was freeze-dried. The pellet was further homogenized with 125 mL of 0.8% acetic acid (pH 3.5) for 20 min and centrifuged. Another two fractions were taken: the supernatant (gluten fraction) and pellet. This step was repeated with 75 mL of 0.2% acetic acid. The supernatant was then combined with the previous gluten fraction and freeze-dried. The pellet was homogenized with 150 mL of water for 20 min and centrifuged. The final supernatant was discarded, and the two layers evident in the pellet were separated by spatula. The upper layer was considered the tailings fraction and the bottom layer was considered the prime starch fraction. Both fractions were freeze-dried. Protein ($N \times 6.25$) was determined according to Lowry et al (1951). Starch, ash, and lipid were determined according to McCready et al (1950). AACC approved methods 08-01 and 30-26 were used (AACC 1995).

Cryo-Scanning Electron Microscopy Observation

Scanning electron microscopy (SEM) observation of the tailings fraction was performed using a Hitachi S-570 model (20 kV) with a cryo-system. To prepare samples, a small amount of tailings fraction was put on the sample stage and frozen by liquid nitrogen. The frozen sample was cracked by mechanical strike, and the temperature of sample was raised to -60°C ; water was sublimated. The sample was coated with gold and observed by SEM.

Viscosity and WBC

A tailings fraction sample (13.85 g, dry weight 1.0 g, pH 3.5) was mixed with 50 mL of water. The pH was adjusted (2.0–10.0) by additions of 17*N* acetic acid or 5*N* NaOH. The mixture was shaken vigorously for 60 min at room temperature. The viscosity of the flour-water suspension was measured (Viscotester VT-03 and -04, Rion Co., Japan). The mixture was then centrifuged at $1,700 \times g$ for 10 min. The weight of the pellet was measured, both wet and after drying at 70°C for 12 hr. WBC was calculated as (wet wt. [g] – dry wt. [g])/dry wt. (g) $\times 100\%$. The WBC stability of the tailings fraction was measured after tailings (pH 3.5) were shaken at 5, 25, and 37°C for 3 hr at a speed of 200 rpm/min.

Effect of Salts on WBC of Tailings Fraction

NaCl, KCl, MgCl₂, and CaCl₂ were used in this study. A tailings fraction sample (13.85 g, dry weight 1.0 g, pH 3.5) was mixed with 50 mL of water. Concentration (mM) of NaCl in the solution was changed from 1.0 to 5.0 mM. Concentration of other salts was adjusted to 5.0 mM. Dialysis was performed against a large amount of water. After centrifugation, the WBC of the tailings fraction was determined as above.

Colloid Titration

Methyl glycol chitosan (MGch), polyvinyl sulfate (PVSK), and toluidine blue solutions were purchased from Wako Pure Chemical Industries and Kishida Chemical, Japan. A MGch solution (1 mL of 1/1,000N, F = 1.005) was mixed with 30 mg of sample (protein in sample was not adjusted), and the pH was adjusted from 2.0 to 11.0 with 1N HCl or 1N NaOH; the total volume was adjusted to 21 mL with water. Titration was performed with 1/2,000N PVSK solution (F = 1.000) with stirring. The end of reaction was determined using toluidine blue: blue color changed to reddish-purple color. After titration, the pH of the solution was checked again. Blank titration was performed in the same way but without sample. The protein (g) equivalence between the titration value of sample and that of a blank test at the same pH obtained from the pH vs. titration value curve (Yoshino and Matsumoto 1966) was determined as:

$$\text{Equivalence/protein (g)} = \text{normality of PVSK} \times \text{difference in titer} \times 10^{-4} / \text{amount of protein (g)}$$

Protease Treatments of Tailings Fraction

Two crystallized acid proteases were used: pepsin A (optimum pH 2.0) and bromelain (optimum pH 4.5). A tailings fraction sample (13.85 g, dry weight 1.0 g, pH 3.5) was mixed with 50 mL of water

and 1 mL of pepsin or bromelain solution (pepsin: 0.01, 0.1, 1.0, 10.0, 100.0 mg each; or bromelain: 1.0 mg/1 mL of water adjusted to pH 3.5). The mixture was then incubated at 37°C for 30 min. Heat-denatured pepsin or bromelain were prepared by boiling for 5 min. After the protease reaction, WBC and viscosity of the tailings fraction were measured.

HPLC and PAGE Analysis of Proteins

For HPLC analysis, a Shimadzu Liquid Chromatograph LC-3A, connected to a Shodex Protein KW 803 column (5009010) was used. The column was equilibrated with 1% SDS or 1% SDS containing 1% 2-mercaptoethanol (2-ME). Protein was first extracted from the tailings fraction (22 g) with 75 mL of 0.2% acetic acid solution (pH 3.5) using the mortar and pestle method. This step was repeated eight times. Protein was freeze-dried after dialysis. Protein was further extracted from the extracted tailings fraction (41.55 g) using 150 mL of water at pH 8.5 adjusted with 1.0N NaOH solution and treated as above. Protein (1.33 mg/mL) was dissolved in 1% SDS. When reducing agent (1% 2-ME) was used, samples were boiled for 5 min before loading on the column. A volume of solution equivalent to 13.3 µg of protein was injected into the HPLC column. PAGE analysis was performed by the method of Laemmli (1970) without 2-ME. Protein (10 µg) was loaded on to the gels. PAGE patterns were checked by staining with Coomassie brilliant blue and high-speed scanning (Shimadzu TLC Scanner CS-920).

RESULTS AND DISCUSSION

Acetic Acid Fractionation of Wheat Flour (pH 3.5)

Total recoveries using both techniques were almost complete (Table I). However, a greater amount of the flour was fractionated into the tailings fraction when using the mortar and pestle method as compared to the blender method (Table I); this was reversed for the prime starch fraction. This seemed to indicate that some of the prime starch fraction was included in the tailings fraction when the fractionation was performed using the mortar and pestle. In addition, the protein contents in tailings fraction produced by the mortar and pestle method were higher than those achieved in the same fraction using the blender method (Table I). These differences in the protein contents of the tailings fraction were reflected in differences of recoveries of the gluten fraction. Lipid content in the gluten fraction was also higher using the mortar and pestle method. The vigorous homogenation of the blender method may have caused a greater release of the gluten-bound lipids. From these results, the difference between two homogenation methods was clear. The blender method appeared to break up weak binding forces that were present among the flour fractions, whereas the mortar and pestle method appeared able to separate flour fractions while maintaining the integrity of any weak binding forces

Acetic acid fractionation

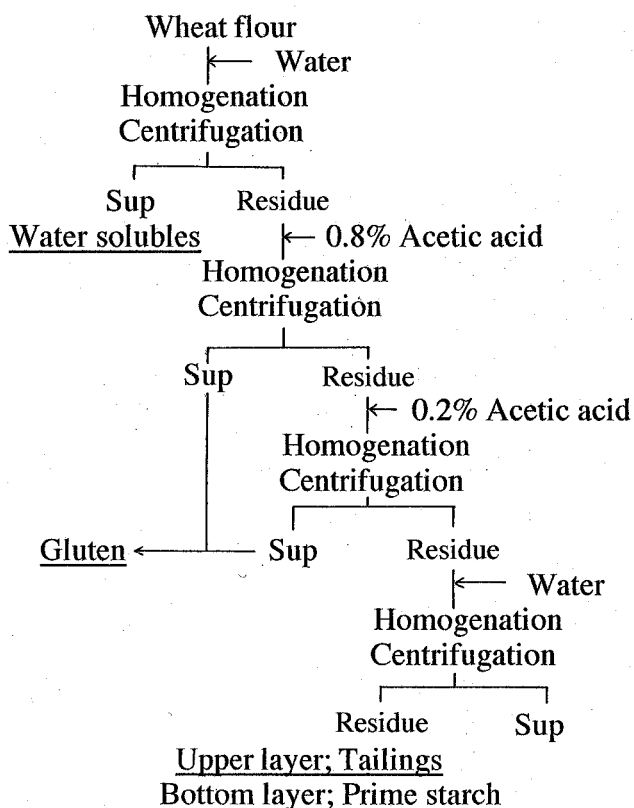


Fig. 1. General procedure for acetic acid fractionation of wheat flour.

TABLE I
Results (%) of Acetic Acid Fractionations^a

Fractions	Yield	Protein	Lipid	Starch	Ash
Mortar and pestle					
Wheat flour	100	15.7	0.9	63.5	0.4
Water solubles	8.3	29.7	0.3	14.3	3.4
Gluten	10.2	80.1	1.7	2.4	0.9
Prime starch	30.5	0.7	0.1	86.0	0.2
Tailings	43.9	11.5	0.3	82.7	0.3
Recovery	92.9				
Waring blender					
Wheat flour	100	15.7	0.9	63.5	0.4
Water solubles	8.3	41.8	0.2	20.8	3.4
Gluten	16.0	75.8	0.1	9.8	0.8
Prime starch	45.0	0.3	0.1	92.1	0.2
Tailings	24.8	1.8	0.0	78.2	0.2
Recovery	95.0				

^a Values are average of three determinations. Expressed on a water-free basis.

present. Consequently, we suggest that the mortar and pestle method can separate flour fractions and, as far as possible, maintain the native states. After acetic acid fractionation of wheat flour, only the tailings fraction collected by the mortar and pestle method showed the same pH-dependent change in WBC and viscosity as the unfractionated wheat flour (Seguchi et al 1997). Only the tailings fraction separated by the mortar and pestle method was analyzed in this study.

Cryo-SEM Observation of Tailings Fraction

To clarify the differences in WBC of tailings fraction at pH 3.5 and 5.0, the sizes of water crystallines were compared. Cryo-SEM photographs of tailings fraction at pH 3.5 and 5.0 are shown in Fig. 2. The tailings fraction at pH 3.5 contained a much higher

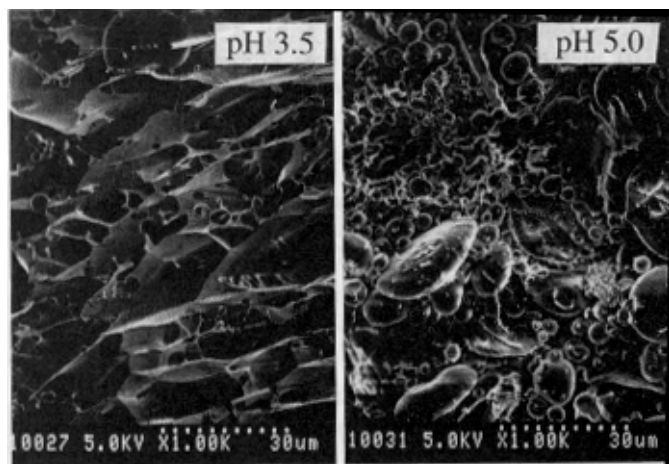


Fig. 2. Scanning electron microscope observation of tailings fraction at pH 3.5 and 5.0.

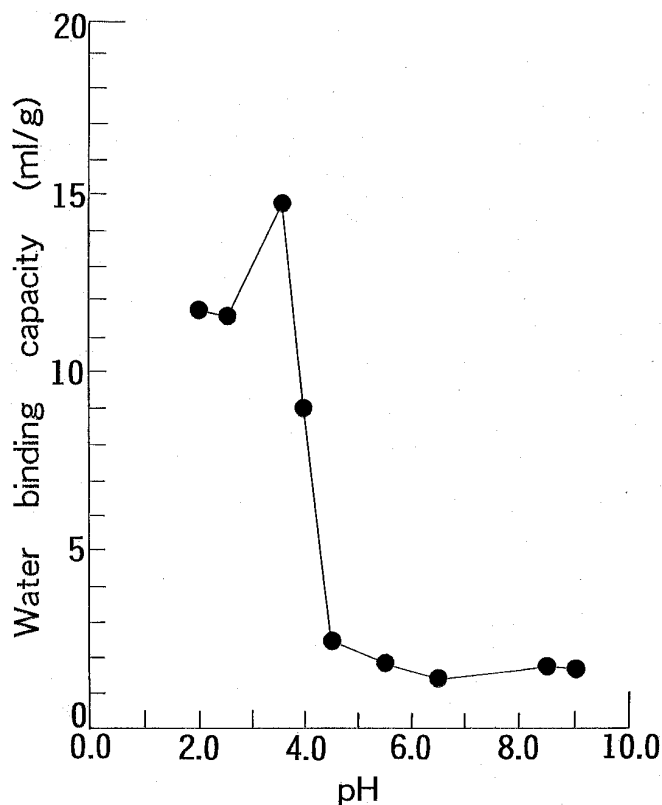


Fig. 3. Water-binding capacity of tailings fraction at various pH levels with acetic acid.

amount of water and larger sized water crystallines were observed. On the other hand, the tailings fraction at pH 5.0 did not contain as much water and smaller sized water crystallines were observed.

WBC of Tailings Fraction

Fig. 3 shows the WBC of tailings fraction at various pH levels. WBC increased greatly between pH 5.0 and 3.5. This result is similar to results obtained for flour dough at various pH levels (Seguchi et al 1997). When the pH of the tailings fraction was altered from pH 3.5 to another pH level and then returned to pH 3.5, the WBC of the tailings fraction did not recover its original high WBC. Therefore, the pH-dependent WBC of the tailings fraction was not fully reversible. The viscosity of the tailings fraction also showed the same irreversible characteristics under same pH change. To clarify the characteristics of these higher pH-dependent WBC and viscosity values, the stability of the tailings fraction WBC at 5, 25, and 37°C was tested for 3 hr (Fig. 4). Throughout this study, the pH of samples was adjusted to pH 3.5. All treatments showed a slight decrease in WBC over time. There seemed to be little temperature dependence in WBC at the three temperatures studied. WBC was rather stable even after 3 hr at all three temperatures. From those results, it was clear that various additions such as salt and enzyme to the pH-dependent WBC and viscosity could be examined.

Effect of Salts on the WBC of Tailings Fraction

Because salt (NaCl) is an essential component in breadmaking formulas, the effects of salt on the higher pH-dependent WBC and viscosity were studied. Fig. 5A indicates the effect of NaCl on the WBC of the tailings fraction. WBC gradually decreased as NaCl concentration increased, and a plateau region was observed at concentrations of >4 mM NaCl. The effects of other salts, such as KCl, MgCl₂, and CaCl₂, were also tested at a concentration of 5.0 mM (Fig. 5B). The results indicated that the WBC of the tailings fraction decreased after salt additions. However, the salt effects were reversible when the salt was excluded, which was in contrast to the apparently irreversible effects of pH change on WBC. The same results were obtained for every salt addition and exclusion by dialysis. Clearly, salt (NaCl) influences the pH-dependent WBC and viscosity of tailings fraction in dough and is related to breadmaking performance.

Colloid Titration of Flour Fractions

To clarify any differences in electrostatic charge between tailings and other fractions, such as gluten and water solubles, colloid titration experiments were performed at various pH levels (Yoshino and Matsumoto 1966). Fig. 6 shows the results of the colloid titra-

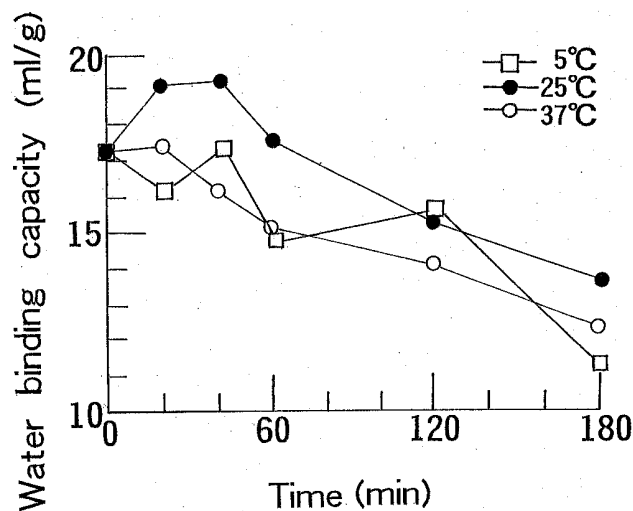


Fig. 4. Stability of the water-binding capacity of tailings fraction at 5, 25, and 37°C.

tion of wheat flour, water solubles, gluten, and tailings fraction. Prime starch fraction did not show any electrostatic charge and was omitted from this study. Wheat flour, water solubles, and gluten fractions indicated the same isoelectric points at ~pH 6.0–8.0. As pH decreased, the amount of positive charge sharply increased. On the other hand, the tailings fraction had a lower isoelectric point at ~pH 3.0. Higher positive charges below the isoelectric point were not observed. However, when the pH was increased in the alkaline region, the tailings fraction showed higher negative charges that were not observed in the wheat flour or in the gluten and water solubles fractions. This may suggest that specific proteins in the tailings fraction have some relationship to the high WBC and viscosity of the tailings fraction observed at low pH.

Effects of Protease on Tailings Fraction

The tailings fraction was incubated with the acid proteases pepsin and bromelain. Recrystallized commercial pepsin, separated from porcine stomach, showed a single band in PAGE analysis. WBC of the tailings fraction decreased with increased amounts of pepsin (Table II). When heat-denatured pepsin was used, the WBC did not decrease. Incubation of the tailings fraction with bromelain gave similar results (Table II). Bromelain addition decreased the WBC of tailings fraction, but heat-denatured bromelain did not. Bovine serum albumin was used in a further attempt to establish the mechanism of the protein effect on the WBC. However, it had almost no effect, although a slight reduction, simply due to a dilution effect, was observed (Table II). The control tailings fraction contained a much higher amount of water and showed gel-like soft textures. However, after pepsin treatment, it decreased markedly in volume and became rather solid in texture.

Viscosity Changes of Tailings Fraction After Pepsin Treatment

Fig. 7 shows the viscosity changes of tailings fraction after pepsin treatment. The viscosity of the untreated tailings fraction sharply increased between pH 3.5 and 5.0. The viscosity of pepsin-

treated tailings fraction was low at all pH levels observed. From those results, it was concluded that the changes of WBC and viscosity were both caused by some component of the proteins in the tailings fraction.

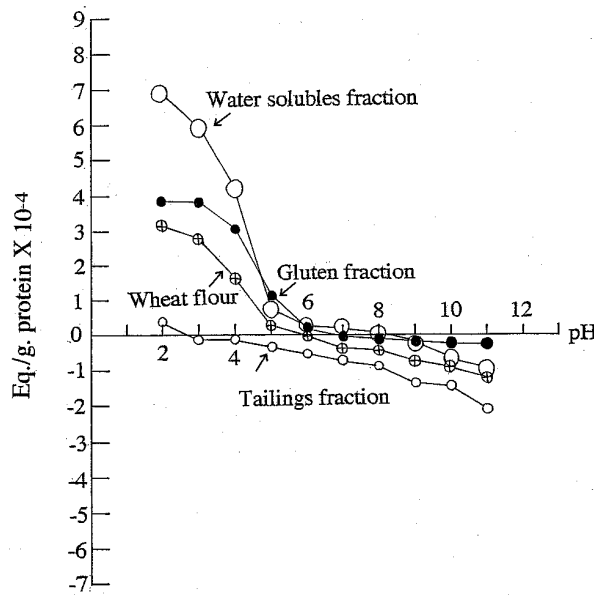


Fig. 6. Colloid titration curves of wheat flour, water solubles, gluten, and tailings fraction.

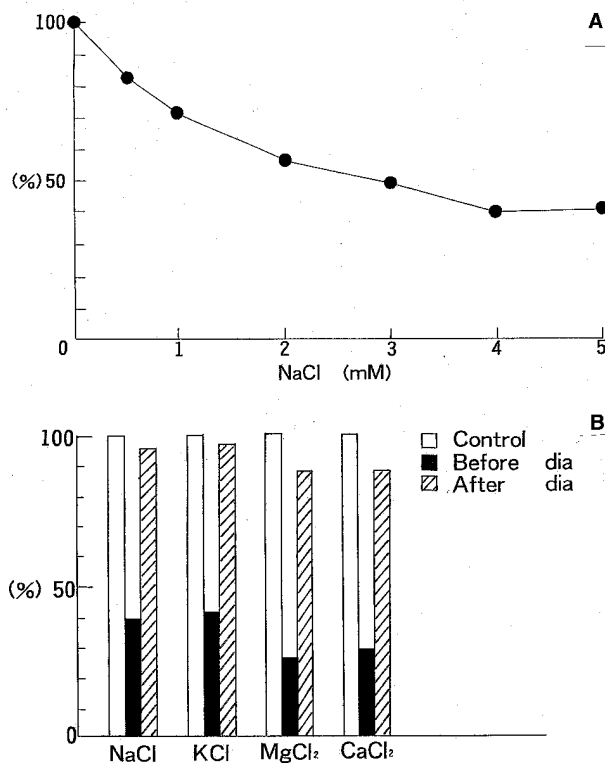


Fig. 5. Effect of salts on the water-binding capacity of tailings fraction. Concentration of NaCl was changed from 1–5 mM (A). Effects of NaCl, KCl, MgCl₂, and CaCl₂ on the water-binding capacity of tailings fraction when added and excluded by dialysis (B).

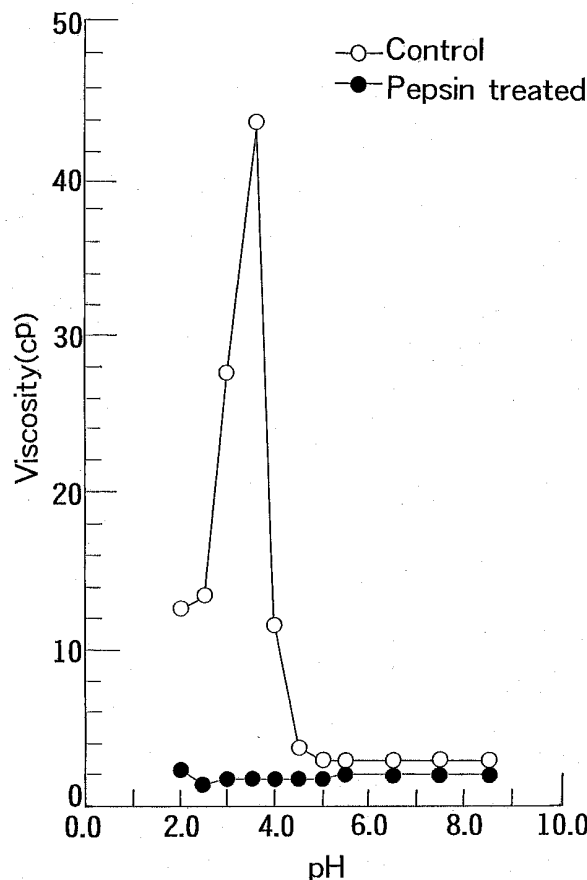


Fig. 7. Viscosity changes of tailings fraction in control and after pepsin treatment.

HPLC Analysis Proteins in Tailings Fraction

Proteins from the tailings fraction were extracted and analyzed by HPLC. Fig. 8 shows the HPLC profiles of the extracted proteins at pH 3.5 and 8.5. No clear differences in the high molecular weight region of these HPLC profiles was observed at 1% SDS (Fig. 8A). There was no change in elution times of the peak at 6.5 min related to the extraction pH with HPLC at 1% SDS without reduction. However, differences could be observed when these

TABLE II
Effects of Enzymes on Water-Binding Capacity (WBC) of Tailings^a

Treatment (mg)	WBC (%)
Control	100.0
Pepsin	
0.01	81.3
0.1	74.3
1.0	13.1
10.0	11.9
100.0	14.7
Heated pepsin	
1.0	96.9
Bromelain	
1.0	14.3
Heated bromelain	
1.0	99.7
Bovine serum albumin	
100.0	83.7

^a Values are average of three determinations.

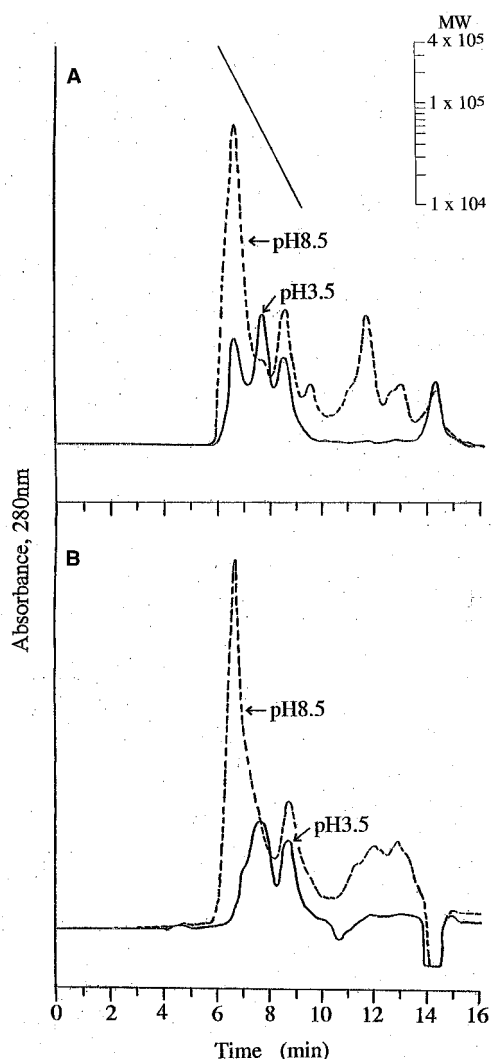


Fig. 8. HPLC profiles of the extracted proteins at pH 3.5 and 8.5 in tailings fraction. Column was eluted with 1% SDS (A) and 1% SDS containing 1% 2-mercaptoethanol (B).

proteins were reduced by 2-ME; that is, the main peak of the extracted proteins at pH 8.5 was not reduced. Otherwise, the same peak of the extracted proteins at pH 3.5 was reduced (Fig. 8B). The M_r of this main protein was ~200 kDa. This water-soluble protein at pH 8.5 may relate to the higher WBC and viscosity of tailings fraction at pH 3.5. Proteins that relate to higher pH-dependent WBC and viscosity are not dissolved at pH 3.5 and have a low level of disulfide bonds. Those proteins may have an important role in breadmaking when flour-water suspension is altered slightly with acetic acid (Seguchi et al 1997).

CONCLUSIONS

Gaseous acetic acid increased WBC and viscosity of wheat flour dough. The changes may be related to improved breadmaking performances (Seguchi et al 1997). Those higher pH-dependent WBC and viscosity characteristics of dough would be due to the presence of the acid-insoluble proteins with a low level of disulfide bonds (>200 kDa M_r).

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