Chemical Changes During Sponge-Dough Fermentation

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ABSTRACT

It was demonstrated that differences in mixing tolerance between unfermented and fermented dough were not caused by organic acids produced during fermentation. Foaming activity of doughs significantly increased during fermentation. As fermentation time was increased, the amounts of acetic-acid-soluble proteins (soluble glutenin) decreased remarkably, whereas proteins soluble in 70% ethanol increased. Two hydrophilic polypeptides of the soluble glutenin increased (detected by reversed-phase high-performance liquid chromatography), and these polypeptides were characterized by their high contents of glycine. Surface hydrophobicity of acetic-acid-soluble proteins in doughs increased gradually with fermentation time when hydrophobicity was determined by hydrophobic fluorescence probe *cis*-parinaric acid. Molecular weight profiles by gel filtration and sodium dodecyl sulfate-polyacrylamide gel electrophoresis of all proteins from the doughs at various fermentation times showed no change. Specific lipoxygenase activity (units per milligram of protein) increased constantly during fermentation, whereas proteinase activity decreased. Those results suggested that rheological changes during fermentation were due to a decrease in surface hydrophobicity of soluble glutenin induced by binding of oxidation products of lipoxygenase.

The effects of sponge-dough fermentation on rheological properties of dough were examined by a number of investigators (Landis and Freilich 1934, Freilich and Frey 1939, Ikezoe and Tipples 1968, Barber et al 1980). Preston and Kilborn (1982), Kilborn and Preston (1982), and Casutt et al (1984) used the extensigram to measure dough rheological properties during fermentation. They showed that fermentation decreases extensigram length, maximum height, and area; for flours of higher inherent strength and levels of salt, fermentation increases values for length, maximum height, and area at all fermentation times, and the rate of decrease in extensigram properties is lower during fermentation. Similarly, Pizzinatto and Hoseney (1980) reported that fermentation of cracker sponges changes the extensibility. Most of these reports are concerned with the effects of fermentation on rheological or physical properties, but only a few studied the mechanism of change in rheological properties.

The objective of the present study was to examine effects of fermentation on gluten protein and to relate the results to changes in rheological properties caused by fermentation.

MATERIALS AND METHODS

Wheat Flour

The flour was an unbleached commercial blend milled by the Nisshin Flour Milling Co., Ltd. Protein content was 13.0%, moisture 14.4%, and ash 0.52% as determined by AACC approved methods (1983). Flour was stored in the cold (−20°C) before use.

Preparation of Fermented Dough

Flour (300 g) with 2% yeast was mixed with water of 57% absorption in the farinograph bowl (24°C) for 4 min, and the resulting dough was placed into a fermentation room at a temperature of 27°C and a relative humidity of 80%. After various fermentation times (ranging from 0 to 4 hr), the dough was frozen at −40°C with liquid nitrogen. The frozen dough was lyophilized, pulverized, ground, and sieved by impact mill (ultracentrifugal mill with the 0.5-mm filter, Retsch).

Operation of Mixograph

Mixograms were obtained in an air-conditioned room maintained at 25 ± 1°C using a mixograph (National Mfg. Co, Lincoln, NE) operating at 87 rpm at spring setting 9 (Johnson et al 1946). Lyophilized flour (30 g, 14% moisture basis) from fermented dough was mixed with 20 ml of distilled water.

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Determination of Foaming Activity

The foaming activity for flours prepared from fermented dough was measured according to the modified stirring method of Kitabatake and Doi (1982). The flour (3.5 g) and 40 ml of 50 m*M* acetate buffer (pH 5.5) in a water-jacketed 50-ml stainless steel container were agitated at 4°C in a rotating six-bladed knife homogenizer (Ace type, Nihon Seiki Kasisha Ltd.). The rotor speed was adjusted to 10,000 rpm. After stirring for 3 min, all of the foam and liquid was immediately transferred to a measuring cylinder by gentle pouring and pipette, and the volume was measured. Foaming activity (FA) was defined by the expression

$$FA = (F/L - 1) \times 100$$

where F is the volume of foam plus liquid, and L is the volume of the liquid phase of the foam, which is calculated when the weight of foam in the vessel and the density of the liquid are known. The FA is dimensionless. The ratio of FA to soluble protein in 50 m*M* acetate buffer (pH 5 5) was expressed as FA/% protein.

Sequential Extraction of Fermented Flour Proteins

The protein contained in the flours from each fermented dough at various fermentation times was sequentially extracted according to a modified Osborne solubility fractionation procedure (Bietz and Wall 1975).

Gel Filtration of Fermented Flour Protein

Gel filtration on Sephacryl 5-300 was performed as described by Okada et al (1986). A column (Pharmacia, 2.5 × 57 cm) was equilibrated with 50 m*M* Tris-HCl buffer (pH 7.0) containing 0.5% sodium dodecyl sulfate (SDS). Protein was extracted by 50 m*M* Tris-HCl buffer (pH 7.0) containing 0.5% SDS and 1.6 m*M* *N*-ethylmaleimide. The suspension was stirred for 60 min at room temperature and was then centrifuged at 28,000 × *g* for 20 min at 25°C. The clear supernatant was collected. Flour protein extract (5 ml) was loaded onto the column, running 35 ml/hr with upward flow at 25°C. The effluent was collected in 5-ml fractions. The protein concentration of the effluent was estimated by the difference in absorbance at 280 and 350 nm. Absorbance at 350 nm was used to correct for turbidity.

SDS-Polyacrylamide Gel Electrophoresis (PAGE)

The SDS-PAGE technique used in this study on dried fermented doughs was described by Ng and Bushuk (1987) to determine the polypeptide subunits in flour protein.

Separation of Gluten Protein by Chromatography

The 70% ethanol-soluble proteins from dried fermented doughs were prepared according to the method described by Bietz et al (1984); glutenin subunits were prepared from dried fermented

doughs according to the method described by Burnouf and Bietz (1984). The protein solutions were filtered through 0.45 μm and the filtrates (50 μl) were directly analyzed at 40°C by reversed-phase high-performance liquid chromatography (RP-HPLC) using a chromatograph (Hitachi model 655A-12) equipped with solvent-delivery systems controlled by a model L-5000LC controller, a model 655A-40 automatic sample injector, and a model 655A-21 ultraviolet monitor set at 210 nm. The packed column (200 × 3 mm i.d.) was a silica-based reversed-phase support column (C18) with 300 Å pores (Gasukuro Kogyo Inc., Tokyo). Data were recorded and quantitated with a Hitachi Chromato Integrator D-2000. Solvent A was 12% acetonitrile with 0.05% trifluroacetic acid (TFA) in distilled water, and solvent B was 80% acetonitrile with 0.05% TFA. Proteins were eluted at 1 ml/min using a gradient from 15 to 600% solvent B over 55 min. These conditions were similar to those used by Bietz et al (1984), except that the TFA concentration was reduced from 0.1 to 0.05% in an attempt to avoid protein deamination as described previously by Heubner and Bietz (1985).

Amino Acid Analysis

For amino acid analysis, protein was hydrolyzed in evacuated tubes by 6*N* hydrochloric acid at 110°C for 24 hr. Amino acids were determined on a automatic amino acid analyzer (Hitachi model 835).

Measurement of Protein Hydrophobicity

Protein hydrophobicity was determined according to the method of Kato and Nakai (1980) and Kato et al (1981) using the hydrophobic fluorescence probe *cis*-parinaric acid. Ethanolic solutions of 3.6 m*M* *cis*-parinaric acid were purged with nitrogen, and equimolar butylated hydroxytoluene was added as an antioxidant. *cis*-Parinaric acid solution (10 μl) was added to 2 ml of protein solution in 10 m*M* acetic acid. The parinaric acid-protein conjugates were excited at 325 nm, and relative fluorescence intensity was measured at 420 nm in a spectrofluorometer (Hitachi Fluorophotometer, F-3000). The relative fluorescence intensity reading was adjusted to 1.0 when 10 μl of *cis*-parinaric acid solution was added to 2 ml of 10 m*M* acetic acid in the absence of protein. The hydrophobicity of extracted protein from the flour was expressed as fluorescence intensity/% protein.

Assay of Lipoxygenase Activity

Samples (3.5 g) of lyophilyzed flours prepared from fermented dough were homogenized with 60 ml of 50 m*M* acetate buffer (pH 4.5) for 5 min at 10,000 rpm by a rotating six-bladed knife homogenizer (Ace type, Nihon Seiki Kaisha Ltd.), and these suspensions were centrifuged for 20 min at 15,000 × *g*. The protein content of the crude enzyme in the supernatant was determined by the method of Lowry et al (1951) using bovine serum albumin as a standard. Lipoxygenase activity was determined according to the modified spectrophotometric method of Kenneth (1964) and Walsh et al (1970). The substrate was prepared in a nitrogen atmosphere by dissolving 100 mg of pure linoleic acid in a mixture of 0.12 ml Tween 20, 2.5 ml 50 m*M* phosphate buffer (pH 7.0), and 0.32 ml 1.0*M* sodium hydroxide. After the linoleic acid was dissolved, the mixture was diluted to 50 ml with the 50 m*M* phosphate buffer (pH 7.0). The substrate was sealed under nitrogen in a syringe bottle and stored at 5°C in the dark prior to use as a stock solution. Reaction mixtures consisted of 2.5 ml of phosphate buffer (pH 4.5), 90 μl of stock substrate, and 5 μl of lipoxygenase solution. The progress of the reaction was recorded continuously against a blank containing no enzyme in a double-beam spectrophotometer at 234 nm. A unit of lipoxygenase was defined as that activity that increases the 234 nm absorbance by 1 absorbance unit in 1 min in 2.5 ml of substrate solution, pH 4.5, using a 1 cm quartz cell.

Assay of Proteolytic Activity

Proteolytic activities (acid and neutral or alkaline proteinase activity) of flour on casein substrate were determined by the following methods. In a Waring Blendor for 5 min, 1 g of flour was dispersed in 20 ml of buffer solution (100 m*M* acetate buffer, pH 4.7, or 100 m*M* phosphate buffer, pH 7.5). The mixture was centrifuged at 14,500 × *g* for 10 min at 0°C and the supernatant was filtered through no. 2 Tokyo Roshi filter paper. This filtrate was used as proteinase extract. Casein substrate was prepared by the modified method of Kageyama et al (1955). Then 1 ml of protease extract in 2 ml of the substrate solution was incubated at 37°C for 60 min. The undigested casein was precipitated with 0.4*M* trichloroacetic acid (TCA), and the amount of TCA-soluble product was determined colorimetrically at 660 nm on a spectrophotometer (Hitachi 220A) by the Folin-Ciocalteu method (Folin and Ciocalteu 1927) using tyrosine as a standard. Proteolytic activities of flours were expressed as micromoles of liberated tyrosine per minute per gram of flour.

RESULTS AND DISCUSSION

Mixograph Studies of Fermented Dough

As fermentation time was increased, the strength of the fermented dough decreased (Fig. 1). The mixogram after 4 hr of fermentation showed less resistance to extension and a shorter peak time than that of unfermented dough. Similar effects of fermentation time on the extensigraph properties of fermented doughs were reported previously (Preston and Kilborn 1982). It is possible that lowering of pH by organic acids produced during fermentation could affect the rheological properties of dough, causing the decrease in mixing tolerance. Hoseney and Brown (1983), investigating the effects of lowering pH on mixograph properties, found only a slight change in the mixogram for a change in pH of 5.58 to 5.52. However, in our study the change of pH was small and similar to those reported by Hoseney and Brown during fermentation; the pH value was 5.72 for unfermented dough and 5.26 for dough fermented 4 hr. When

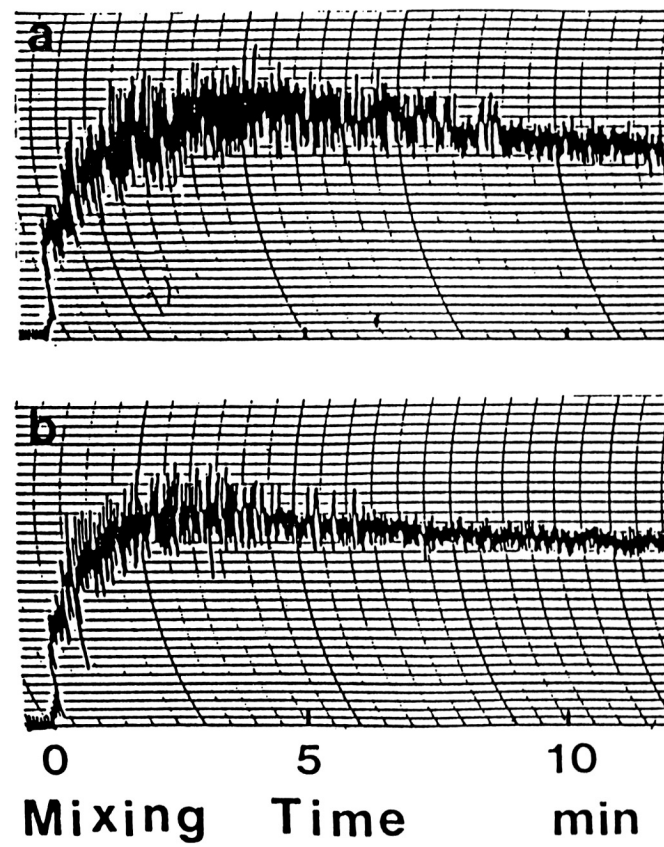

Fig. 1. Effect of fermentation on mixograms of freezed-dried flours made from dough with different fermentation time: **a,** dough fermented 0 hr; **b,** dough fermented 4 hr.

we adjusted the pH value of the unfermented dough to 5.2 by addition of dilute lactic acid, the mixogram changed only slightly. Also, after adding dilute sodium hydroxide to dough fermented 4 hr to raise the pH to 5.7, there were no differences in mixograms. These results demonstrated that factors other than acidity played a major role in modifying the physical properties of the dough.

Changes of Foaming Activities During Fermentation

FA of dough increased with large changes between 1 and 2 hr of fermentation time. As shown in Figure 2, the ratio of FA to soluble protein in 50 m*M* acetate buffer (pH 5.5) increased gradually. These results suggested that the increase in FA might be due to a change in hydrophobicity rather than more protein being solubilized, and rheological changes of dough might be caused by a change of the surface hydrophobicity of gluten protein. Kato et al (1981) reported an increase in foaming power with increasing surface hydrophobicity.

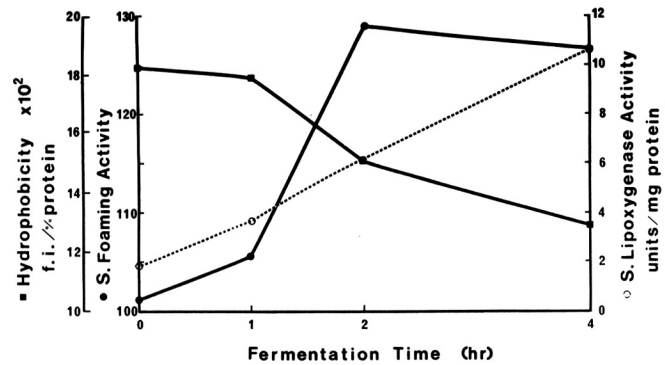

Fig. 2. Changes in foaming activity, lipoxygenase activity, and hydrophobicity of protein during fermentation. The hydrophobicity of protein was expressed as fluorescence intensity per percent protein.

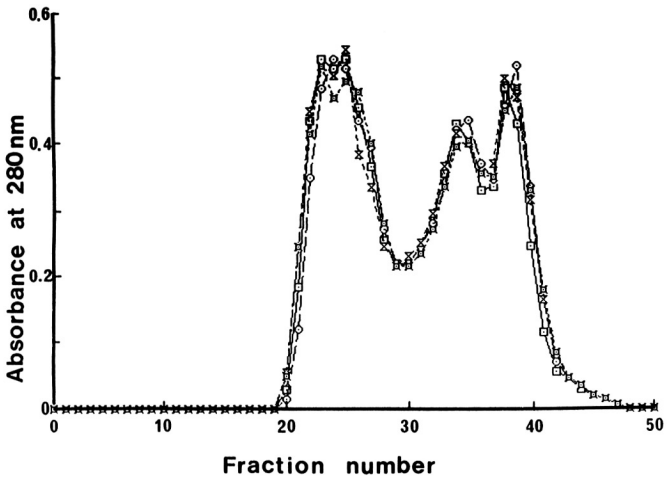

Fig. 3. Elution profiles of gluten protein from the fermented doughs at various fermentation times by gel filtration on Sephacryl S-300: □ = 0 hr, ⊙ = 1 hr, ⧗ = 2 hr, ⧖ = 4 hr.

Fractionation of Proteins from Fermented Dough

Osborne protein fractions from fermented dough were prepared (Table I). The total recoveries of flour protein in the fractions were 95.4, 96.7, 97.2, and 99.0% at various fermentation times (0, 1, 2, and 4 hr, respectively). The results showed almost the same recovery of extraction by this procedure. As fermentation time was increased, 70% ethanol-soluble protein increased and acetic-acid-soluble protein decreased significantly. On the other hand, the amount of protein extracted with water and NaCl solution changed only slightly. The fermentation evidently caused a change in the solubility of gluten proteins; a large decrease occurred in the acetic-acid-soluble fraction and an increase in the 70% ethanol-soluble fraction. Each protein fraction was subjected to SDS-PAGE, but the differences between proteins extracted from unfermented and fermented dough were small (data not shown).

Changes in the protein of fermented doughs were determined

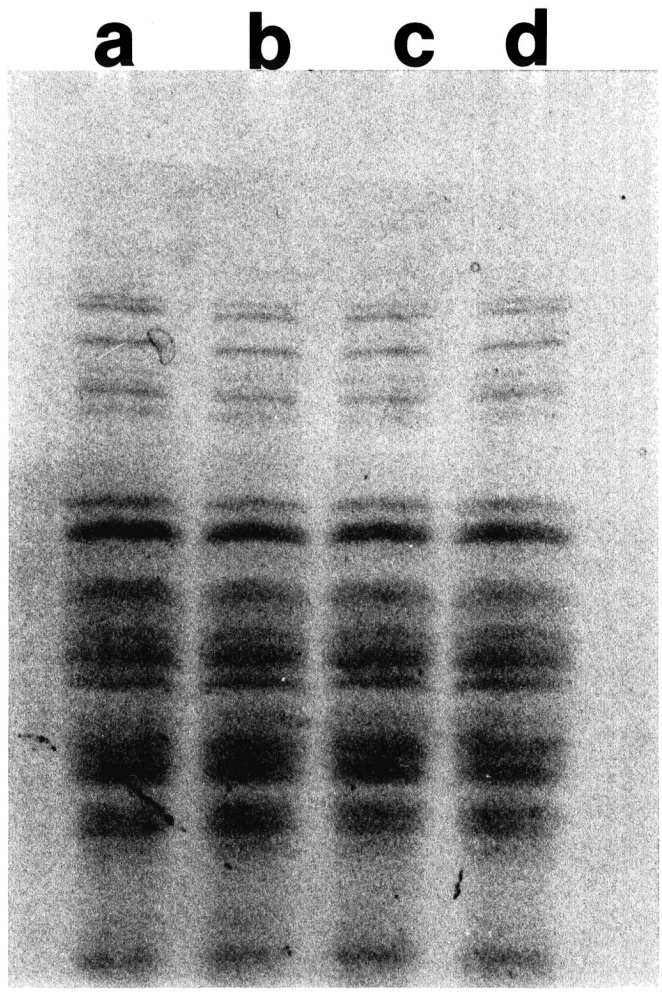

Fig. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis patterns of reduced glutenin from fermented doughs at various fermentation times: **a** = 0 hr, **b** = 1 hr, **c** = 2 hr, **d** = 4 hr.

TABLE I
Effects of Fermentation on Osborne's Protein Fractions[a]

Fermentation Time (hr)	Percent (%) of Total Protein					
	Water Soluble	0.5*M* NaCl Soluble	70% EtOH Soluble	50 m*M* Acetic Acid		Total Recovery
				Soluble	Insoluble	
0	11.83	2.69	25.19	19.62	36.06	95.4
1	11.50	1.74	29.77	16.24	37.41	96.7
2	11.76	2.43	30.13	13.31	39.55	97.2
4	10.59	2.38	42.56	9.20	34.25	99.0

[a]Values are the average of two replications. All duplicates were within 5% of the mean.

by gel filtration profiles (Fig. 3) and SDS-PAGE (Fig. 4). Since the protein extracted was almost 95% of total flour protein, the small differences in chromatographic and electrophoretic results indicate that the change in molecular weights during fermentation was negligible. A weak and decreasing acidic proteolytic activity (proteinase) in the doughs was detected (Table II). These results suggested that proteinase activity evidently could not change the molecular weight of the proteins. Thus, during fermentation the decomposition of gluten proteins to lower molecular weights apparently did not occur.

However, the possibility still exists that even if there is only weak proteolytic activity, it could break the large glutenin polymers into smaller ones that are still large enough not to be distinguishable by gel filtration and SDS-PAGE. Further studies may be necessary to ascertain this possibility.

We used RP-HPLC for detecting differences among flour proteins from unfermented and fermented dough. First of all, 70% ethanol-soluble proteins of unfermented and fermented dough were subjected to RP-HPLC (Fig. 5). The RP-HPLC pattern of 70% ethanol-soluble proteins from unfermented dough was very similar to that from fermented dough. These results and those of the SDS-PAGE patterns of ethanol-soluble protein (not shown) extracted by the modified Osborne method (Bietz and Wall 1975), revealed that 70% ethanol soluble proteins were not altered qualitatively during fermentation. Secondly, preparations of the total protein subunits of the reduced and cyanoethyl glutenin were subjected to RP-HPLC (Fig. 6). As fermentation time was increased, the amounts of two peaks (P1 and P2) that eluted early and had hydrophilic properties increased gradually. The area ratio of peak P1 plus P2 to total glutenin area from unfermented dough was 9.5:100, which increased in the dough fermented 4 hr to 28.6:100. The retention times of peaks P1 and P2 indicate they are a part of the high molecular weight subunits of glutenin and peak P3 low molecular weight subunits (Burnouf and Bietz 1984). When the protein of peaks P1, P2, and P3 from the 4-hr fermented dough were subjected to SDS-PAGE, the protein of peaks P1 and P2 exhibited low mobility corresponding to one of the high molecular weight subunits of glutenin (data not shown).

The protein in these peaks was hydrolyzed by 6*N* HCl and amino acids were determined on the amino acid analyzer (Table III). Peak P3 had a typical amino acid composition of gluten with high contents of glutamic acids and proline. Peaks P1 and P2, however, were characterized by higher amounts of glycine, whereas glutamic acid and proline were relatively high in peak P2 but considerably lower in peak P1.

Changes in Surface Hydrophobicity and Lipoxygenase Activity During Fermentation

In order to investigate the effect of fermentation on the surface hydrophobicity of dough proteins, the surface hydrophobicity was determined using the hydrophobic fluorescence probe, *cis*-parinaric acid. Even though during fermentation the solubility of proteins in 10 m*M* acetic acid solution did not change much more than 10 mg/ml, the surface hydrophobicity of the acetic acid soluble proteins decreased gradually as shown in Figure 2.

TABLE II
Changes in Proteolytic Enzyme (proteinase) Activity During Fermentation

Fermentation Time (hr)	Proteinase Activity[a,b]	
	Acidic (pH 4.7)	Neutral (pH 7.5)
0	0.181	not detected
1	0.127	not detected
2	0.119	not detected
4	0.101	not detected

[a]Values were expressed as micromoles of liberated tyrosine per minute per gram of extracted protein.
[b]Values are the average of replications. All duplicates were within 5% of the mean.

Lipoxygenase activity was determined because its activity in fermented dough may be related to the hydrophobicity of the acetic-acid-soluble proteins. As the fermentation time was increased, specific lipoxygenase activity (units per milligram of protein) in the flours from fermented dough increased as shown in Figure 2. These results indicated a possible relationship between lipoxygenase activity and the decrease in surface hydrophobicity of soluble proteins in dilute acetic acid and the increase in foaming activity of fermented dough during fermentation.

CONCLUSION

Mixograph and pH studies showed that a decrease in mixing tolerance of fermented dough was not caused by organic acids produced during fermentation. Foaming activity of doughs significantly increased during fermentation. This phenomenon did not appear to be due to a change in molecular weight of the proteins, because SDS-PAGE and gel filtration on

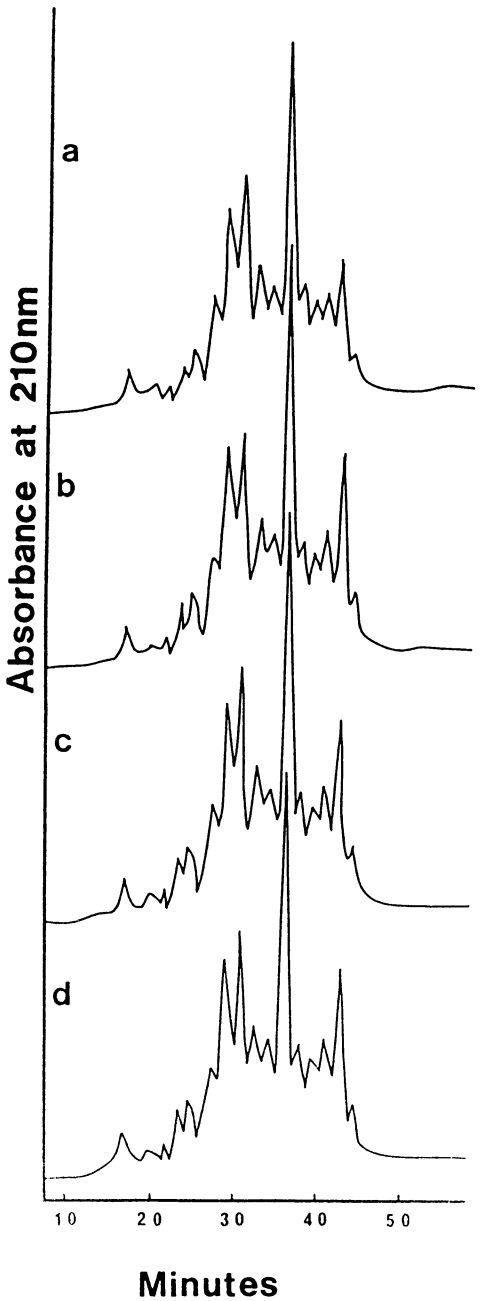

Fig. 5. Reversed-phase high-performance liquid chromatography analysis of 70% ethanol-soluble proteins from doughs fermented for various times: **a** = 0 hr, **b** = 1 hr, **c** = 2 hr, **d** = 4 hr.

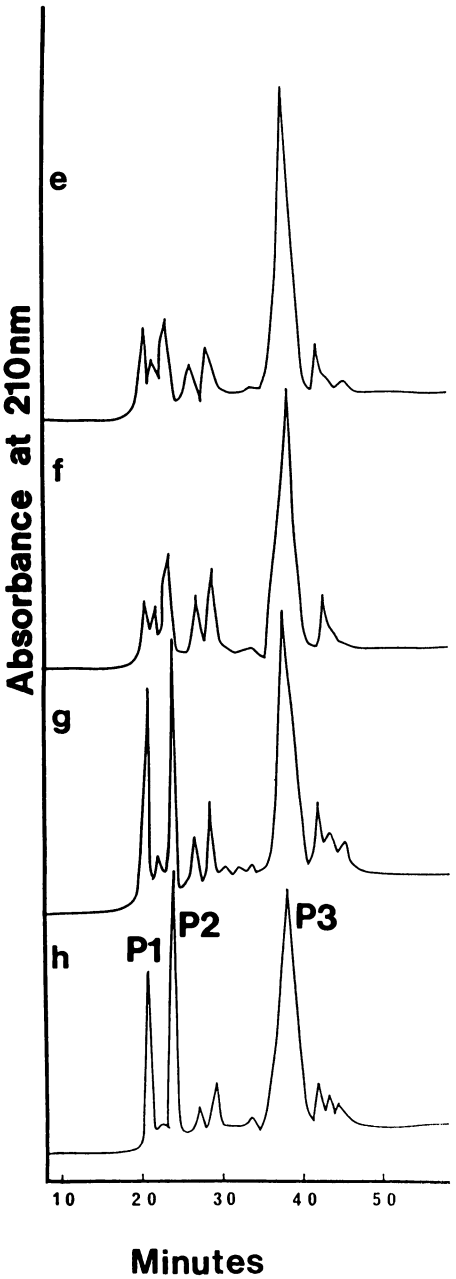

Fig. 6. Reversed-phase high-performance liquid chromatography analysis of reduced glutenin from doughs fermented for various times: **e** = 0 hr, **f** = 1 hr, **g** = 2 hr, **h** = 4 hr.

Sephacryl 5-300 showed no detectable change of molecular weights of proteins in unfermented and fermented dough. Also proteolytic activity in the dough was low.

The fermentation caused a change in protein solubility; there was a large decrease in acetic-acid-soluble proteins and an increase in 70% ethanol-soluble proteins. Furthermore the surface hydrophobicity of dilute acetic-acid-soluble proteins decreased gradually as fermentation time was increased. These results suggested that the decrease in surface hydrophobicity of glutenin might occur as the result of the conformational change of glutenin protein without changing molecular weights.

Lipoxygenase is an enzyme that catalyzes the oxidation of polyunsaturated lipid and when added as soy flour is reported to affect the rheological properties of dough (Frazier et al 1973). Possibly this enzyme is bound to the hydrophobic site in the lipid-binding protein ligolin that was reported by Frazier et al (1981) to be strongly associated with glutenin. Referring to these reports and the present results, we hypothesize that part of the lipoxygenase bound by the hydrophobic site is released with lipid binding on mixing before fermentation, and the activated lipoxygenase might catalyze the oxidation of polyunsaturated lipids during fermentation. Then the oxidized lipid (peroxide of unsaturated fatty acids) might be bound and affect the hydrophobicity of glutenin to induce conformational changes in gluten protein. As the result of this, more lipoxygenase might be released. However, it is obvious that additional studies are necessary to ascertain these suggestions.

TABLE III
Composition of Amino Acids

Amino Acid	Mole Percent of Amino Acid[a,b]		
	P1	P2	P3
Aspartic acid	7.12	4.13	3.90
Threonine	4.08	3.37	3.61
Serine	11.83	7.65	8.81
Glutamic acid	16.03	33.74	31.97
Proline	8.75	11.36	12.09
Glycine	17.81	10.26	6.53
Alanine	4.65	3.63	4.31
Valine	4.01	3.06	5.53
Methionine	0.36	0.20	nd[c]
Isoleucine	2.90	3.09	3.74
Leucine	6.06	5.19	8.06
Tyrosine	2.78	2.22	1.52
Phenylalanine	3.14	5.28	4.12
Lysine	3.20	2.05	1.53
Histidine	2.60	1.77	2.12
Arginine	4.67	2.99	2.52
Tryptophan	nd	nd	nd

[a]Values are the average of two replications. All duplicates were within 5% of the mean.
[b]Proteins corresponding to peak P1, P2, and P3 shown in Fig. 7 were fractionated from chromatographic elution.
[c]Not detected.

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