Studies on Heavily Ground Flour Using Roller Mills. II. Chemical Alteration of Proteins, Particularly Globulin, During Dough Mixing

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ABSTRACT

The chemical changes in proteins of flour during heavy grinding and dough mixing were studied by gel filtration on Sephacryl S-300, by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and by determining the rate of aggregation of proteins, stored at −20° C in 0.5% sodium dodecyl sulfate solution containing 1.6×10^{-3} *M* *N*-ethylmaleimide. Overgrinding of flours caused little change in the elution profiles of extracts of flour or dough according to gel filtration. On the other hand, the rate of aggregation of proteins, stored at −20° C in 0.5% sodium dodecyl sulfate solution containing 1.6×10^{-3} *M* *N*-ethylmaleimide, increased with more passes through the rolls and with dough mixing. The results suggested that the conformation of flour protein is changed by overgrinding and mixing, so that denaturation of flour protein is more easily caused by frozen storage. Wheat flour globulin was fractionated into three fractions, namely, a high-molecular-weight fraction (HMW-globulin), a medium-molecular-weight fraction (MMW-globulin), and a low-molecular-weight fraction (LMW-globulin) by gel filtration on Sephacryl S-300. MMW-globulin was especially reactive and polymerized into HMW-globulin by forming S-S bonds during mixing; that of overground stream X (a late-break flour) was more reactive than others. Globulins in wheat flour may play an important role as binders between proteins during dough mixing, thus affecting the rheological properties of dough.

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The effects of starch damage on flour baking quality have been studied by many workers (Ponte et al 1961, Schiller and Gillis 1964, Tipples and Kilborn 1968, Evers and Stevens 1984). Schlesinger (1964) concluded that the mechanical action of ball milling was not sufficient to alter protein structure. In contrast, D'Appolonia and Gilles (1967) reported that overgrinding flour caused a decrease in the total flour nitrogen contained in water-soluble material, an increase in nonprotein nitrogen in the water solubles, an increase in amino groups, and an increase in low-molecular-weight (LMW) protein. In our previous study (Okada et al 1986), the alteration in flour characteristics through overgrinding was also studied. We found that, as the number of passes through the rolls increased, the bromate requirement was less, the sulfhydryl (SH) content of the water-soluble fractions and the SH/disulfide (SS) ratio decreased, and the valorimeter value of the farinogram increased. These changes may result from the oxidation of flour components during overgrinding by roller milling.

Globulins, although minor components in wheat flour proteins, have been studied by many workers because of their interesting chemical properties. Pence and Elder (1953) studied physical and chemical properties of purified albumin and globulin proteins. Pence et al (1954) suggested that the ratio of albumins to globulins in flour may be related to protein quality. Nimmo et al (1968), Fisher et al (1968), and Redman and Fisher (1968) studied the fractionation of purothionin from crude globulins. Patey et al (1976) found that albumins are stable both qualitatively and quantitatively, whereas the globulin fraction, principally the purothionins, is unstable during long-term storage of flour. Terada et al (1978) reported that wheat flour globulin was polymerized at alkaline pH values and reversibly depolymerized by reduction at neutral or slightly acidic pH.

Wheat flour globulin is known to be reactive protein. We consider that globulin in wheat flour may affect the rheological properties of dough. The purpose of the work reported here was to determine the chemical alteration of proteins, mainly globulin protein, in overground flour during dough mixing.

MATERALS AND METHODS

Wheat Flour

Two commercial mill streams were used: stream A (an early reduction flour) and stream X (a late-break flour), both prepared for the previous study (Okada et al 1986).

Overground flours were prepared with the Miag Vario-Roller mill as described previously (Okada et al 1986). Mill streams A and X were repassed through the mill 1, 3, and 5 times. An extremely overground sample was obtained by repassing stream X through the mill 10 times.

Farinograph Operation

A Brabender farinograph was operated as described in AACC method 54-21 (1983). The thermostat was maintained at 30° C, and a large mixing bowl containing 300 g of flour was used.

Preparation of Dried Doughs

Doughs were mixed to the peak time and mixing was continued for another 12 min (overmixing). After an appropriate mixing time, the dough was immediately frozen by immersion in liquid nitrogen, lyophilized, and finely ground.

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Gel Filtration on Sephacryl S-300

Gel filtration on Sephacryl S-300 was performed as described previously (Okada et al 1986). 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.

Determination of the Turbidity of Flour Protein Solutions

Flour was suspended in 0.5% sodium dodecyl sulfate (SDS) solution containing $1.6 \times 10^{-3} M$ *N*-ethylmaleimide (NEMI). The suspension was stirred with a magnetic stirrer for 60 min at room temperature and was centrifuged at $28,000 \times g$ for 20 min at 25° C. The clear supernatant (containing about 8 mg of protein per milliliter) was collected, frozen, and stored at −20° C. This flour protein solution was thawed before measuring absorbance at 350 nm for turbidity on a Hitachi 220A spectrophotometer against a blank. The change in turbidity of a flour protein solution due to frozen storage was indicated by expressing the turbidity of a frozen-and-thawed flour protein solution as a percentage of the turbidity of the original, unfrozen flour protein solution. The change in turbidity of a protein solution was used as an index of the aggregation of the protein.

Preparation of Globulins

Globulins were prepared by a modified Osborne procedure described by Chen and Bushuk (1970) except that the 0.5*M* sodium chloride solution contained excess NEMI (10 mol per mole of SH in the flour).

Reduced and reoxidized globulins were prepared by the method of Kanazawa and Yonezawa (1974) except that 0.5% SDS was used as solvent.

Purification of Flour Proteins

Untreated flour proteins used for this study of subunits were purified from Osborne fractions (Chen and Bushuk 1970) by gel filtration on Sephacryl S 300.

SDS Polyacrylamide Disc Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis (PAGE) was performed on a column gel (5 mm × 70 mm × 12 tube) at 3 mA per tube for 2.5–3.0 hr with the buffer system of the method of Orth and Bushuk (1973). The concentration of acrylamide in the gel was 7.5%. The gels were scanned at 590 nm with a dual-wavelength scanner CS-900 (Shimadzu Seisakusho Ltd., Japan).

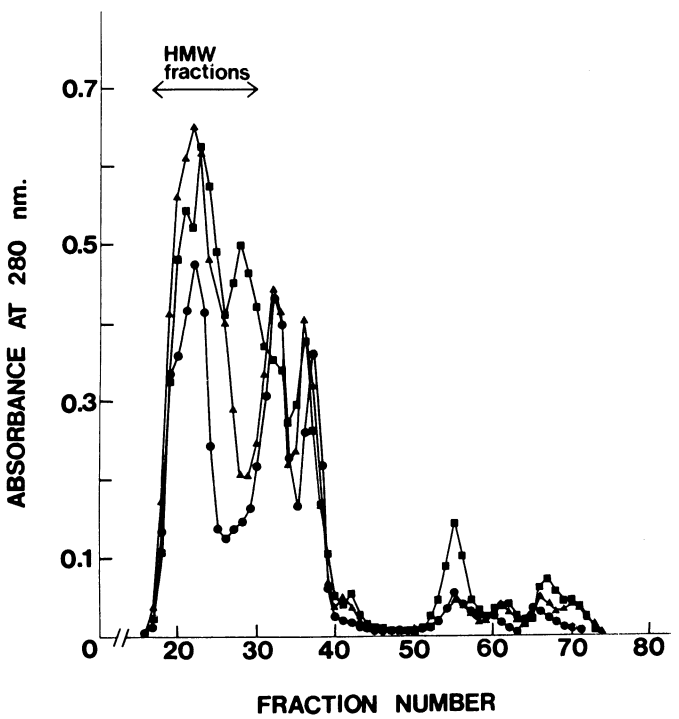

Fig. 1. Gel filtration profiles of proteins extracted from flour or dough of untreated stream X: ●—● no mixing, ▲—▲ peak time, ■—■ overmixing.

RESULTS AND DISCUSSION

Gel Filtration Profiles of Proteins Extracted from Flour and Dough

Figure 1 shows the gel filtration profiles of proteins extracted from flour and dough of untreated stream X. Proteins extracted from flour were fractionated to glutenin, gliadin, and albumin. The elution profiles of proteins extracted from dough showed increases in the high-molecular-weight (HMW) fraction compared with those of proteins extracted from flour. Gel filtration profiles of proteins extracted from streams A and X were similar (data not shown). Changes in the proportion of the HMW fraction during dough mixing (Table I) were expressed as a ratio of the HMW fraction compared to the sum of the gliadin and albumin fractions. The HMW fraction similarly increased in both streams with dough mixing, whereas overground stream X caused more of an increase in the proportion of the HMW fraction than overground stream A.

Aggregation of Protein during Freezing Storage

As shown in Figure 2, the rate of change in turbidity of flour protein solutions stored at −20° C increased with more passes through the rolls and with dough mixing. The rates of turbidity increase for stream A were greater than those for stream X. The results also suggest that effects of overgrinding on the aggregation of protein were similar to those of dough mixing.

Hashizume and co-workers (1969, 1971, 1974), who studied the changes in the properties of soybean protein produced by freezing, found that soybean protein dissolved in solution was rendered insoluble by freezing and thawing, and that the insolubilization of protein was promoted by the presence of free SH groups in the protein, by heat denaturation, and by the addition of small amounts of urea before freezing. They concluded that denaturation of soybean protein by freezing may be caused by intermolecular reactions through S-S bonds as a result of concentration by freezing. In the current study, we suggest that this cryoaggregation was the result of exposure of hydrophobic groups due to denaturation of flour protein by overgrinding and mixing, because formation of new S-S bonds in protein would not be caused in an excess of NEMI. This conclusion is partly based on the report of Doi (1984), that denaturation of ovalbumin is caused without intermolecular reactions through S-S bonds as a result of concentration by freezing.

Polymerization of Globulin

Sephacryl S-300 gel filtration profiles of the globulin fraction are shown in Figure 3. Although medium-molecular-weight (MMW) globulin was a small peak, the other two globulin fractions had large peaks appearing at the elution volumes of HMW and low-molecular-weight (LMW) proteins. As the HMW-globulins become insoluble for 0.5*M* NaCl during preparation of globulin, this protein has not been examined by many workers. Pence and Elder (1953) suggested that these insoluble proteins are mainly denatured albumin. To clarify the mechanism that causes the protein to become insoluble in 0.5*M* NaCl during the preparation, globulin was purified in the absence and presence of NEMI.

TABLE I
Proportions of High-Molecular-Weight Proteins During Dough Mixing[a]

Flour	No Mixing	Peak Time	Overmixing[b]
Stream A			
Untreated	1.00	1.77	1.56
Overground[c]	1.00	1.26	1.31
Stream X			
Untreated	1.00	1.89	2.18
Overground[d]	1.00	2.34	2.18

[a] Values are the average of duplicate determinations.
[b] Peak time plus 12 min.
[c] With five passes through the rolls.
[d] With 10 passes through the rolls.

Changes in the proportion of each globulin fraction during preparation were investigated by gel filtration on Sephacryl S-300. As shown in Table II, the proportion of HMW-globulin increased remarkably, whereas in the absence of NEMI that of MMW-globulin decreased compared to when NEMI was present. Globulins extracted from either untreated flour or overground flour showed the same behavior. It is concluded from the above results that this polymerization of HMW globulin is caused by S-S bonds.

TABLE II
Effect of *N*-ethylmaleimide (NEMI) on Preparation of Globulin[a]

Flour	High-Molecular-Weight Globulin (%)	Medium-Molecular-Weight Globulin (%)	Low-Molecular-Weight Globulin (%)
Untreated[b]			
No additives	49.9	27.8	22.3
NEMI[c]	31.6	42.4	26.0
Overground[d]			
No additives	47.8	28.2	24.0
NEMI[c]	30.8	41.8	27.4

[a] Values are the average of duplicate determinations.
[b] Stream X.
[c] Adding 1.9×10^{-5} mol/g of flour.
[d] Stream X overground with 10 passes through the rolls.

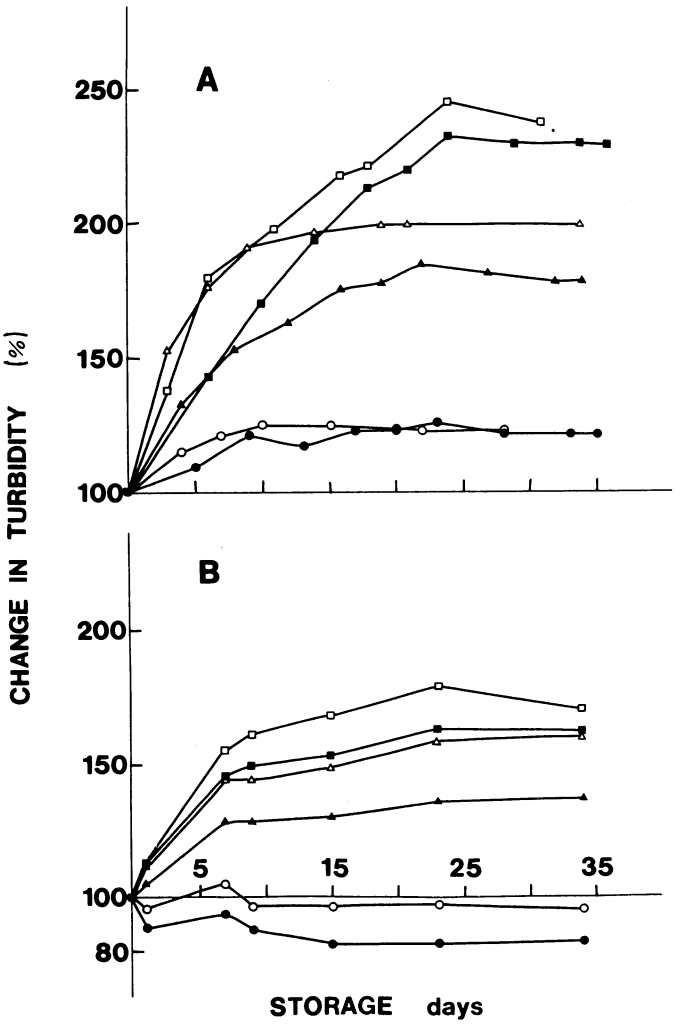

Fig. 2. Changes of turbidity percentage of original for protein extracts of flour or dough from both streams. **A,** stream A. No mixing: ●—● untreated, o—o five passes. Peak time: ▲—▲ untreated, △—△ five passes. Overmixing: ■—■ untreated, □—□ five passes. **B,** stream X. No mixing: ●—● untreated, o—o 10 passes. Peak time: ▲—▲ untreated, △—△ 10 passes. Overmixing: ■—■ untreated, □—□ 10 passes.

Alteration of the Globulin Fraction Proportion During Dough Mixing

Figure 4 shows the alteration in the proportions of globulin fractions in both streams during dough mixing on farinograph. At the peak time on the farinograph, the proportion of HMW globulin increased markedly and that of MMW globulin decreased considerably, whereas the level of the LMW fraction hardly changed. MMW globulin of overground stream X was more reactive than others.

Graveland et al (1979) reported that glutenins adsorb globulins. However this phenomenon was not observed in the presence of SDS, which occupies all hydrophobic regions of proteins. Because the solvent used in this study was SDS, the polymerization of MMW globulin into HMW globulin may be caused by intermolecular reactions through S-S bonds.

The proportions of the globulin fractions in streams A and X were 2.6 and 3.2% of the total protein, respectively. These are very small amounts; they are significant if it is assumed that globulins play an important role as binders between proteins during dough mixing. Our conclusions are similar to those of Redman and Elton (1968), that in wheat flour doughs the proteins will be present in very concentrated solution or colloidal suspension, and it is possible that intermolecular disulfide crosslinking between proteins such as purothionin and the major dough proteins may occur, creating an optimum structure.

The densitometer scan of SDS-PAGE for MMW globulin extracted from untreated stream X is given in Figure 5. With increased mixing, the subunits of R_f 0.34, 0.41, and 0.44 increased, those of R_f 0.46, 0.48, and 0.52 decreased, peak 0.56 was

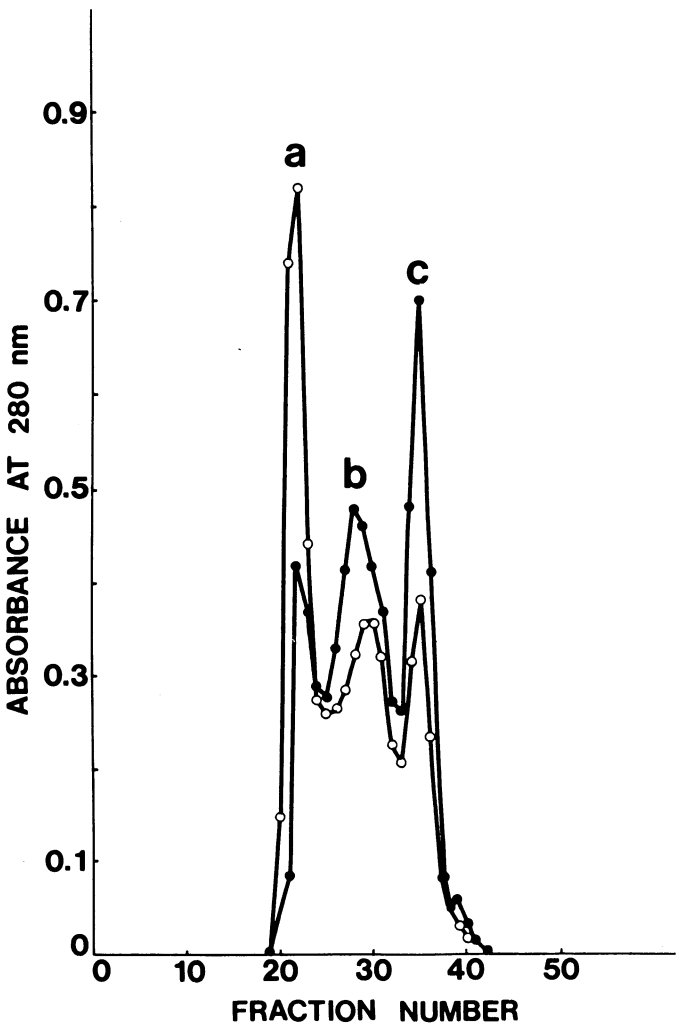

Fig. 3. Sephacryl S-300 gel filtration profiles of globulin fractions: o—o insoluble in 0.5*M* NaCl, ●—● soluble in 0.5*M* NaCl; **a,** HMW globulin; **b,** MMW globulin; **c,** LMW globulin.

unchanged, and peak 0.59 disappeared at the peak time on the farinograph. After over mixing, peaks at R_f 0.34 and 0.44 decreased, and those at R_f 0.50, 0.56, and 0.63 increased as compared with the profile for the peak time. The SDS-PAGE patterns for stream A were generally similar to those for stream X (data not shown). Thus SDS-PAGE as well as gel filtration indicated changes in the globulin fraction with dough mixing.

Comparison of Subunits of Glutenin and HMW Globulin

Both glutenin and HMW globulin elute at the void volume on gel filtration using Sephacryl S-300, indicating their molecular weights to be more than a few hundred thousand. Figure 6 shows the densitometer scan of SDS-PAGE of reduced glutenin and reduced HMW globulin extracted from stream X. Glutenin had more subunits than HMW globulin; they shared some subunits of similar mobility but the latter lacked subunits of R_f 0.30, 0.46, 0.51, 0.59, and 0.81. The results of SDS-PAGE suggested that glutenin and HMW globulin are distinct proteins.

Comparison of Subunits of Albumin and LMW Globulin

Albumin was eluted at the same position as LMW globulin on gel filtration on Sephacryl S-300 (Fig. 3). A closer comparison was made between albumin and LMW globulin after reduction and alkylation by SDS-PAGE. As shown in Figure 7, albumin lacked the subunits of R_f 0.81 (of about 19,000 mol wt) and R_f 0.86 (about 16,000 mol wt), thus distinguishing it from LMW globulin. The SDS-PAGE patterns of LMW globulin after reduction were similar to those for unreduced LMW globulin. These results provide formation relevant to the observation (Fig. 4) that the proportion of LMW globulin changed little as a result of dough mixing.

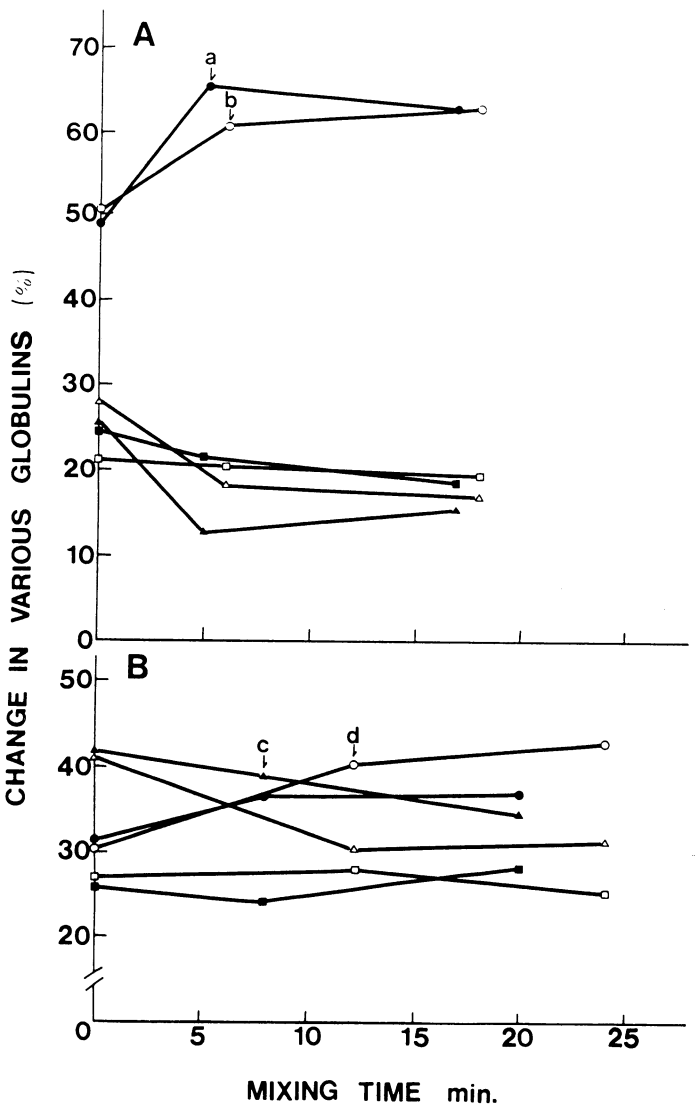

Fig. 4. Alterations in the proportion of the globulin fraction in both streams; **a, b, c,** and **d** are peak times. **A,** stream A. Untreated: ●—● high-molecular-weight (HMW) globulin, ▲—▲ medium-molecular-weight (MMW) globulin, ■—■ low-molecular-weight (LMW) globulin. Five passes: o—o HMW globulin, △—△ MMW globulin, □—□ LMW globulin; **B,** stream X. Untreated: ●—● HMW globulin, ▲—▲ MMW globulin, ■—■ LMW globulin. Ten passes: o—o HMW globulin, △—△ MMW globulin, □—□ LMW globulin.

Reduction and Reoxidation of MMW Globulin

As already described, MMW globulin polymerized into HMW globulin during dough mixing. To better understand this phenomenon, the reduced and reoxidized MMW globulins of stream X were analyzed by SDS-PAGE. On reduction (Fig. 8), subunits of R_f 0.36, 0.46, and 0.59 disappeared, and those with R_f 0.32, 0.42, 0.65, 0.69, 0.81, and 0.90 appeared. The SDS-PAGE

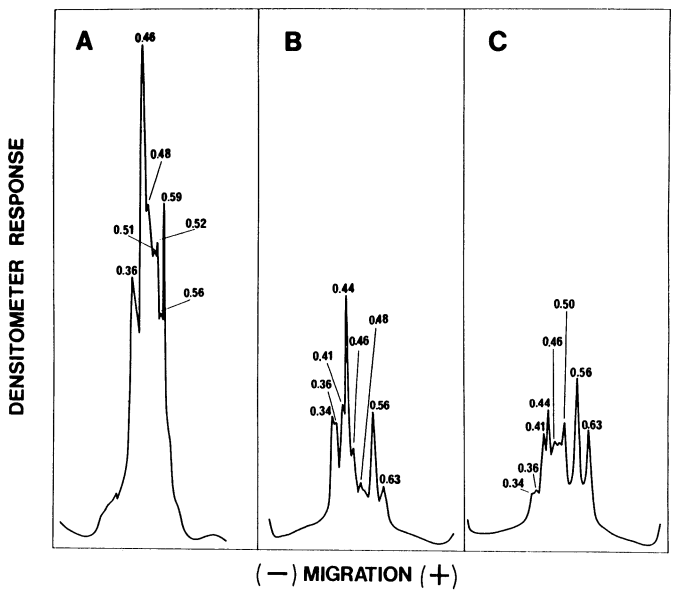

Fig. 5. Sodium dodecyl sulfate-polyacrylamide gel electrophoretic scan patterns for medium-molecular-weight globulin extracted from untreated stream X: **A,** no mixing; **B,** peak time; and **C,** overmixing.

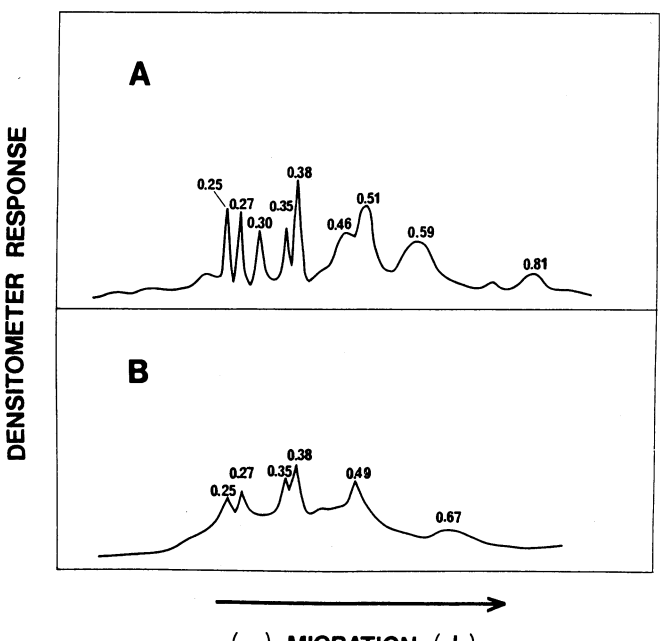

Fig. 6. Sodium dodecyl sulfate-polyacrylamide gel electrophoretic scan patterns for reduced glutenin (**A**) and reduced high-molecular-weight globulin (**B**) from untreated stream X.

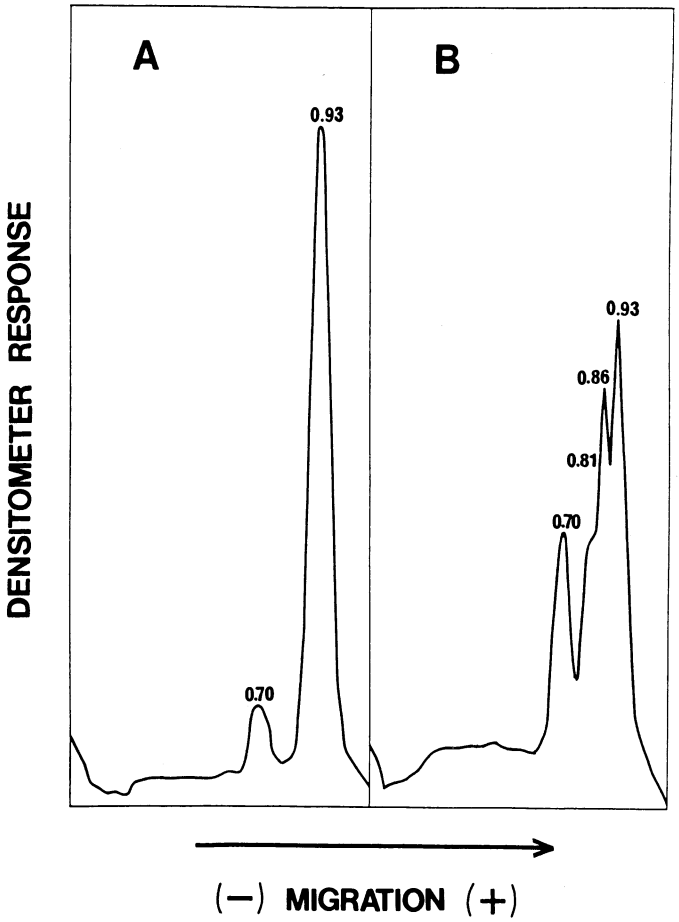

Fig. 7. Sodium dodecyl sulfate-polyacrylamide gel electrophoretic scan patterns for reduced albumin and reduced low-molecular-weight globulin from untreated stream X: **A,** reduced albumin; **B,** reduced low-molecular-weight globulin.

profile of reoxidized MMW globulin was similar to that of the unreduced MMW globulin except in the HMW and LMW regions. Terada et al (1978) reported that incubation of wheat-flour globulin with a mixture of alkali carbonates resulted in a decrease in SH groups of the globulin. In addition, the component of molecular weight about 77,000 disappeared and a new component, whose molecular weight was more than one million, increased significantly. In this study, it was also clear that the MMW globulin fraction consisted of the proteins containing reactive inter-S-S bonds.

CONCLUSION

The effect of overgrinding and dough mixing of flour on the structure changes of proteins was not clearly shown in the studies with gel filtration. However, changes in protein conformation due to overgrinding and mixing were observed on the basis of cryoaggregation. This aggregation of protein after low-temparature storage was probably the result of hydrophobic interactions of flour protein, promoted by overgrinding and mixing.

Wheat-flour globulins were studied after fractionation into three fractions. The MMW globulin was especially reactive and polymerized into HMW globulin by forming S-S bonds during dough mixing, that of overground stream X (the outer part of the endosperm) being more reactive than of other streams. Our previous paper (Okada et al 1986) reported that the baking properties of reground stream X were improved compared to stream X before regrinding. The polymerization of reactive globulin described above may effect improvement in the breadmaking properties of overground stream X.

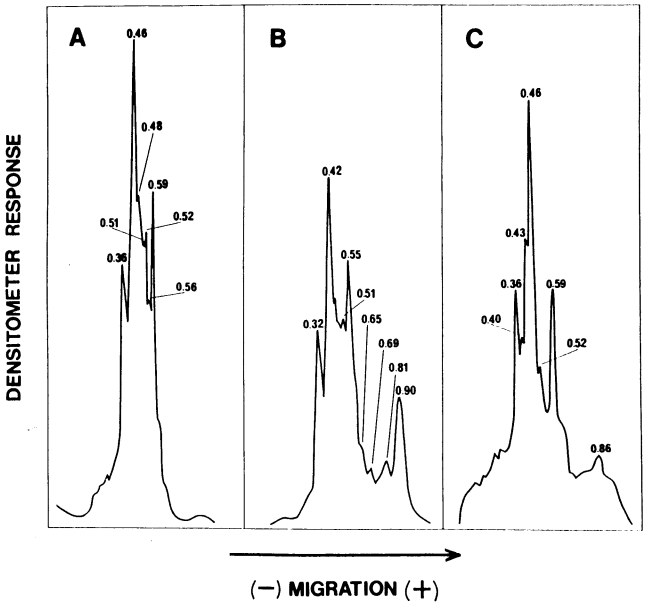

Fig. 8. Sodium dodecyl sulfate-polyacrylamide gel electrophoretic scan patterns for reduced and reoxidized medium-molecular-weight (MMW) globulin from untreated stream X: **A,** untreated MMW globulin; **B,** reduced MMW globulin; and **C,** reoxidized MMW globulin.

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