Disaggregation of Glutenin with Low Concentrations of Reducing Agent and with Sonication: Solubility, Electrophoretic, and Scanning Electron Microscopy Studies[1]

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ABSTRACT

Doughs obtained from a hard red spring wheat flour (Len) were mixed in a farinograph, with and without the reducing agent dithiothreitol (DTT), and freeze-dried. The proteins from the dough were extracted. The proteins from Len flour were also extracted by using sonication. Solubility of the proteins increased markedly from about 82% in the control to about 95–100% for both the sonicated and the DTT-treated samples. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the protein extracts, with and without reducing agent, of both farinograph-mixed and sonicated procedures showed an increase in the amount of glutenin aggregates with lower molecular weight that remained at the 12% resolving gel origin and a concomitant decrease of the high molecular weight glutenin aggregates at the 4% stacking gel origin, as DTT concentration or sonication time increased. The portion of the gel showing heavy streaking, where the high molecular weight subunits usually appear, was cut out and eluted with sample solvent containing reducing agent. SDS-PAGE of the eluted samples under reducing conditions revealed that both the DTT-treated and sonicated samples contained high molecular weight subunits of glutenin resulting from the proteins that caused the streaking. Scanning electron micrographs of the DTT-treated doughs revealed major alterations in the protein matrix network as DTT concentration was increased, even though SDS-PAGE of unreduced extracts did not reveal high molecular weight glutenin subunits. These results indicate that the larger glutenin protein aggregates can be dissociated into smaller aggregates without release of monomeric high molecular weight glutenin subunits, thereby affecting rheological and breadmaking properties.

It is well established that the glutenin protein fraction plays an important role in determining differences in breadmaking quality among wheat cultivars (for review articles see Wrigley and Bietz 1988 and MacRitchie et al 1990 and references cited within). The work of Payne et al (1979; 1981a,b) on the relationship of high molecular weight (HMW) subunits of glutenin and breadmaking-quality differences resulted in a great deal of research on this subject. However, research on the native glutenin molecule is more difficult because of its large size and the difficulty of its solubility in aqueous solvents.

The purpose of the present research was to use published procedures to isolate native glutenin and to provide information on its physicochemical properties that would be useful for understanding its structure-function relationships.

MATERIALS AND METHODS

Flour Treatments and Extraction

The hard red spring wheat sample used in this study was Len. The protein content was 14% mb (as is). Flour (50 g) containing low amounts of dithiothreitol (20, 40, and 80 μmol of DTT) was mixed into dough in a farinograph according to the procedure of Ng et al (1991). N-ethyl-maleimide (NEMI), a sulfhydryl blocking agent, was added to flours containing DTT (100 μmol/50 g) to prevent oxidation of sulfhydryl groups to disulfides. Flour in aqueous solution was sonicated. Proteins were isolated according to the procedure of Singh et al (1990a,b).

Protein Determination

Standard procedures were used to determine the amount of protein in the various samples (AACC 1983). Protein was determined on 10-ml liquid extracts of the samples listed in Table

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TABLE I
Effect of Sonication and Low Concentrations of the Reducing Agent Dithiothreitol (DTT) on Protein Extractability and Solubility (%) of Flour and Dough from the Spring Wheat Cultivar Len

		Magnetic Stirring, hr[b]				Sonication, sec[c]		
	Stirring[a]	0.25	0.5	1	2	15	30	60
Flour	65 (±0.5)	...	75 (±0)	77.3 (±1.2)	81.6 (±1.2)	90 (±0)	96.3 (±1.4)	99.7 (±0.5)
Dough (control)	63 (±0)	73 (±0.5)	...	83 (±0.7)	93 (±2.0)	...	...	...
Dough with additives								
N-ethyl-maleimide (NEMI)	69 (±0)	95 (±1.0)	...	97 (±0.5)	99 (±0.8)	...	...	...
NEMI plus 20 μmol of DTT	77.5 (±0.25)	98 (±1.0)	...	100 (±0)	100 (±0)	...	...	...
NEMI plus 40 μmol of DTT	82.5 (±0.5)	96 (±0.7)	...	100 (±0)	99.7 (±0.4)	...	...	...
NEMI plus 80 μmol of DTT	87 (±1.0)	100 (±0)	...	100 (±0)	100 (±0)	...	...	...

[a] Flour was dispersed in extracting solution with a few turns of a spatula.
[b] Stirred on a magnetic stirrer with a stirring bar.
[c] Sonicated according to Singh et al (1990b). Samples were dispersed with a spatula, sonicated, and centrifuged immediately after sonication.

I. Buffer (10 ml) used for extracting the proteins was used as a blank. After centrifugation, pellets were freeze-dried and ground in a mortar with pestle. A 1-g sample was used for protein determination. DTT-treated dough samples were freeze-dried, ground, and extracted (Table I). Protein of extracts and pellets were determined as above.

Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970), as modified by Khan et al (1989). Proteins were eluted from cut gel pieces by immersing the pieces in SDS sample buffer, either with the reducing agent β-mercaptoethanol or with no reducing agent. Extraction proceeded for 24–36 hr, with intermittent vortexing. Samples were then centrifuged, and the supernatant used for electrophoresis.

To obtain enough protein for electrophoresis, cut gel pieces from three patterns of the same sample were used for extraction. We kept the number of cut gel pieces consistent for each extracted gel whenever qualitative or quantitative comparisons were made.

Scanning Electron Microscopy

Immediately after being mixed in the farinograph, dough pieces were viewed under cryogenic conditions according to the procedure of Berglund et al (1990, 1991). Scanning electron microscopy (SEM) was used to view and photograph samples with a JEOL JSM 35 scanning electron microscope at an accelerating voltage of 15 kV.

RESULTS AND DISCUSSION

Protein Disaggregation and Solubility

Figure 1 shows the effect of small amounts of the reducing agent DTT on the farinograph curve: 20 μmol (Fig. 1C) shows some loss of dough integrity; 40 μmol (Fig. 1D) shows that dough integrity is completely lost. These results are identical to those of Ng et al (1991), confirming interlaboratory reproducibility. It would seem, therefore, that the proteins, especially the gluten proteins, have been extensively altered or disaggregated to result in such drastic rheological changes as that shown in Figure 1D.

Table I compares the solubility of the DTT-treated and sonicated samples. The DTT-treated samples were extracted by stirring with a magnetic bar for different time periods. After only 15 min of stirring (Table I), ~98% of the proteins were solubilized from DTT-treated samples, a result similar to that obtained after 1 or 2 hr of stirring. The control dough (no DTT and no NEMI) sample showed an increase in solubility from 73% (after 15 min of stirring) to 93% (after 2 hr of stirring). Figure 1D showed that dough integrity was completely lost at the 40-μmol DTT treatment, indicating a breakdown of the protein network to such an extent that solubility would be enhanced, as shown in Table I. Therefore, for DTT-treated samples, 0.5 hr should be sufficient time for almost complete extraction of proteins.

Singh et al (1990a) reported increased solubility of wheat proteins upon sonication. Table I shows the increase in solubility of proteins from flour as sonication time was increased. At sonication for 30 and 60 sec, ~95% of the proteins were solubilized. The control sample, which represented proteins solubilized by stirring with a spatula without sonication, showed only ~65% solubility. These results are very similar to those of Singh et al (1990a). It would seem, therefore, that upon sonication, the large moleclar weight (size) wheat proteins are disaggregated or depolymerized, resulting in greater solubility in aqueous solvents. Extractability of both sonicated and DTT-treated samples was very similar: 95–100% solubility of protein material.

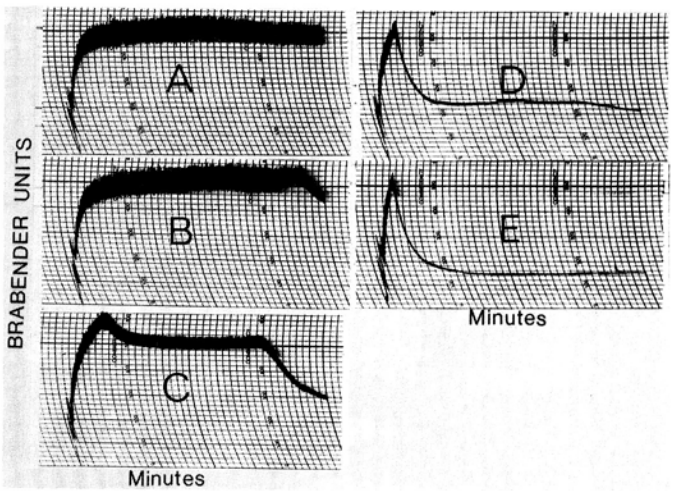

Fig. 1. Farinograms of flour-water doughs of a hard red spring wheat (Len). **A,** control (flour-water). **B,** control plus N-ethylmaleimide (NEMI). **C,** control plus NEMI plus 20 μmol of dithiothreitol (DTT). **D,** control plus NEMI plus 40 μmol of DTT. **E,** control plus NEMI plus 80 μmol of DTT.

Also note in Table I that the control flour sample showed an increase in solubility as stirring time increased from 30 min (75%) to 2 hr (81.6%). Dough samples showed greater protein solubility (93%) than did flour samples (81.6%) for the same extraction period (2 hr).

Characterization of Solubilized Proteins

To obtain information on protein disaggregation, we used SDS-PAGE to analyze the proteins extracted from sonicated and DTT-treated samples. The proteins were extracted with sample buffer without reducing agent and subjected to SDS-PAGE without reducing agent. The absence of reducing agent in the extraction and SDS-PAGE procedures ensured that any disulfide bond breakage or other types of disaggregation of proteins would be caused only by sonication or DTT treatments.

Figure 2 compares the SDS-PAGE patterns of unreduced sonicated and DTT-treated samples. In the sonicated samples, there was a decrease in the amount of protein that remained at the 4% stacking gel origin (Fig. 2, cut 1), and a concomitant increase at the 12% resolving gel origin as sonication time increased, from

no sonication (pattern a) to 120 sec of sonication (pattern e). In Figure 2, cut 3, the amount of streaking increased as sonication time increased, which was also as observed by Singh et al (1990a). No HMW subunits of glutenin were visible in unreduced samples (Fig. 2, patterns a–e), but they were clearly visible in the reduced samples (patterns k–m). Cuts 4 and 5 showed only very slight increases in band intensity as sonication time increased.

The DTT-treated samples (Fig. 2, patterns f–j) were similar to those of the sonicated samples: cut 1 showed a decrease in the amount of protein remaining at the 4% stacking gel origin, and cut 2 showed an increase as DTT concentration increased from 0 to 80 μmol. The DTT-treated and sonicated samples at cut 3 were also similar, in that there was an increase in faint bands and streaking as the amount of DTT increased from 0 to 80 μmol. Cut 4, however, showed a more marked increase in band intensity in the DTT-treated samples from 0 to 80 μmol of DTT than it did in the sonicated samples. Unlike the sonicated samples, the upper part of cut 5 also showed an increase in band intensity that was similar to that of cut 4. Cut 3 in the unreduced DTT samples was similar to the sonicated samples, in that no HMW subunits of glutenin were visible as those in the reduced glutenin patterns (patterns k–m).

These SDS-PAGE patterns of sonicated and DTT-treated samples indicated that the disaggregation or depolymerization of glutenin proteins were somewhat similar for cuts 1–3. Cuts 4 and 5, which contain gliadins, albumins, and globulins, as well as low molecular weight (LMW) subunits of glutenin, did not show an increase in band intensities as sonication time increased. On the other hand, the DTT-treated samples indicated that disaggregation involved some disulfide bond breakage because DTT is a reducing agent. Disulfide bond breakage was indicated by the increase in intensity of bands in cut 4 and the beginning of cut 5 as the amount of DTT increased from 0 to 80 μmol. The bands contributing to increased intensity were some of the LMW subunits of glutenin. These subunits were released, perhaps, as a result of reduction of the more rheologically accessible disulfide bonds of glutenin as proposed by Ng et al (1991) and Gao et al (1992).

Cut 3 of Figure 2 showed a number of faint bands in both the sonicated and the DTT-treated samples with molecular weights higher than 100,000, which is higher than the HMW subunits of glutenin. These faint bands could be smaller disulfide-bonded aggregates of glutenin, which themselves were cleaved from larger disulfide-bonded aggregates by sonication and DTT. Some of these bands may also be the triticin proteins identified by Singh and Shepherd (1985). Therefore, sonication may result in cleavage of rheologically accessible disulfide bonds, resulting in disaggregation or depolymerization of glutenin and increased solubility. Singh et al (1990a) suggested that increased protein solubility and streaking may be a result of disulfide bond cleavage.

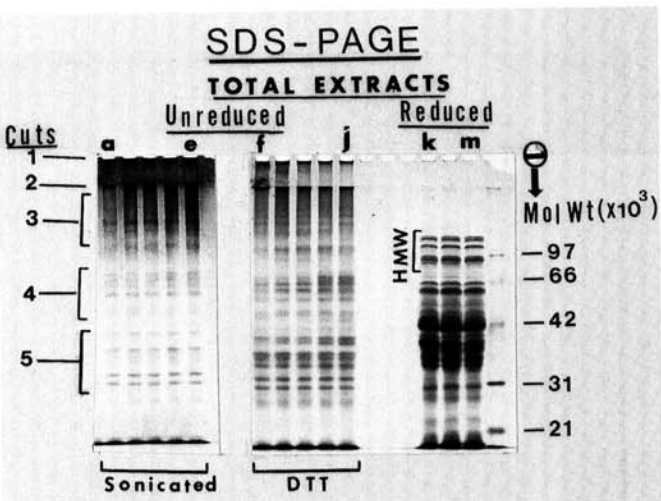

Fig. 2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of total protein extracts. Unreduced sonicated flour samples (patterns a–e): 0, 15, 30, 60, and 120 sec of sonication, respectively. Dough samples treated with the reducing agent dithiothreitol (DTT) (patterns f–j): control (flour-water), control plus N-ethylmaleimide (NEMI), control plus NEMI plus 20, 40, and 80 μmol of DTT, respectively. Reduced samples (patterns k–m): protein extracts from dough containing 20, 40, and 80 μmol of DTT, respectively. Cuts 1–5 refer to sections of gel from which proteins were eluted for subsequent analyses.

Elution of Unreduced Proteins and Characterization by SDS-PAGE

The cuts labeled 1–5 in Figure 2 (nonreduced glutenin extracts run under nonreducing conditions) were made for each sample and the protein from the gel pieces were eluted with sample buffer containing reducing agent β-mercaptoethanol. The same number of gel pieces for each sample were eluted with equal volumes of buffer, and equal volumes of extract were applied to the SDS gel for each sample for quantitative comparisons.

Figure 3 shows the SDS-PAGE patterns of the cut gel pieces of the sonicated samples run under reduced conditions. The horizontal labeling (cuts 1–5) of Figure 3 correspond to the vertical labeling of Figure 2 (cuts 1–5). In Figure 3, the patterns from left to right include: total reduced proteins from a flour sample included for comparison purposes; two lanes from gel pieces of dough (farinograph) and flour samples without sonication; and four lanes of proteins eluted from the 4% gel origin (15, 30, 60, and 120 sec of sonication). The patterns became fainter as soni-

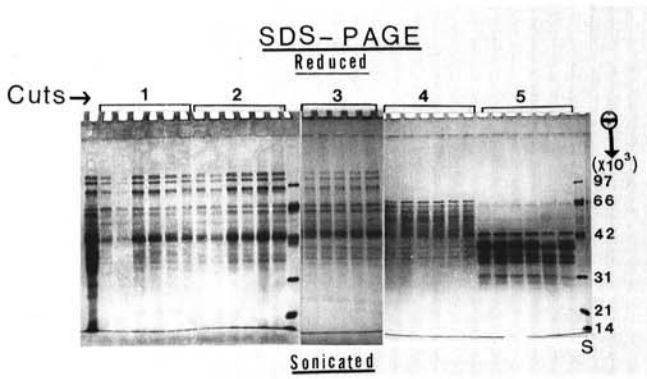

Fig. 3. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions of the samples eluted from cut gel pieces (cuts 1–5 at top correspond to cuts 1–5 in Fig. 2). **1,** Total flour proteins, dough extract, flour extract, and extracts sonicated at 15, 30, 60, and 120 sec. **2–5,** Dough extract, flour extract, and extracts sonicated at 15, 30, 60, and 120 sec. S = standard proteins.

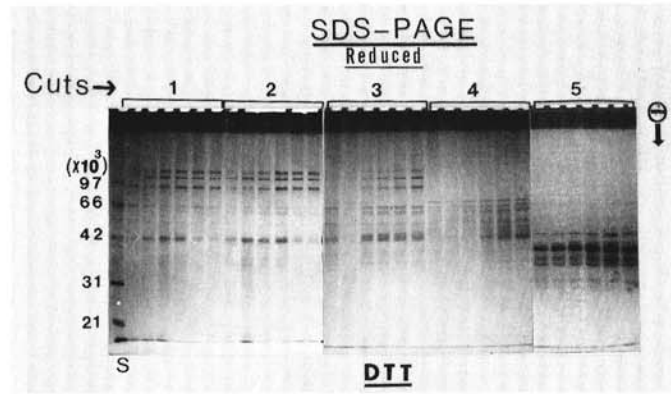

Fig. 4. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions of the samples eluted from cut gel pieces (cuts 1–5 at top correspond to cuts 1–5 in Fig. 2). **1–5,** Flour extract, dough extract, extract of dough containing N-ethylmaleimide (NEMI), and extracts of dough containing NEMI plus 20, 40, and 80 μmol of dithiothreitol (DTT).

cation time increased; the HMW aggregates of glutenin were depolymerized into smaller aggregates, which then entered the 4% stacking gel and migrated to the 12% resolving gel interphase (Fig. 3, cut 2). This region, eluted from the 12% gel origin, showed an increase in staining intensity of the bands as sonication time increased from 0 to 120 sec, confirming the depolymerization of larger glutenin aggregates into smaller ones. Cut 3 also showed an increase in staining intensity, but only of the HMW subunits of glutenin; the LMW subunits stayed relatively constant in staining intensity. This region had showed streaking in unreduced samples of total extracts (Fig. 2). The streaking material, therefore, seemed to be LMW unreduced glutenin aggregates that overloaded the 12% resolving gel origin, resulting in the streaking. Cuts 4 and 5 were relatively constant in staining intensity, indicating that LMW subunits of glutenin were not depolymerized by disulfide bond breakage as sonication time increased.

Figure 4 shows the SDS-PAGE patterns of the proteins eluted from the cut gel pieces of the DTT-treated samples. The trend in staining intensity for cuts 1 (4% origin), 2 (12% origin), and 3 (streaking material) were identical to those of the sonicated samples discussed above. As DTT concentration increased from 0 to 80 μmol, the HMW aggregates of glutenin were depolymerized by disulfide bond breakage into LMW aggregates, which were, however, still large enough not to enter a 12% resolving gel. Cut 4 and the beginning of cut 5, however, differed from the sonicated samples (shown in Fig. 3), in that staining intensity increased as DTT concentration increased. This meant that the reducing agent DTT, unlike sonication, broke disulfide bonds to release LMW subunits of glutenin.

The DTT-treated samples showed a decrease in staining intensity in cut 1 and an increase in staining intensity in cuts 2 and 3 because of disulfide bond breakage as DTT concentration increased. This same trend in staining intensity for cuts 1–3 were noticed for the sonicated samples as sonication time increased. Therefore, it can be concluded that sonication may have broken disulfide bonds. However, it would seem that sonication did not break disulfide bonds to the same extent that DTT did, because cuts 4 and 5 in the sonicated samples (where the LMW subunits of glutenin appear) did not show an increase in staining intensity. However, the DTT-treated samples did show an increase in staining intensity in this region, indicating release of LMW glutenin subunits.

Fractionation of Protein Samples by Multistacking SDS-PAGE

The multistacking SDS-PAGE procedure of Khan and Huckle (1992) was used to fractionate and compare unreduced sonicated and DTT-treated samples (Fig. 5).

Figure 5 shows patterns from total protein extracts. Both sonicated and DTT-treated samples were extracted with the SDS-phosphate buffer system of Singh et al (1990a,b) by gently stirring with a spatula. The multistacking gels ranged from 4% (largest pore size; retains HMW glutenin aggregates) to 12%. The 14% gel is the origin of the resolving gel (smallest pore size; retains the LMW glutenin aggregates). Pattern 1 represents the control (no sonication and no DTT). Patterns 3–8 compare the DTT-treated and sonicated samples side-by-side, from the lowest to the highest DTT concentration and sonication time. Patterns 9–11 compare all sonicated samples. Patterns 12–14 compare all DTT-treated samples.

As pointed out by Khan and Huckle (1992), all streaking in the 14% resolving gel was eliminated (Fig. 5). At the same time, the streaking materials (proteins) were retained at the various interphases of the stacking gels, depending on the size of the protein (glutenin) aggregates. Perhaps the amount of streaking in a sample (extract) may be an important indicator in determining rheological and breadmaking-quality differences among wheat cultivars. The glutenin fraction, therefore, consists of a mixture of aggregates of varying molecular sizes with, perhaps, other components such as carbohydrates and lipids associated with them.

Figure 5, patterns 3–8 also shows that sonicated samples contained more protein (darker stained bands) in their extracts than did DTT-treated samples when equal volumes of extracts were applied. Table I, however, showed that more vigorous stirring was required to extract equivalent amounts of protein from the DTT-treated (ground dough) samples as compared to that required for the sonicated samples. Sonicated samples required only gentle stirring with a few turns of a spatula. As shown in Figure 2 (unreduced sonicated and DTT-treated extracts), the region where the LMW glutenin subunits appear (arrows in Fig. 5) did not show an increase in band intensity in the sonicated samples (patterns 9–11), while the DTT-treated samples (patterns 12–14) did show an increase.

SEM Characterization of Doughs

SEM performed under cryogenic conditions was used to examine the ultrastructure of dough pieces taken from control and DTT-treated samples. The control dough (no NEMI and no DTT) showed normal development of gluten (Fig. 6A,B). Starch granules were tightly embedded in the gluten network, which was reticulate in nature. There were very few, if any, holes or air pockets present. When NEMI, the sulfhydryl blocking agent, was present in the dough (Fig. 6C,D), the gluten was less well developed. Pockets of air or trapped gases were evident. The gluten that lined the holes had apparently air-dried and formed a continuous sheet. There seemed to be less contact between starch and gluten. When treated with 20 μmol of DTT (Fig. 7A,B), the number and the size of the air pockets in the dough had both increased. The gluten was less well developed, and the starch granules appeared to be less firmly embedded and attached by only very thin strands. When treated with 40 μmol of DTT (Fig. 7C,D), a tremendous increase in the volume of air spaces occurred. The air spaces were lined with continuous sheets of gluten as a result of air drying. Essentially the only area that showed gluten development was at the margins of the numerous air pockets. The gluten in other portions of the sample was poorly developed. It should be noted that, at this level of DTT treatment, the dough lost its integrity completely, as shown in Figure 1D.

Low and high magnification micrographs (Fig. 7E,F) show dough treated with 80 μmol of DTT. At this concentration, there did not appear to be any normal development of gluten. The

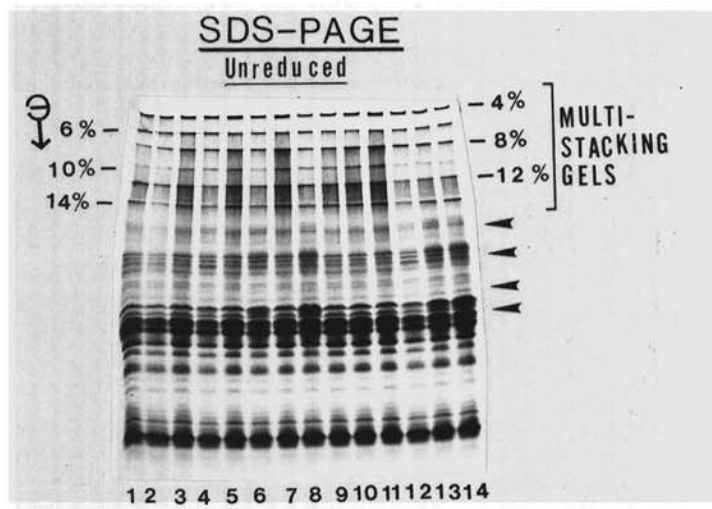

Fig. 5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under nonreducing conditions using multistacking gels of protein extracted without reducing agent. Flour extract (1), dough extract (2), extract of flour sonicated for 15 sec (3), extract of dough containing N-ethylmaleimide (NEMI) plus 20 μmol of dithiothreitol (DTT) (4), extract of flour sonicated for 30 sec (5), extract of dough containing NEMI plus 40 μmol of DTT (6), extract of flour sonicated for 60 sec (7), extract of dough containing NEMI plus 80 μmol of DTT (8), extract of flour sonicated for 15 sec (9), extract of flour sonicated for 30 sec (10), extract of flour sonicated for 60 sec (11), extract of dough containing NEMI plus 20 μmol of DTT (12), extract of dough containing NEMI plus 40 μmol of DTT (13), extract of dough containing NEMI plus 80 μmol of DTT (14). Arrows show region of gel where band intensity increased in DTT-treated samples.

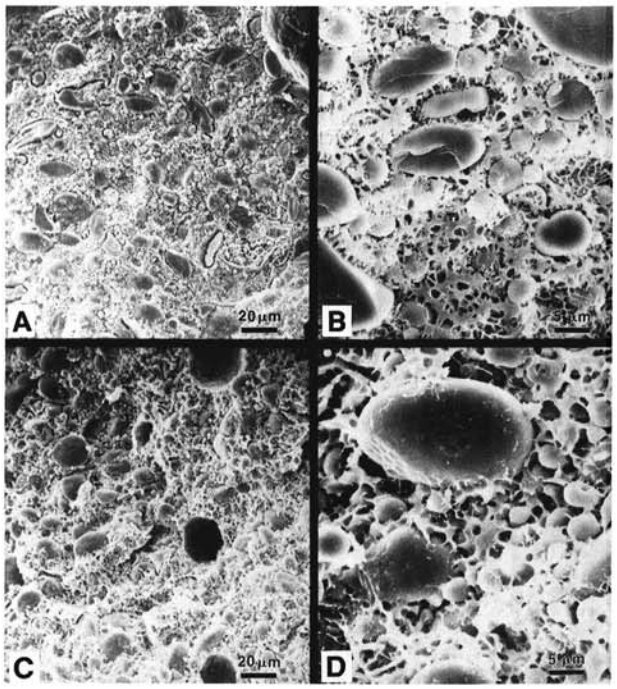

Fig. 6. Scanning electron micrographs of dough at low (**A,C**) and high (**B,D**) magnifications; control (**A,B**), control plus N-ethylmaleimide (**C,D**).

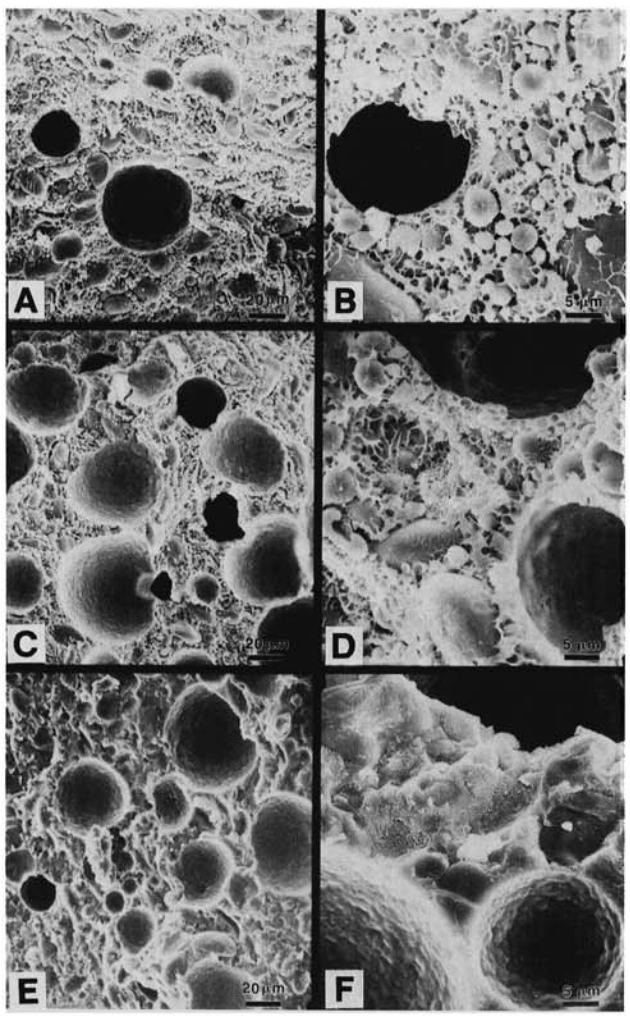

Fig. 7. Scanning electron micrographs of dough at low (**A,C,E**) and high (**B,D,F**) magnifications containing N-ethylmaleimide plus 20 μmol of dithiothreitol (DTT) (**A,B**), 40 μmol of DTT (**C,D**), and 80 μmol of DTT (**E,F**).

starch granules appeared to be embedded in a very dense, continuous matrix interspersed with large gas spaces. The sample appeared to be extremely dry. As shown in Figure 1E, the dough completely lost its integrity when treated with 80 μmol of DTT.

CONCLUSIONS

The SDS-PAGE and SEM data of this study clearly showed that sonication and low concentrations of DTT altered the three-dimensional structure of the glutenin proteins. Large glutenin aggregates were depolymerized by disulfide bond breakage and breakage of other types of secondary forces into smaller aggregates (without release of monomeric HMW glutenin subunits), enhancing solubility. The multistacking SDS-PAGE procedure of Khan and Huckle (1992) showed that glutenin is a mixture of aggregates of varying molecular sizes. The SEM data from control and DTT-treated dough samples dramatically illustrated the importance of protein-protein or protein-starch interactions for maintaining gluten networking and dough integrity. It is clear that interactive properties of gluten proteins with other dough components are important for an understanding of dough properties and quality differences.

ACKNOWLEDGMENT

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