Use of Sonication and Size-Exclusion High-Performance Liquid Chromatography in the Study of Wheat Flour Proteins. I. Dissolution of Total Proteins in the Absence of Reducing Agents[1]

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ABSTRACT

Total proteins from a very strong wheat flour, Mexico 8156, were almost completely extracted without chemical reduction of disulfide bonds by applying mechanical shear with an ultrasonic probe in 2% sodium dodecyl sulfate solution at pH 6.9. Proteins from a very weak flour, Israel M68, were even easier to solubilize using this procedure. The increased solubility of flour proteins by sonication, compared with simple stirring, was similar to that achieved by mixing flour dough in a mixograph. However, sonication is more efficient, and hence it required much less time (30 sec) to achieve complete extraction of proteins. Furthermore, a very small quantity of flour sample, equivalent to half an endosperm (11 mg), is required for the study of wheat seed proteins using sonication in combination with size-exclusion high-performance liquid chromatography. By this method, the total unreduced flour proteins were fractionated into three distinct peaks of decreasing size range, representing mainly glutenin, gliadin, and albumin-globulin, respectively.

Baking studies employing classical flour reconstitution techniques have demonstrated directly that gluten proteins are the prime factors governing wheat quality (Finney 1943, MacRitchie 1978). Traditionally, gluten proteins have been divided into two main classes based on their solubility in 70% ethanol. The alcohol-soluble class is gliadin and the residue is considered to be glutenin, a part of which can be solubilized in 0.1*M* acetic acid (Osborne 1907). However, it has long been realized that distinction between solubility classes is not sharp and that there is much overlap of components. As a consequence, there has been a tendency to define these classes on the basis of molecular size. Thus, proteins larger than 100 kDa were considered to be mainly glutenin, those between 100 and 25 kDa mainly gliadin, and proteins smaller than 25 kDa were classed as albumin and globulin (Meredith and Wren 1966, Bushuk and Wrigley 1971). This definition minimized the overlap of components but did not eliminate it completely. Bietz and Wall

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(1972) showed that some albumins and/or globulins are considerably larger than 25 kDa. Furthermore, dimers of some low molecular weight (LMW) glutenin subunits (30–50 kDa) have molecular weights similar to those of ω-gliadins (69-78 kDa; Bietz and Wall 1972, 1975; Graveland et al 1984), and minor triplet band proteins (globulin) form oligomers of 150–160 kDa and larger size (Singh and Shepherd 1985). Recently all gluten proteins, apart from minor globulin components, have been classified as prolamins (Shewry et al 1986), and their subdivision into gliadin and glutenin is based on functional properties: i.e., glutenins are of aggregating (more accurately polymeric) type and gliadins are nonaggregating or monomeric.

While confusion regarding classification is being reduced by recent molecular biological research (Kreis et al 1985, Shewry et al 1986), one of the greatest obstacles in studying the wheat seed storage proteins and relating their properties to dough characteristics is the difficulty of completely dissolving them without using conditions that chemically alter the proteins. Although it is possible to completely solubilize all the wheat flour proteins in dilute NaOH solutions (Dimler et al 1944), many covalent bonds, particularly disulfide bonds, are broken at high pH, and the protein structure is significantly altered (Cecil and McPhee 1959). Complete dissolution of flour proteins is also achieved by inclusion of reducing agents (e.g., 2-mercaptoethanol or dithiothreitol) in the solvent, but such reduced proteins are useful only for the study of individual polypeptides. Any information on how these polypeptides interact to form large glutenin polymers is lost.

To date there is no solvent system that will consistently solubilize total flour proteins (>95%) from wide-ranging wheat cultivars without reducing disulfide bonds. Danno et al (1974), Danno (1981), and Bottomley et al (1982) compared various solvents for the extraction of unreduced proteins. They studied dilute acetic acid, aluminium lactate buffer (pH 3.1), acetic acid/urea/cetyltrimethylammonium bromide (AUC) solvent of Meredith and Wren (1966), urea/cetyltrimethylammonium bromide buffered to pH 5.5 with sodium citrate (CUC), and sodium dodecyl sulfate (SDS); of these, SDS was the most efficient solvent. However, protein extractability with SDS is also quite variable, and usually proteins from strong wheat flours are less extractable than those from weak flours (Danno and Hoseney 1982, Moonen et al 1982, Bietz 1984, Dachkevitch and Autran 1989). Although higher pH of the SDS solution (pH 8.0 in Bottomley et al 1982) or vigorous mechanical mixing in a blender (Danno 1981) increased extractability to over 90% in a limited number of cultivars, no consistent system has been developed that would extract 95% or more flour proteins from diverse wheat cultivars.

In a separate development, Mecham et al (1962, 1965) discovered that extractability of flour proteins in dilute acetic acid was dramatically increased when flours were mixed into dough. To explain this phenomenon, they postulated that dough mixing decreases the size of protein aggregates. This theory was later supported by Tsen (1967, 1969) and Tanaka and Bushuk (1973b) who concluded that depolymerization of very large glutenin molecules by scission of disulfide bonds is the most probable mechanism for the reduction in size of protein molecules. Tsen (1967) clearly demonstrated using gel filtration chromatography that increase in extractability is almost entirely due to solubilization of previously insoluble glutenin, and smaller proteins (gliadin and albumin/globulin) are not affected.

MacRitchie (1975) developed a simple model for this phenomenon based on Bueche's (1960) theory of mechanical degradation of polymers. He also suggested that the depolymerization of large glutenin molecules ($MW > 5 \times 10^3$ kDa) via scission of disulfide bonds is the most likely reason for increased solubility of proteins, but more importantly, he argued that none of the shorter chains in the sample will be broken. All chains with molecular weights higher than a certain critical size "M_{max}" will be broken down into LMW material. The new LMW material formed by this breakdown will, in general, be within the molecular weight range M_{max} to $M_{max}/3$, due to the fact that chains break preferentially near their centers (Bueche 1960). Consequently, molecular weight distribution of the original high molecular weight (HMW) glutenin polymers will be narrowed by shear degradation, but LMW proteins (smaller glutenin polymers, gliadins, and albumins/globulins) will not be affected. This model is consistent with the gel filtration results of Tsen (1967, 1969), Tanaka and Bushuk (1973a), and Danno and Hoseney (1982).

The cited studies suggest that dough mixing could be a preliminary step for more efficient solubilization of unreduced proteins without affecting their size-based fractionation into polymeric glutenin, monomeric gliadin, and albumin/globulin. However, the disadvantage is that the mixograph requires a relatively large quantity of flour (minimum 10 g). Also, a long mixing time followed by freeze-drying and grinding of doughs is required for increased protein extractability. In the present study we tried to achieve similar shear degradation of large gluten polymers using ultrasonic probes in order to solubilize total proteins from small flour samples.

MATERIALS AND METHODS

Wheat Flour Samples

White flours milled on a laboratory Buhler mill, from cultivars Mexico 8156 (75% extraction, 11.7% protein) and Israel M68 (66.1% extraction, 10% protein), were defatted with chloroform according to MacRitchie and Gras (1973) and air-dried. Unless stated otherwise, air-dried defatted flours were used throughout this study because they were easier to suspend in the solvent buffer uniformly. Nondefatted Mexico 8156 and Israel M68 flours were also used to study protein extractability with and without sonication.

Dough Mixing

A 10-g mixograph was used for mixing Mexico 8156 flour into dough for 5, 10, and 20 min at a mixing speed of 90 rpm. The amount of water added was 60% (13% flour moisture basis). Doughs were frozen immediately after mixing and then freeze-dried and ground into fine powder in a coffee grinder.

Sonication

A Branson sonifier (model B-12 cell disrupter, Branson Sonic Power Company, Danbury, CT) was used with a 12-mm diameter macrotip probe for large-scale sonication (using 40 ml of extracting buffer in 50-ml polycarbonate centrifuge tubes), and a 3-mm diameter stepped microtip probe for small-scale sonication (using 1 ml extracting buffer in 1.5 ml Eppendorf tubes). The sonifier generated ultrasonic vibrations with a frequency of 20 kHz. The sonication power setting was varied between 1 and 10 (outputs 4–20 W) depending on the purpose of experiment (see Results and Discussion). The solid-to-solvent ratio for the large-scale sonication was 1:20, and it was varied between 1:10 and 1:90 for the small-scale sonication. Before sonication, flour samples were gently but uniformly dispersed in extracting buffer with a spatula to avoid loss of sonic energy in dispersing otherwise unevenly wetted flours. After sonication, the Eppendorf tubes were cooled in a waterbath to room temperature. Longer sonications (>30 sec) generated excessive heat and therefore were done in 30-sec bursts with intermittent cooling. A 15°C water bath was used for cooling the 50-ml tubes, and longer sonications were done in 1-min bursts. In any case, temperature produced during 30 sec (small tubes) and 1 min (large tubes) of sonications did not exceed 60°C. Two replicates were done for each sonication treatment.

Protein Extraction

Total unreduced proteins from normal flours, freeze-dried dough powders, and sonicated flour samples were extracted with 2% (w/v) SDS in 0.05*M* sodium phosphate buffer pH 6.9 (Bietz 1984, Huebner and Bietz 1985). Sonication was carried out directly in the protein-extracting buffer. All samples (with or without sonication) were rotated for 2 hr at 16 rpm, or for 30 min at 45 rpm, on a sample rotator and then centrifuged at 12,000 × *g* (Eppendorf tubes) or 48,000 × *g* (50-ml tubes) for 20 min.

The clear supernatants were used for high-performance liquid chromatography (HPLC) fractionation and protein determination using the bicinchoninic acid method of Smith et al (1985) with 2 hr incubation at 37°C. Flour residues from the large-scale extractions were analyzed for amount of unextracted nitrogen using Kjeldahl procedure.

Proteins from Mexico 8156 flour were also extracted sequentially with 0.05*M* NaCl (25°C) and 70% ethanol (4°C, to minimize glutenin extraction) solutions to obtain traditional Osborne (1907) albumin/globulin and gliadin fractions. Salt and ethanol extractions were done twice by vortexing the flour suspension for 30 sec and then centrifuging for 10 min at 2,000 $\times g$ to recover the supernatants (Bietz and Wall 1975). The residue (mainly glutenin) and total proteins were completely extracted with 2% SDS in sodium phosphate buffer (pH 6.9) by a 30-sec sonication at power setting 5 (output 10 W). All sequential extractions were done in Eppendorf tubes with 1 ml of solvents from 100 mg of flour. Before HPLC fractionation, the salt and ethanol extracts were also dissolved in the SDS-sodium phosphate buffer and centrifuged at 12,000 $\times g$ for 20 min.

Size-Exclusion HPLC

Size-exclusion (SE)-HPLC was carried out using a Waters HPLC system, comprising a model 510 pump, a model 481 variable wave length detector, and a model 712 WISP automated sample injector. A model 840 chromatography system was used for control of the pump and for acquisition and reprocessing of data from the detector. The Waters Protein-Pak 300 column, used in these experiments has a claimed separation range of 10–500 kDa for globular proteins and 2–150 kDa for random coils. Standards comprising β-galactosidase (116 kDa), ovalbumin (45 kDa), trypsin inhibitor (20.1 kDa), and cytochrome c (12.4 kDa) were run to assist in determining the molecular weight range of wheat proteins. A 0.05*M* sodium phosphate buffer (pH 6.9) containing 0.1% SDS was used as the eluent with a flow rate of 0.5 ml/min. Protein samples were applied in the same buffer but containing 2% SDS (Bietz 1984, Huebner and Bietz 1985). The elution buffer was filtered through 0.2 μm polyvinylidene difluoride membranes (Durapore, Millipore Corp., Bedford, MA) and degassed under vacuum before use. In addition, there was an in-line filter (0.2 μm) between the sample injector and the column. Absorbance was measured at 210 nm. Samples were prepared as described above and, where necessary (depending on the solid-to-solvent ratio during extraction), were diluted with extracting buffer to give a final concentration of 0.5–1 mg protein/ml. After being filtered (0.45-μm Millex HV filters, Millipore Corp.), 20 μl of each sample was applied to the column. In measuring the size of each peak, the lowest points on the troughs (valleys) were used as cutoff points between peaks. An 18-min fixed time cutoff point was used for the last peak, because this included all the proteins larger than 5 kDa. Areas of the peaks were calculated and expressed in arbitrary units based on peak heights measured in millivolts and peak width measured in centiminutes. Two replicates were done for all the measurements.

SDS-Polyacrylamide Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis (PAGE) of both unreduced and reduced proteins was done to check the distribution of protein components in different solubility and HPLC fractions. The procedure was similar to that described earlier (Singh and Shepherd 1985), except that before loading on gels the final sample buffer contained 3*M* urea, and the separating gel acrylamide concentration (indicated in Figure legends) was varied according to need. Both salt and ethanol extractions were done at 25°C (Bietz and Wall 1975), and before electrophoresis these were dissolved (without and with sonication) in SDS-PAGE sample buffer (65 m*M* tris-HCl pH 6.8 containing [w/v] 4% SDS, 15% glycerol, 0.001% bromophenol blue, and 3*M* urea.) Sodium phosphate buffer impaired the electrophoretic separation. Glutenin (residue following salt and ethanol extractions) and total proteins were extracted by 30 sec sonication directly in the SDS-PAGE sample buffer. The HPLC fractions corresponding to three main peaks, and a fourth fraction corresponding to material eluting after 18 min, were collected, dialyzed against deionized water for 24 hr, and freeze-dried. One milligram of freeze-dried fractions was dissolved in 0.1 ml of sample buffer for electrophoresis.

SDS-PAGE was also used to check the effect of long sonication on molecular weight distribution and extractability of wheat proteins. Mexico 8156 flour (100 mg) was sonicated for 0.5, 1, 2, 4, and 8 min at power setting 5 (output 10 W) in Eppendorf tubes with 1 ml of SDS-PAGE sample buffer, with and without 6*M* urea, and then homogenized for 1 hr at room temperature with occasional vortexing. Control samples were treated in a similar way but without sonication. After a 20-min centrifugation at 12,000 $\times$ *g*, supernatants were diluted with sample buffers without or with 6*M* urea, respectively, to get a final 3*M* urea concentration. Proteins from the residues were extracted with 1 ml of sample buffer containing 3*M* urea and 2% mercaptoethanol. Equal volumes of extracts were loaded on the gel.

TABLE I
Extractability of Unreduced Proteins from Defatted Mexico 8156 Flour in 2% Sodium Dodecyl Sulfate (SDS) Solution at pH 6.9 (solid-to-solvent ratio 1:20)

Treatment	% Protein Recovery[a]	% HPLC[b] Area			Absolute HPLC Area[c] $\times 10^{-5}$				Area/%PR[d]
		Peak 1	Peak 2	Peak 3	Peak 1	Peak 2	Peak 3	Total	
Stirring only[e]									
120 min	65.4	20.2	53.5	26.3	56	148	73	277	4.23
Mixograph (speed 90 rpm)									
5 min	78.2	37.5	40.6	21.9	118	128	69	315	4.03
10 min	84.6	38.0	40.3	21.7	128	136	73	337	3.98
20 min	85.3	38.3	39.8	22.0	127	132	73	332	3.89
Sonication									
Setting 10									
2 min	97.1	37.4	41.4	21.3	135	149	77	361	3.72
4 min	97.6	35.8	42.7	21.5	130	155	78	363	3.72
Setting 7									
2 min	94.6	37.9	41.2	21.0	130	142	72	344	3.64
4 min	95.6	36.4	41.7	21.9	129	148	78	355	3.71
Setting 3									
10 min	94.9	36.6	41.7	21.8	131	149	78	358	3.77

[a]Based on Kjeldahl N in the residues (standard errors (± SEM) were less than 1% of the means).
[b]High-performance liquid chromatography.
[c]Arbitrary unit for HPLC peak area was millivolts × centiminutes; values are presented as nearest integers (±SEMs were less than 2% of the means).
[d]PR = Protein recovery.
[e]Samples after mixograph and sonication treatments were also stirred for 120 min in an identical way.

RESULTS AND DISCUSSION

Dough Mixing and Large Scale Sonication

Proteins from Mexico 8156 flour, ground freeze-dried doughs, and sonicated flour samples were extracted at room temperature for 2 hr by gentle rotation (16 rpm) of tubes containing 2 g of flours in 40 ml of extracting buffer (2% SDS, pH 6.9). After centrifugation, residues were analyzed for protein extractability (Table I). Only 65.4% of the total flour protein was extracted with simple stirring by rotation, which increased to 85.3% after 20 min of dough mixing. The extractability increased by 12.8% after the first 5 min of dough mixing, and by 6.4% after the next 5 min; a further 10 min of mixing resulted in only minor increase. The most likely explanation for these results is that, initially, very large glutenin polymers required much less energy for their shear degradation, but as the size of polymers was reduced, much higher energy was required for a further degradation (Bueche 1960, MacRitchie 1975). These results also agree with those of Danno and Hoseney (1982) except that our maximum protein extractability was 85.3%, even after 20 min of dough mixing, compared with about 95% after only 15 min of dough mixing by Danno and Hoseney (1982) using four different flours. The difference may be because Mexico 8156 is

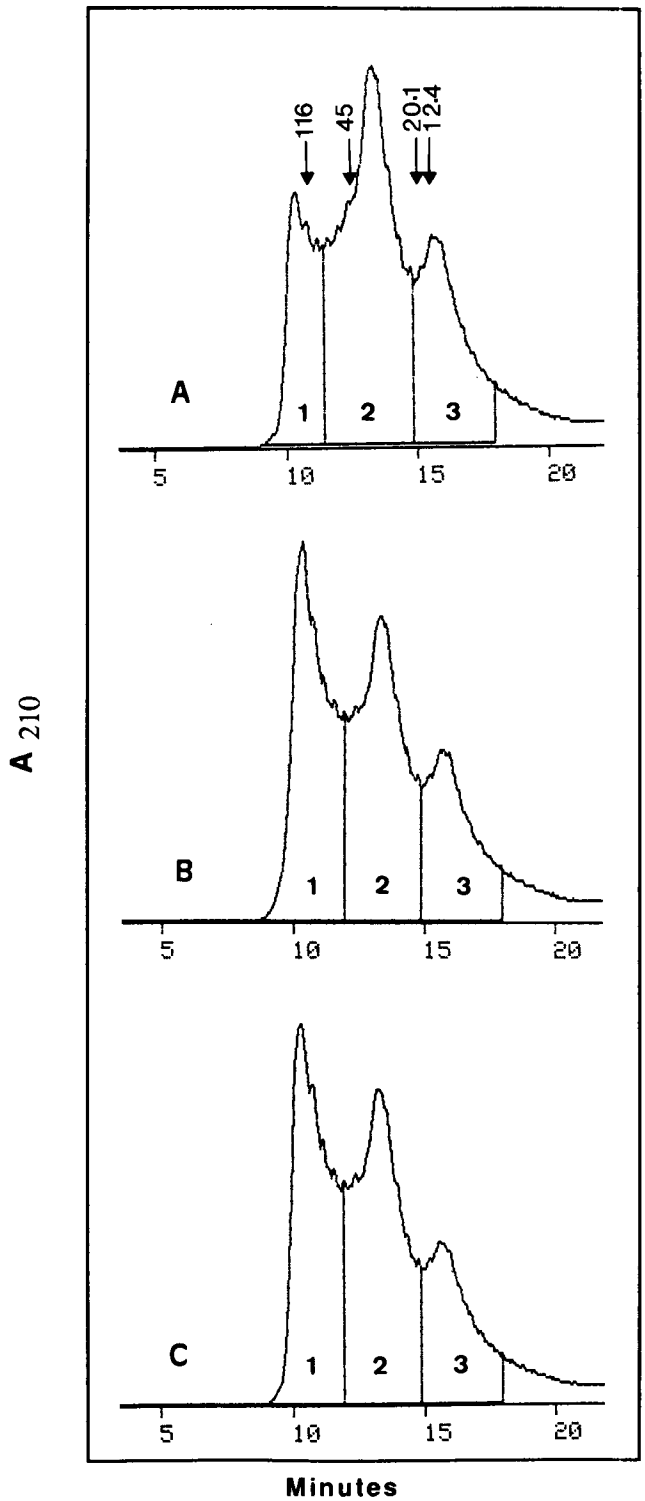

Fig. 1. Size-exclusion high-performance liquid chromatograms of unreduced proteins extracted with buffer containing sodium dodecyl sulfate from Mexico 8156 flour. Solid-to-solvent ratio 1:20 (2 g of flour or dough powder in 40 ml extracting buffer). **A,** Flour; **B,** freeze-dried dough mixed for 20 min; **C,** flour sonicated for 2 min with macrotip probe at power setting 7 (output 15 W). Chromatograms were divided into three peaks as indicated. A_{210} = Absorbance at 210 nm. Minutes = elution times. Arrows with molecular weights (12.4–116 kDa) indicate the elution times for standard proteins.

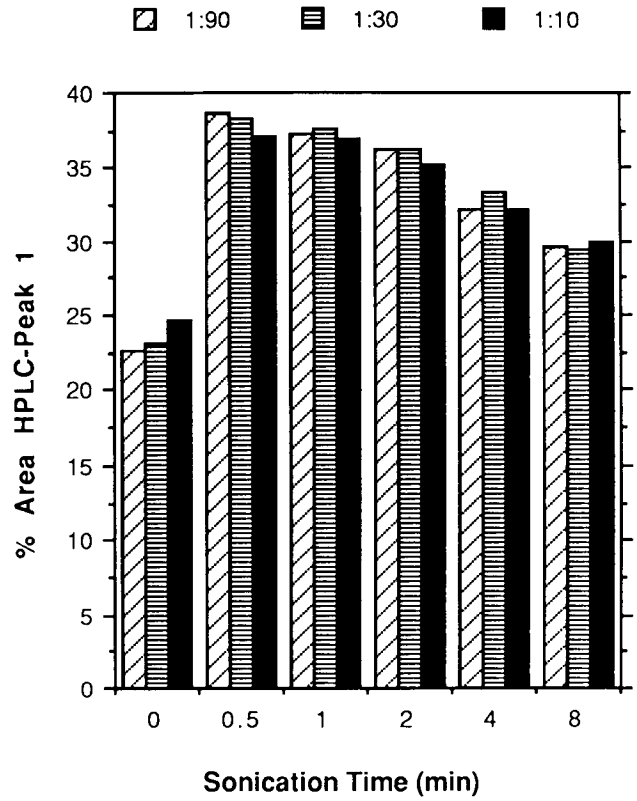

Fig. 2. Effect of solid-to-solvent ratio (1:10, 1:30, 1:90) and sonication time on percent area peak 1 of the size-exclusion high-performance liquid chromatograms of unreduced protein extracts from Mexico 8156 flour. Flours were sonicated in Eppendorf tubes with 1 ml of extracting buffer using a microtip probe at power setting 5 (output 10 W).

TABLE II
Protein Recoveries[a] from Nondefatted Mexico 8156 and Israel M68 Flours by Sonication at Power Setting 5 (output 10 W) for Different Times (solid-to-solvent ratio 1:90)

	Percent Protein Recovery (±SEM[b])	
Sonication Time	**Mexico 8156**	**Israel M68**
Stirring only[c]	64.5 ± 2.5	79.4 ± 0.8
5 sec	86.7 ± 3.3	91.7 ± 5.9
15 sec	95.5 ± 6.6	95.3 ± 0.3
30 sec	97.3 ± 2.8	92.8 ± 6.5
1 min	97.0 ± 3.0	96.0 ± 0.7
2 min	95.3 ± 0.7	102.5 ± 2.3
4 min	101.5 ± 1.3	102.8 ± 4.3
8 min	94.5 ± 2.1	108.1 ± 3.9

[a]Based on bicinchoninic acid protein determinations on supernatants.
[b]SEM = Standard error of the means.
[c]Sonicated samples were also stirred in an identical way.

an extremely strong flour, and hence prolonged mixing or higher mixing speed may be required for a better protein extraction (MacRitchie 1975).

In contrast to dough mixing, sonication dramatically increased both the speed and extent of protein extractability from Mexico 8156 flour (Table I). Protein extracts were fractionated by SE-HPLC into three main peaks of decreasing size-range, representing mainly glutenin, gliadin, and albumin/globulin, respectively (these fractions are characterized in detail later in this paper). Typical SE-HPLC patterns of proteins extracted from normal flour, dough powder, and sonicated flour samples are shown in Figure 1. These chromatograms show that effects of dough mixing and sonication on the extractability and size distribution of proteins were quite similar.

Percentage and absolute areas of the HPLC peaks are presented in Table I. A major increase due to dough mixing or sonication,

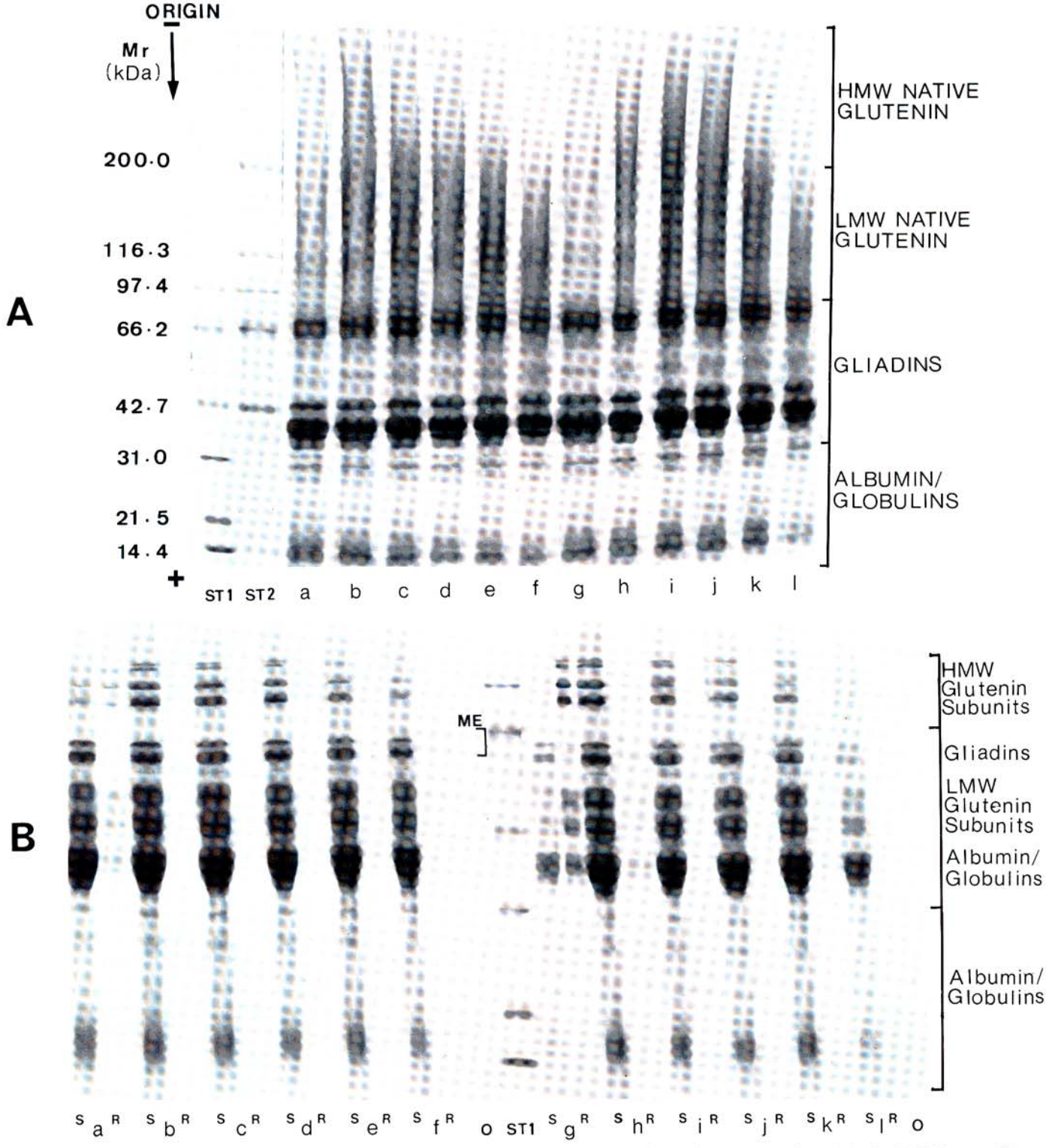

Fig. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoretic (SDS-PAGE) patterns of proteins extracted from Mexico 8156 flour, solid-to-solvent ratio 1:10. **A,** Unreduced proteins (4–20% gradient acrylamide gel, 1% cross-link). **B,** Reduced proteins (10% acrylamide gel, 2% cross-link). Proteins were extracted directly in SDS-PAGE sample buffer without (a–f) or with (g–l) 6*M* urea, in the absence of reducing agents, by sonication at power setting 5 (output 10 W) for: controls (**a** and **g**); 30 sec (**b** and **h**); 1 min (**c** and **i**); 2 min (**d** and **j**); 4 min (**e** and **k**) and 8 min (**f** and **l**). Reduced sample buffer without added protein (**o**). **ST1** and **ST2** are Bio-Rad molecular weight standards. **ME** indicates the position of faint mercaptan bands (Tasheva and Dessev 1983). **S** = supernatants; **R** = residues.

compared with stirring only, was in the proportion of peak 1 (glutenin), which confirms the earlier findings of Tsen (1969), Tanaka and Bushuk (1973a), and Danno and Hoseney (1982). A decrease in the proportions of peak 2 (gliadin) and peak 3 (albumin/globulin) is a consequence of the increase in peak 1. This is evident from the data for absolute peak areas representing total amounts of protein. Thus, there was a more than twofold increase in the amount of glutenin (peak 1), whereas the amounts of gliadin (peak 2) and albumin/globulin (peak 3) were affected only marginally (Table I). It appears that the amount of peak 2 and to some extent peak 3 material was relatively higher with sonication than with dough mixing. However, this could be due to oversonication (considered in detail below) resulting in some smaller glutenin polymers that overlapped with gliadins, due to the relatively lower resolving power of SE-HPLC compared with SDS-PAGE. Lower absolute areas for peak 2 of the dough powders, compared with control flour using stirring only (Table I), result because cutoff points (lowest points on the troughs) between the first and second peaks for the former were slightly higher, resulting in slight underestimation of peak 2 area and a corresponding overestimation of peak 1 area. Cutoff points for sonicated flours and dough powders were identical. Ratios of total absolute areas of HPLC peaks to percent protein recoveries were close to a constant (Table I) showing that HPLC can accurately quantify gluten proteins (Bietz 1986).

Small Scale Sonication

Effect of solid-to-solvent ratio and sonication time. After encouraging results with the large-scale sonication, we attempted to reproduce these effects using much smaller flour samples with 1 ml of extracting buffer in Eppendorf tubes. Three different solid-to-solvent ratios (1:10, 1:30, and 1:90) and five sonication times (0.5–8 min) were used. A sonication power setting of 5 (output 10 W) was used because results in Table I indicated that higher sonication power gives better protein extraction in a shorter time, and the power limit for the microtip was at setting 7. Effect of sonication power setting on glutenin extractability is examined further below. Both control and sonicated flour samples were homogenized at room temperature by gentle rotation of the tubes at 16 rpm for 2 hr. Supernatants were fractionated by SE-HPLC, and changes in the proportion of glutenin (peak 1) are shown in Figure 2. For a given sonication time, the solid-to-solvent ratio did not significantly affect the glutenin extraction. However, a minor trend was that without sonication proportionately more glutenin was extracted with lower solvent ratio (1:10), whereas with short sonication (30 sec) higher solvent ratios (1:30, 1:90) gave slightly better extraction. There was no clear difference with sonications longer than 30 sec. Subsequent studies were carried out with a 1:90 ratio, as this required just 11 mg of flour, ideal for a nondestructive, single-seed analysis of wheat seed proteins by SE-HPLC (Bietz 1984, 1986).

Figure 2 shows that there was a dramatic increase in the proportion of peak 1 after short 30-sec sonication. This proportion was maximum at 30 sec; subsequently, it slowly but steadily declined with increasing sonication time. The decline is thought to be due to oversonication resulting in formation of much smaller glutenin molecules, some of which were not clearly resolved from gliadins by SE-HPLC. This was also apparent from a progressive increase in retention time for peak 1, indicating smaller molecular size. Retention times for the maximum of peak 1 after 1-, 2-, 4-, and 8-min sonications were 10.48, 10.50, 10.68, and 10.93 min, respectively. As expected, the rate of increase in the proportion of peak 1 during the first 30-sec of sonication was much faster, when polymers to be solubilized are thought to be much larger ($>20 \times 10^3$ kDa; MacRitchie 1975, Graveland et al 1984) compared with a slower rate of decline due to oversonication. Altered HPLC profiles upon longer sonications (2 min or more) were not due to changed protein extractability, because this was more than 94.5% for each of the sonication treatments over 30 sec (Table II).

Additional evidence for oversonication causing excessive depolymerization came from SDS-PAGE of proteins extracted by sonication for 0–8 min (Fig. 3). Since equal volumes of extracts were loaded for each treatment, changes in the relative amounts of protein present in different regions of the gel could be compared. Figure 3A shows unreduced (native) proteins fractionated in a 4–20% gradient acrylamide gel of high porosity (1% cross-link), which allowed protein molecules of up to 500 kDa to enter the separating gel. SDS-PAGE of unreduced extracts separated total wheat endosperm proteins into a cathodal streak, representing predominantly glutenin (also including some albumin/globulin), and many faster moving discrete gliadin and albumin/globulin bands (Singh and Shepherd 1985), as labeled in Figure 3A. Protein fractionation is similar to SE-HPLC but resolution is clearly much greater with SDS-PAGE. A problem with the latter is that very large molecules unable to penetrate the pores of separating gel could not be analyzed. Nevertheless, lanes a and b in Figure 3A show that 30 sec of sonication caused a significant increase in the intensity of the cathodal streak, suggesting extraction of additional glutenins of large molecular size. Longer sonications did not show much effect until 4 and 8 min (Fig. 3A, lanes e and f) when the amount of streak near gel origin (HMW native glutenin) was significantly reduced, indicating a selective degradation of larger glutenin molecules. Sonication in SDS-buffers containing 6*M* urea gave similar results (Fig. 3A, g–l). Gliadin and albumin/globulin profiles were not significantly affected by these treatments (Fig. 3A), suggesting that in the presence of SDS most changes due to sonication were confined to large glutenin polymers.

SDS-PAGE of reduced protein extracts and corresponding residues (Fig. 3B) confirmed that unextracted proteins in the

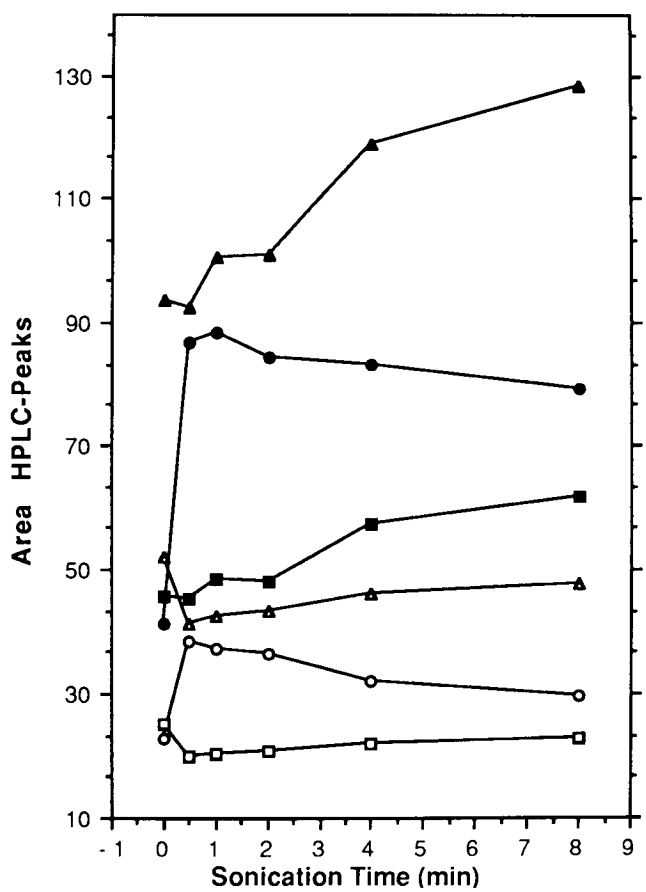

Fig. 4. Effect of oversonication on percent (open symbols) and absolute (closed symbols) areas under the three peaks (fractions) of size-exclusion high-performance liquid chromatograms of unreduced protein extracts from Mexico 8156 flour, solid-to-solvent ratio 1:90. Peak 1 (circles); peak 2 (triangles); peak 3 (squares). Flours were sonicated in Eppendorf tubes with 1 ml of extracting buffer at power setting 5 (output 10 W). Peak areas are expressed in arbitrary units based on peak heights measured in millivolts (absorbance at 210 nm) and peak width measured in centiminutes (elution time)

residues of control (nonsonicated) samples were predominantly glutenin (compare SDS-insoluble gel protein or glutenin I; Moonen et al 1982, Graveland et al 1984). There was no protein left in the residues after sonications of 30 sec or longer (Fig. 3B, b–f). Inclusion of 6*M* urea caused more protein to remain in the residue without sonication, and a longer 1-min sonication was required to solubilize all the proteins (Fig. 3B, g–l), further suggesting that in the presence of SDS the action of ultrasonic vibrations (mechanical shear degradation of polymers) differs from the action of urea (a denaturant), which is thought to break the hydrogen bonds. Thus, disruption of hydrogen bonds is not a likely explantion for increased protein solubility in SDS buffers after sonication.

An examination of absolute areas for three HPLC peaks (Fig. 4) also suggested that reduction in the proportion of peak 1 upon long sonication was not due to a decreased solubility, rather it was due to excessive depolymerization of glutenin, as discussed above. The absolute area of peak 1 increased very dramatically in the beginning (a more than two-fold increase after the first 30 sec of sonication) and then after a short period of minor increase (next 30 sec), it started to decrease. However, this decrease was accompanied with a corresponding increase in the areas under peaks 2 and 3. Prolonged sonication (4 and 8 min) resulted in a much higher increase in peaks 2 and 3 (Fig. 4), suggesting a pronounced tailing-off of smaller glutenin polymers into gliadin and albumin/globulin peaks. It is shown above by SDS-PAGE of oversonicated samples (Fig. 3A) that the extraction of gliadin and albumin/globulin was not significantly affected by sonication.

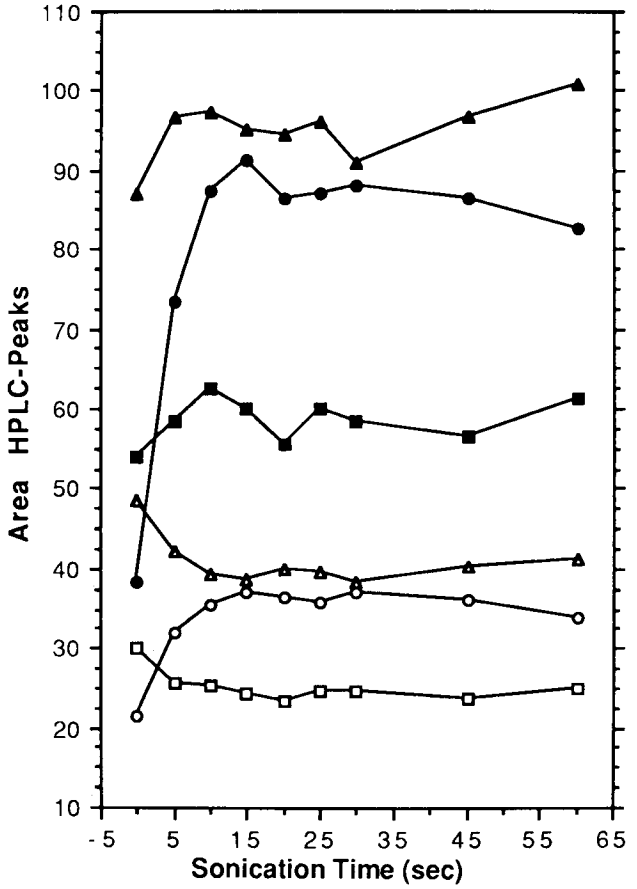

Fig. 5. Effect of short sonications on percent (open symbols) and absolute (closed symbols) areas under the three peaks (fractions) of size-exclusion high-performance liquid chromatograms of unreduced protein extracts from Mexico 8156 flour, solid-to-solvent ratio 1:90. Peak 1 (circles); peak 2 (triangles); peak 3 (squares). Flours were sonicated in Eppendorf tubes with 1 ml of extracting buffer at power setting 5 (output 10 W). Peak areas are expressed in arbitrary units based on peak heights measured in millivolts (absorbance at 210 nm) and peak width measured in centiminutes (elution time).

Effect of shorter sonication times and sonication power. Since increase in the proportion of peak 1 was very dramatic after just 30 sec of sonication, another time series experiment was carried out using much shorter time intervals (Fig. 5). As before, there was an increase in the proportion of peak 1 and a corresponding decrease in proportions of peaks 2 and 3 after sonication. Furthermore, absolute areas under all three peaks increased, although the increase in the amount of glutenin (peak 1) was much more dramatic (more than twofold) than the minor increases in gliadin and albumin/globulin peaks. The percentage of area peak 1 was maximum between 15 to 30 sec, followed by a slow but steady decline as noticed before (Fig. 2). It is interesting that the first 5 sec of sonication caused a huge increase in glutenin extractability. Subsequent 5-sec increments in the sonication time (up to 15 sec) caused smaller and smaller increases in glutenin extractability. This nonlinear increase in protein extractability is very similar to that observed with dough mixing (MacRitchie 1975, Danno and Hoseney 1982, Graveland et al 1984, and Table I of this paper), except for the time scale, suggesting that a similar mechanism (shear degradation) is involved.

Effect of sonication power on the extractabilty of glutenin was examined at sonication times of 15 and 30 sec (Fig. 6). At power settings 1 to 4, when energy outputs were lower (4–8 W), longer sonication time (30 sec) gave better glutenin extraction, but the gap progressively narrowed with increasing sonication power. At power settings 5 and 6 (10 and 12 W), proportions of glutenin for 15- and 30-sec sonications were very similar, and finally, at setting 7 (15 W), an oversonication in 30 sec was apparent from a clear drop in the proportion of peak 1 compared with that at 15-sec sonication. These results indicate that both the power setting on the sonifier and sonication time are critical for achieving consistent results. Here also, a parallel exists between the sonication power setting and mixing speed of the mixograph (MacRitchie 1975). In a fixed time, the higher sonication power or dough mixing speed extracted more protein. However, energy level with the mixograph was not high enough to cause excessive depolymerization, as seen with sonication (Fig. 3A, lanes f and l).

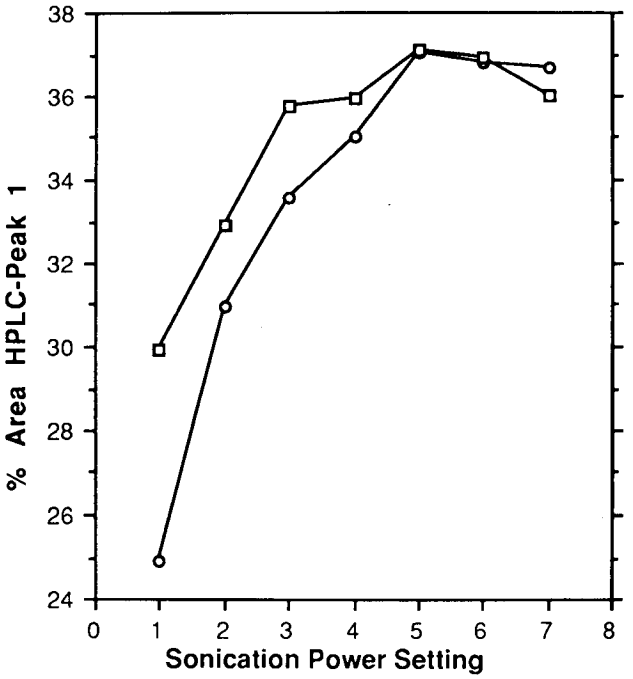

Fig. 6. Effect of sonicator power setting on the extractability of peak 1 proteins (mainly glutenin) as measured by change in percent area peak 1 of the size-exclusion high-performance liquid chromatograms of unreduced protein extracts from Mexico 8156 flour, solid-to-solvent ratio 1:90. Flours were sonicated in Eppendorf tubes with 1 ml of extracting buffer for 15 sec (circles) or 30 sec (squares).

Extractability of total proteins from strong versus weak flours. Mexico 8156 was chosen for this study because it was the strongest wheat flour available to us, and it is known from earlier investigations that proteins from strong flours are more difficult to extract than those from weak flours (Orth and Bushuk 1972, Danno and Hoseney 1982, Moonen et al 1982, Dachkevitch and Autran 1989). For comparison, an extremely weak flour from Israel M68 was also sonicated in a similar way. For results reported so far we used defatted flour, which was a fine powder and therefore easier to uniformly suspend in the extracting buffer. However, if sonication is to be used routinely to extract proteins for HPLC and electrophoresis, it would be desirable to eliminate the defatting step. Thus, in the present experiment normal nondefatted flours were used. Proteins from both control and sonicated flours were extracted by rotating the sample tubes at 45 rpm for 30 min at room temperature. Without sonication, only 64.5% of the total protein was extracted from Mexico 8156 flour, compared with 79.4% from Israel M68 (Table II). This is consistent with the results of Danno and Hoseney (1982), Moonen et al (1982), and Dachkevitch and Autran (1989). A 30-sec sonication gave almost complete extraction of proteins from Mexico 8156 flour; longer sonications (up to 8 min) did not alter extractability significantly (Table II), consistent with our earlier discussion based on SDS-PAGE and HPLC data (Figs. 3B and 4). Similar results were obtained with Israel M68, except that a 5-sec sonication was sufficient to extract 91.7% protein from this flour compared with 86.7% from Mexico 8156. Occasionally, protein extractability appeared to be greater than 100%, due to high standard errors of the means with bicinchoninic acid protein assay (Table II). However, the results indicate that a 30-sec sonication at power setting 5 would extract almost all the proteins from strong as well as weak flours (more genotypes are compared in the following paper).

Mechanism of Protein Solubilization by Sonication

It is the SDS-insoluble gel protein or glutenin I (Moonen et al 1982, Graveland et al 1984) that is made soluble by sonication. There is no doubt that this solubilization is brought about by a reduction in the size of glutenin I molecules, as biochemically they have the same component polypeptides as those present in SDS-soluble glutenin (glutenin II, Graveland et al 1984). While we do not have direct evidence to elucidate the exact mechanism, it must involve disrupting one or more of the major noncovalent (hydrophilic and hydrophobic interactions, hydrogen bonds) and covalent (disulfide bonds) forces that join different polypeptides together. Several researchers have used sonication to solubilize wheat proteins (Godon and Petit 1968, Huebner and Rothfus 1971, Jennings 1978), but no conclusive explanation has been given for the mechanism of protein solubilization. Godon and Petit (1968) studied the effect of ultrasonic vibrations of different frequencies (23–970 kHz) on gum gluten and their acetic acid suspensions and suggested that in suspension a depolymerization of glutenin fraction caused increased protein solubilization. Jennings (1978) showed that ultrasonic treatment of flours completely dispersed gel proteins formed by suspending flours in 1*M* acetic acid or solvent PAW (phenol/acetic acid/water, 1:1:1). He ruled out the possibility of sonication solubilizing gel proteins via breaking hydrogen bonds or hydrophobic interactions because these bonds were already broken in solvent PAW (Bagdasarian et al 1964, Jennings 1978). Based on the negative effect of salts on gel volume, Jennings (1978) suggested that sonication solubilized gel proteins by breaking ionic bonds. However, according to Mecham et al (1962) salt reduces gel volume by delaying the hydration of sediment proteins rather than by increasing their solubility. In the present study, involvement of ionic bonds was ruled out because all hydrophilic and hydrophobic bonds were broken by SDS before sonication. Due to its very heavy binding to proteins (average 1.4 g of SDS per gram of protein), SDS is suggested to disrupt most noncovalent bonds (Tanford 1968, Reynolds and Tanford 1970, Graveland et al 1984). Further, sonication (a physical process) is unlikely to affect chemical equilibria that are involved in noncovalent bonding. Thus, by elimination we conclude that sonication reduced size of glutenin I polymers by breaking covalent bonds.

MacRitchie (1975) estimated that energy levels in the mixograph are sufficient to break disulfide bonds. Energy levels with our ultrasonic probes were higher than in the mixograph. The activation energy for breaking bonds in the theory of mechanical degradation of polymers involves a term for the bond energy. Disulfide bonds will be broken at rates very much below those required to break peptide bonds. Although the energies for peptide bonds are only about double those of disulfide bonds, the probability of their breakdown will be considerably less because the rate expression involves an exponential term containing the bond energy. It is shown above that a remarkable similarity exists between the mixograph and sonication results, except for the higher speed and efficiency of protein extraction with sonication. A nonlinear increase in protein extractability and selective degradation of large glutenin polymers strongly suggests that a shear degradation of disulfide bonds, similar to that described by Bueche (1960) and MacRitchie (1975), is the most likely explanation for increased protein solubility with both dough mixing and sonication. Graveland et al (1980, 1984) suggested that reduction of disulfide bonds at specific positions in the glutenin I polymers takes place during dough mixing by the action of a redox system present in flour. However, according to MacRitchie (1975) disulfide bonds are broken due to shear forces, and the role of redox systems may lie in a subsequent chemical modification at the points of scission. More studies are needed before reaching a firm conclusion on the exact mechanism of disulfide bond scission.

Identity of SE-HPLC Peaks with Osborne Solubility Fractions

Size-exclusion chromatography separates proteins on the basis of molecular size. From comparison with molecular weight standards (Materials and Methods), it is estimated that molecular weight distributions for the three HPLC peaks are >100, 80–25, and 25-5 kDa. These correspond mainly to the known size ranges for glutenin, gliadin, and albumin/globulin, respectively (Meredith and Wren 1966, Bushuk and Wrigley 1971, Huebner and Wall 1976; see Kasarda et al 1976 for review), although there is some overlap of size between the classes (Bietz and Wall 1972). Our conclusions are further supported by the HPLC and SDS-PAGE data presented below.

We separated salt-soluble (albumin/globulin), ethanol-soluble (gliadin), and salt- and ethanol-insoluble (glutenin) fractions and total protein extracts from Mexico 8156 flour by SE-HPLC. The overlap in size distribution of these solubility fractions (some of which is likely to be due to incomplete extraction) is clearly evident from Figure 7. The majority (58.2%) of proteins in the salt-soluble fraction corresponded to peak 3 (Fig. 7A), but a significant proportion (27.3%) also eluted with peak 2; these are likely to be 61–68 kDa albumins and globulins (Bietz and Wall 1972, Cole et al 1981). The remaining 14.5% of proteins in this fraction eluted with peak 1 and therefore must be larger than 100 kDa. These could be native aggregating albumins (Gupta and Shepherd 1987). However, since salt-soluble proteins represent only 15–20% of the total endosperm proteins, peak 1 is only 2–3% of the total flour proteins. The majority (61.8%) of ethanol-soluble proteins eluted with peak 2 (classical α-, β-, γ-, and ω-gliadins); but a significant proportion (30.9%) also corresponded to peak 3 (Fig. 7B). Detailed analysis of this LMW fraction by others has revealed that it consists of hydrophobic lipid-binding albumins, or CM proteins (named for their solubility in chloroform/methanol) (Redman and Ewart 1973, Rodriguez-Loperena et al 1975). A minor proportion (7.25%) of ethanol-soluble proteins was present in the first peak; this HMW fraction is a LMW glutenin contaminant rather than true monomeric gliadin (Bietz and Wall 1980). The salt- and ethanol-insoluble glutenin fraction eluted mainly with peak 1 (Fig. 7C), but it also contained a substantial amount of smaller proteins that could be dimers of LMW glutenin subunits or monomeric gliadins (peak 2) and albumin/globulins (peak 3) not completely solubilized

during salt and ethanol extractions (Bietz and Wall 1972, 1975). SE-HPLC patterns of sonicated salt and ethanol extracts were similar to those of nonsonicated extracts (data not shown).

Overlap of components between solubility fractions is clearly shown by SDS-PAGE of unreduced and reduced proteins (Fig. 8A and B, lanes a–e). SDS-PAGE patterns of salt and ethanol extracts before and after sonication were almost identical (Fig. 8A and B, a and c vs. b and d), suggesting that sonication did not significantly affect these smaller molecules. Most components of these two fractions were monomeric, as inferred from lack of major change in their electrophoretic mobilities after reduction, except for small amounts of streaking near gel origin in the unreduced fractions. These streaks represent HMW albumin/globulin aggregates in salt extracts (Fig. 8A, a and b) and native LMW glutenin in ethanol extracts (Fig. 8A, c and d) separated into their subunits after reduction (Fig. 8B, a–d). The slow and fast moving groups of gliadin bands (labeled "GD" in Figs. 8A and B, c and d) are ω- and α-, β-, and γ-gliadins, respectively (compare Singh and Shepherd 1985). HMW subunits of reduced albumins (labeled "AL" in Figure 8B, lane e) had similar mobility to ω-gliadins but lack of the latter in the glutenin fraction could be established by comparing unreduced fractions (Fig. 8A, c–e). Figure 8 also compares these solubility fractions with preparative HPLC fractions corresponding to three main peaks and material eluting after the 18-min cutoff for peak 3 (tracks g, h, i, and j, respectively). Clearly, HPLC fractions have less overlap of components than solubility fractions, especially HPLC peak 1, which lacked all the LMW albumins prevalent in the glutenin of solubility fractionation (Fig. 8A and B, g vs. e), and peak 3 which had significantly less HMW material (Fig. 8A, a and b vs. i). Purity of the gliadin fraction was not very high with either method. The presence of classical gliadins (α, β, γ, and ω components) in ethanol extracts and HPLC peak 2 was also confirmed using the improved acid PAGE procedure of Khan et al (1985); however, we could not get satisfactory separation of nongliadin fractions with this method (data not shown).

No protein was present in the HPLC fraction eluting after the 18-min cutoff for peak 3 (Fig. 8A and B, lane j); bands seen in this fraction after reduction were due to 2-mercaptoethanol (Fig. 8B, lane h; and Tasheva and Dessev 1983). We should emphasize here that at the time these preparative fractions were collected, the performance of the column had deteriorated significantly, inferred from considerably more peak widening under similar HPLC conditions. This means that for most of our separations the overlap of size between HPLC fractions was less than indicated by Figure 8. The problem of deterioration

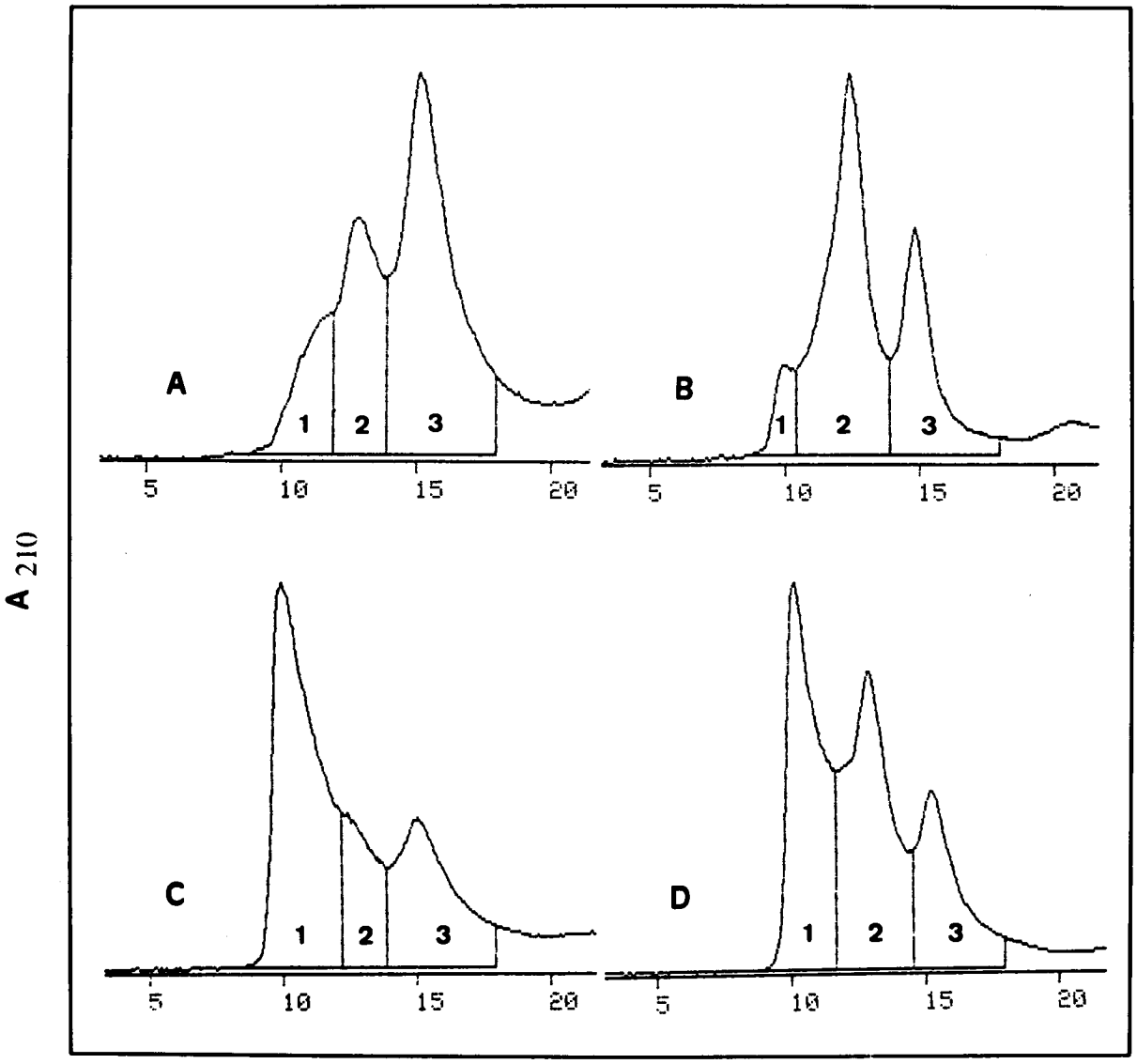

Fig. 7. Size-exclusion high-performance liquid chromatograms of unreduced solubility fractions and total protein extract from Mexico 8156 flour. **A,** albumin/globulin (0.05*M* NaCl extract); **B,** gliadin (70% ethanol extract); **C,** Glutenin (residue after salt and ethanol extractions); **D,** total protein extract. The glutenin fraction and total proteins were extracted by sonication. A_{210} = Absorbance at 210 nm. Minutes = elution times.

in column performance, which we think may be due to use of SDS in the eluent, is currently being investigated using alternative eluents.

It is evident that the solubility fractionation is not sharp and could lead to erroneous conclusions, particularly when several cultivars with variable degrees of overlap between components are being analyzed. However, because of its size-based fractionation, SE-HPLC resolves many of these overlaps, especially those due to incomplete extraction. It is still misleading to designate the three HPLC peaks of total protein extracts (Fig. 7D) as exclusively glutenin, gliadin, and albumin/globulin, but in a broad sense it is true. Peak 1 represents only polymeric proteins that are almost entirely glutenin, except for a minor proportion being triplet proteins (Singh and Shepherd 1985) and

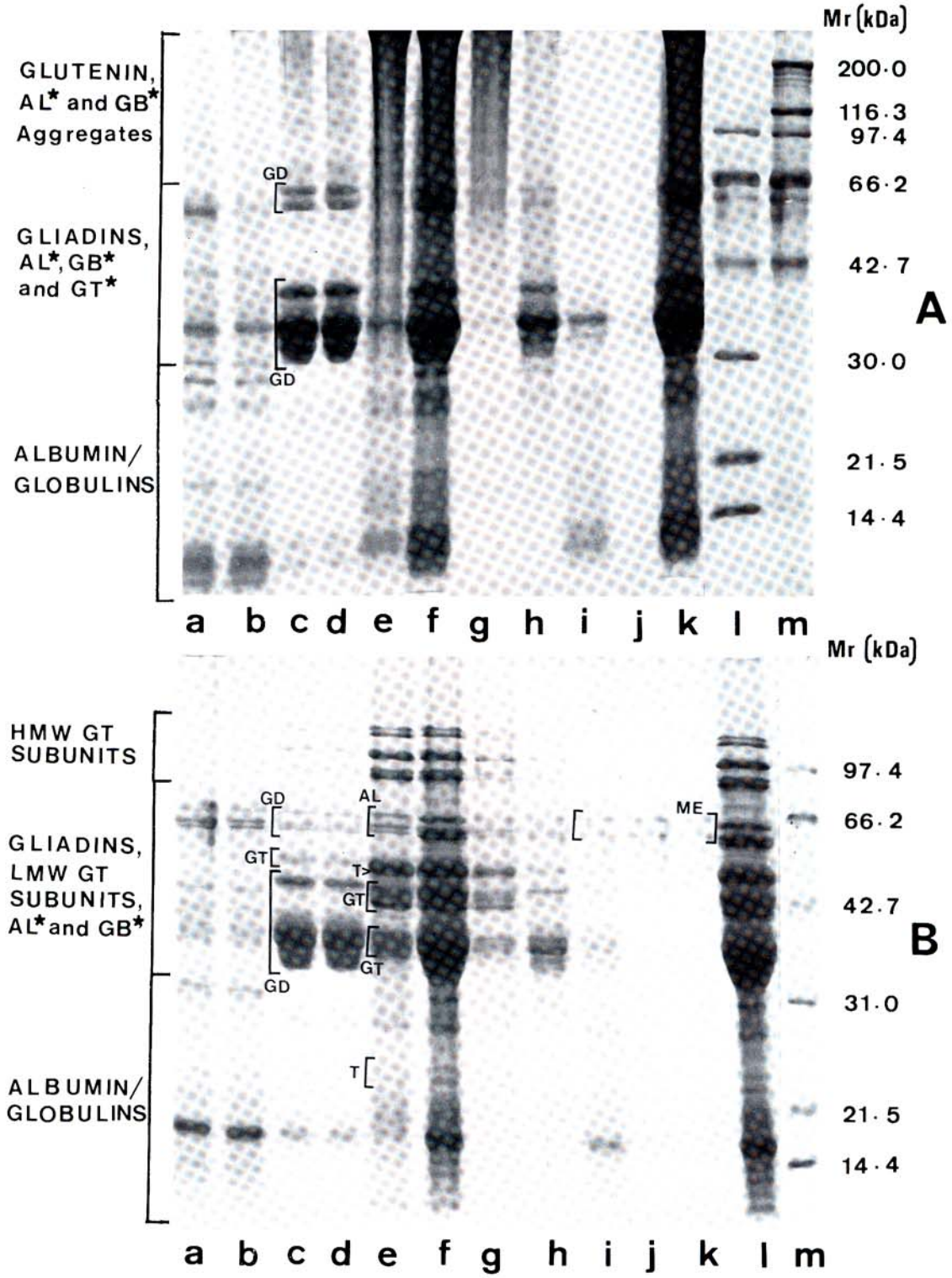

Fig. 8. Sodium dodecyl sulfate-polyacrylamide gel (10% acrylamide, 2% cross-link) electrophoretic patterns of Osborne solubility fractions and preparative size-exclusion high-performance liquid chromatography (SE-HPLC) fractions from Mexico 8156 flour. **A,** Unreduced proteins; **B,** Reduced proteins. Albumin/globulin (0.05*M* NaCl extract) (**a**); sonicated albumin/globulin (**b**); gliadin (70% ethanol extract) (**c**); sonicated gliadin (**d**); sonicated glutenin (salt and ethanol insoluble fraction) (**e**). SE-HPLC fractions: peak 1 (**g**, glutenin); peak 2 (**h**, gliadin); peak 3 (**i**, albumin globulin); material eluting after 18 min (**j**). Sonicated total proteins are f and k in A, f and l in B; Bio-Rad molecular weight standards are l and m in A and m in B; k in B is reduced sample buffer without added protein, showing mercaptan (ME) bands (Tasheva and Dessev 1983). AL = Albumin; GB = globulin; GD = gliadin; GT = glutenin; T = cv. Triplet protein subunits (Singh and Shepherd 1985); * = minor components of total proteins in the indicated size groups.

aggregating albumins (Gupta and Shepherd 1987). On the other hand, peak 3 represents mainly albumin/globulins, including the CM proteins, with some tailing-off of α-, β-, and γ-gliadins from peak 2. Although classical monomeric gliadins are present almost entirely in peak 2, this peak also contains small but significant amounts of LMW glutenin aggregates and HMW albumins.

In addition to the above studies using white flours, we also analyzed endosperm halves from single wheat kernels by grinding them into fine wholemeal powder with pestle and mortar and then extracting total unreduced proteins by sonication for 30 sec at power setting 5 as described above. SE-HPLC patterns of these extracts were similar to those for white flours, showing the suitability of this method for single-kernel analysis of wheat proteins as described by Bietz (1984).

CONCLUSIONS

We conclude from the present study that complete dissolution of unreduced proteins from strong and weak flours is possible by sonication in a 2% SDS solution (pH 6.9). This method has following advantages: 1) A complete extraction is achieved without the need to chemically reduce disulfide bonds. Unlike chemical reduction, shear degradation of disulfide bonds (the suggested mechanism for increased protein solubility) is a selective process. If controlled properly, only the largest glutenin polymers are degraded and the degradation products are still too large to affect the size-based fractionation of total proteins into three main groups representing glutenin, gliadin and albumin/globulin. 2) A very small quantity of flour (11 mg, equivalent to half an endosperm) is required, hence nondestructive single-seed analysis is feasible. The solid-to-solvent ratio (1:10 to 1:90) did not significantly affect the extractabilty or size distribution of protein. 3) A very short time (30 sec) is needed to completely extract proteins. When combined with SE-HPLC, this extraction provides a rapid method (which can be partly automated) of analyzing relative and absolute quantities of major flour protein fractions. Practical application of this procedure is described in the companion paper.

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