Use of Sonication to Probe Wheat Gluten Structure

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ABSTRACT

Variation in power and time of sonication of gluten and glutenin suspensions was used to gain information about the mechanism of molecular breakdown. At low power, an increase in sulfhydryl (SH) content of solubilized gluten could be ascribed to additional glutenin brought into the solution. Higher power sonication of gluten suspensions, at constant protein concentration and with increasing times, progressively shifted the molecular weight distribution (measured by size exclusion HPLC) to lower molecular weights. It was accompanied by a parallel decrease in SH content. This suggested that the freed cysteine residues formed by scission of disulfide (SS) bonds, caused by sonication, reacted to form new intramolecular SS bonds with free cysteines on the same molecular fragments. Circular dichroism measurements appeared to support this conclusion. High-power ultrasound produced SE-HPLC profiles with diffuse peaks corresponding to HMW-GS and LMW-GS. SDS-PAGE patterns of protein fractions obtained in different elution ranges of SE-HPLC confirmed that individual subunits had been produced by sonication. A fraction of the polymeric protein eluting at the void volume of SE-HPLC appeared to be resistant to breakdown by sonication.

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Sonochemistry is an emerging science in which high power ultrasound is used to change reaction pathways and kinetics in unique ways (Maynard 2000). One of its earliest applications was in the selective degradation of polymers. It has been used to break down the glutenin fraction with the largest molecular size in wheat protein to solubilize the total protein and facilitate its characterization (Singh et al 1990). Controlled depolymerization also has the potential to give information about glutenin structure. This approach has been followed previously using partial reduction. By analyzing the degradation products, deductions can be made about how the glutenin subunits are assembled in the final polymers (Ng et al 1991). Ultrasound is another way of achieving this and it has some advantages because effects can be closely controlled by variations of time and intensity. By monitoring the degradation products, some insight can be gained into the mechanism of molecular breakdown. This can lead to deductions about which bonds are more easily broken and how different subunits are joined in glutenin polymers. In the present study, ultrasound intensity and time were varied. Changes in molecular weight distribution were followed by size exclusion (SE) HPLC and the glutenin subunit composition of degradation products measured by SDS-PAGE. Accompanying changes in sulfhydryl (SH) content were also monitored.

MATERIALS AND METHODS

Gluten and Glutenin Samples

Gluten was prepared by hand washing dough from Kansas wheat cultivar Ike after extraction of the lipid with chloroform (MacRitchie and Gras 1973). Glutenin was prepared from the powdered freeze-dried gluten by a fractional extraction procedure based on pH level. Gluten was stirred in water at pH 5.0 (5 g/100 mL) for 30 min and centrifuged at 2,000 × *g* for 15 min. The supernatant was decanted and the extraction procedure repeated three times. A final extraction was made at pH 3.0. This left a highly pure glutenin free from gliadin as shown by SE-HPLC. The solid remaining after the extractions was freeze-dried and will be referred to as glutenin.

Sonication

Sonication was conducted with a sonic dismembrator (model 60, Fisher Scientific, Pittsburg, PA). The sonicator microprobe was placed consistently at one-third the distance from the bottom of the Eppendorf tube.

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SH Content

SH determinations were based on the method of Beveridge et al (1974). Ike gluten suspensions were stirred or sonicated for given times, centrifuged at 20,000 × *g* for 15 min and filtered through 0.45-μm filters. An aliquot (100 μL) was added to the degassed Ellman's reagent (1 m*M* DTNB in Tris-HCl buffer at pH 8) and incubated in the dark for 1 hr. Within 5 min, the absorbance was read for all samples at 412 nm. All concentrations were converted to m*M* equivalents of cysteine using a calibration curve with cysteine.

SE-HPLC

SE-HPLC was conducted using a HP 1100 system (Hewlett Packard) with automatic injection. Protein was extracted from gluten or glutenin (2 mg/mL) suspensions with an aqueous solution adjusted to pH 3.0 with HCl. Proteins were fractionated on a Biosep-SEC-S4000 column (Phenomenex, Torrance, CA). The eluting solvent was acetonitrile and water (1:1) containing 0.05% TFA. Fractions were collected using a fraction collector (Foxy Jr., Isco, Inc., Lincoln, NE) and freeze-dried.

SDS-PAGE

Freeze-dried fractions were dissolved in extraction buffer at pH 6.8 and separated on 10% SDS-PAGE minigels (Novex, San Diego, CA) in a discontinuous system. The minigels were run at a constant voltage of 200V for 1 hr. Gels were stained according to the procedure of Neuhoff et al (1988).

Circular Dichroism

Circular dichroism (CD) spectroscopy was conducted using a spectropolarimeter (Jasco-81, Jasco Inc., Easton, MD). Measurements were made at 26°C in a 0.1-cm cell with a scan speed of 50 nm/min and a resolution of 0.1 nm. Sensitivity was 50 mdeg. Spectra were recorded as an average of four scans. Bandwidth was 1nm, response was 4 sec. The solvent was water adjusted to pH 3.0 with HCl. The solvent blank run was set as the base line for all further data collection. 0.66 mg/mL of gluten was sonicated for different times to obtain the CD spectra. The results presented were mean residue ellipticity (units of degrees cm^2/decimole) plotted as a function of wavelength in nm. The residue weight was assumed to be 110. For reduction studies, 80 μL of 0.8*M* dithiothreitol (DTT) was added to 0.5 mL of the sample and heated at 60°C for 1 min and the CD spectrum recorded. The CD spectrum of the same sample was again recorded after heating at 60°C for additional steps of 1 min.

RESULTS AND DISCUSSION

SE-HPLC Profiles and SH Contents

At low power sonication (6W output), an increase in the SE-HPLC polymeric protein void volume peak was observed with little change

in the profile at higher elution times (Fig. 1). At higher power sonication (15W output) over the time scale used there was a shift in the SE-HPLC profile as illustrated in Fig. 2. The area of the void volume peak decreased and the protein was shifted to longer elution times and thus to lower molecular weights. After a rapid initial increase during the first 15 sec, the SE-HPLC area remained constant (Fig. 3), showing that a maximum amount of protein had been solubilized. Low power sonication was accompanied by an increase in SH content but this could be mainly ascribed to the extra protein (mainly glutenin) that had been solubilized (Fig. 3). At high power sonication, the SH content decreased with increasing sonication time and a corresponding decrease in molecular weight. This effect was contrary to what was expected as sonication is believed to reduce molecular size by splitting interchain SS bonds. This would normally create two new SH groups for each SS bond broken. The observed decrease in SH content with increasing sonication time suggests that the newly formed SH groups react with other free SH groups to form new SS bonds. Figure 4 shows the effect of higher power sonication (15W output) on the SE-HPLC profile of glutenin. There is a progressive decrease in the void volume peak with increasing sonication time and a corresponding increase in profile at longer elution times. The decrease in the void volume area was equal to the increase in area of the new peaks in the chromatogram at higher elution times. This is consistent with results shown in Fig. 5. High power sonication of glutenin showed behavior similar to gluten with respect to a decreasing SH content.

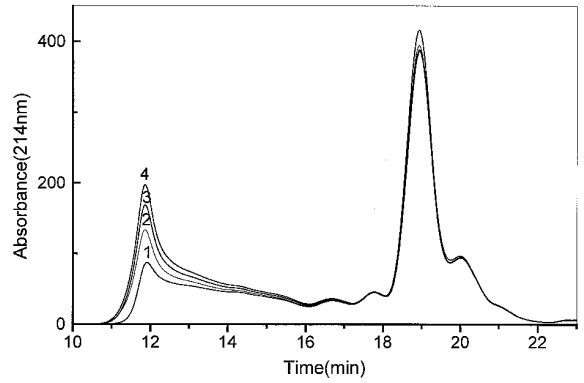

Fig. 1. Size exclusion HPLC of gluten at low power sonication (6W output) for 15, 30, 60, 90 sec (peaks 1–4, respectively). Absorbance units × 10^3.

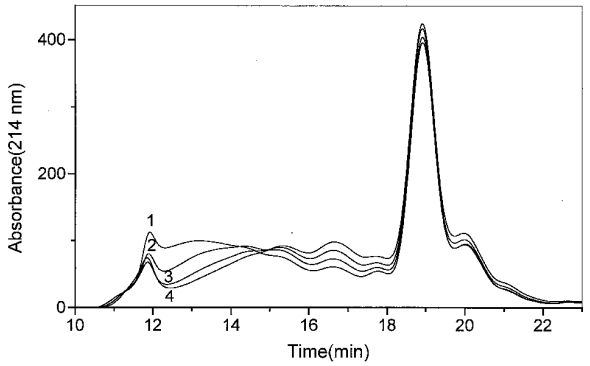

Fig. 2. Size exclusion HPLC of gluten at high power sonication (15W output) for 15, 30, 60, 90 sec (peaks 1–4, respectively). Absorbance units × 10^3.

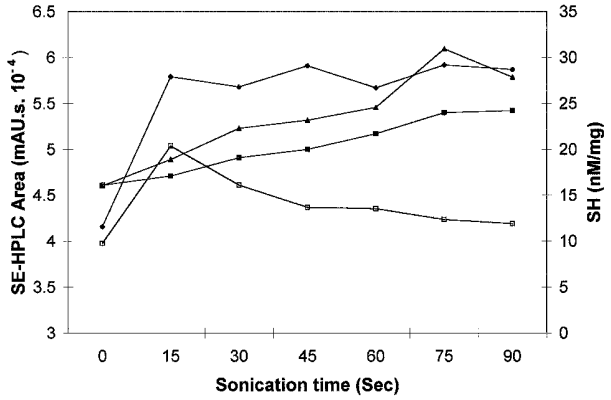

Fig. 3. Size exclusion HPLC area and SH content for low power (6W output) and higher power (15W output) sonication of gluten suspensions for different times. Size exclusion HPLC area low power (■) and high power (◆). SH content low power (▲) and high power (□).

Circular Dichroism

The decrease in SH content and presumed increase in SS bond formation might be expected to result in molecular conformational changes. To check this, CD measurements were made. Gluten shows a broad negative band at 200–240 nm due to different secondary structural conformation. Disulfide bonds, being chiral, have been shown in different systems to contribute to secondary structure in the region of 240 nm (Bekkers et al 1999; DuPont et al 2000). In view of the complexity and heterogeneity of the gluten system, simple spectra are not expected. However, an overall increase in intensity of the CD spectra during sonication suggests that more molecular folding occurs (Fig. 6A). In this folding process, SH groups may become hidden or participate in SS bond formation. If the increase

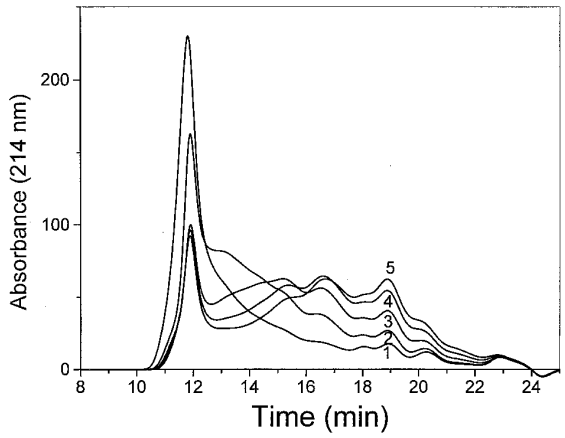

Fig. 4. Change in size exclusion HPLC profile with increasing sonication time for glutenin. Total change of area for 10, 20, 60, 120, 160 sec (peaks 1–5, respectively). Power output 15W. Absorbance units × 10^3.

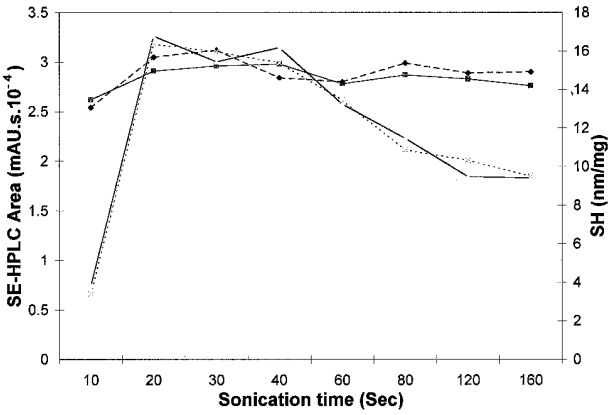

Fig. 5. Size exclusion HPLC area and SH content of glutenin sonicated at higher power (15W) as a function of sonication time. Size exclusion HPLC area duplicate runs (◆,■). SH content duplicate runs (✖,▲).

in intensity of the CD spectrum is due to formation of SS bonds, then it would be expected that subsequent reduction by agents such as mercaptoethanol should decrease the CD intensity in that region. This was found to be the case (Fig. 6B).

Reduction and Sonication

SE-HPLC profiles of Ike glutenin after sonication for 10 min at high power (15W output) and after reduction (using mercaptoethanol) are shown in Fig. 7. In reduced samples, there are two well-defined peaks that occur at elution times correponding to those expected from HMW-GS and LMW-GS. n sonicated samples, two peaks also are evident although they are more diffuse. The peaks occur at elution times that are slightly greater than those for the reduced samples. This suggests that discrete glutenin subunits are produced by sonication. Elution times are influenced not only by the molecular

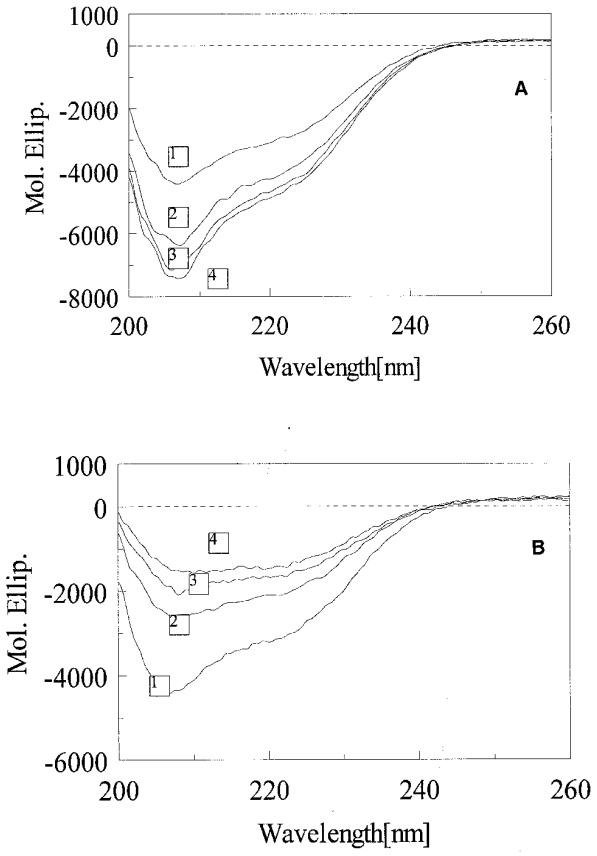

Fig. 6. Circular dichroism spectra for gluten. **A,** Sonication times (15W, no reduction) at 5, 10, 15, 60 sec (peaks 1–4, respectively). **B,** Reduction conditions (5 sec of sonication, 15W) for no reduction (1) and with 0.08*M* DTT at 60°C for 1 min (2); 2 min (3); 3 min (4). Ellipticity units are deg cm^2/decimole.

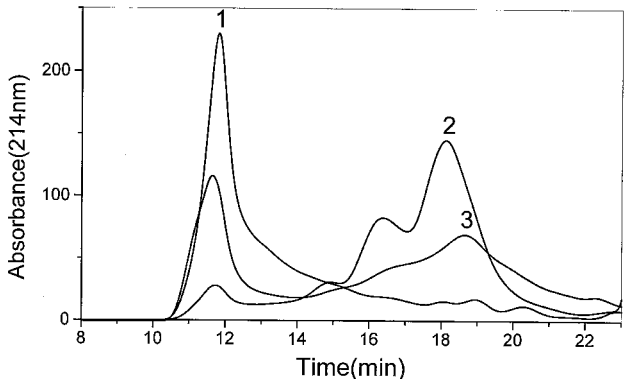

Fig. 7. Comparison of the effects of reduction and sonication on size exclusion HPLC profile of glutenin. Original glutenin (1); glutenin reduced by 2-mercaptoethanol (20 µL/mL) at 80°C for 30 min (2); glutenin sonicated at 15W output for 10 min (3). Absorbance units × 10^3.

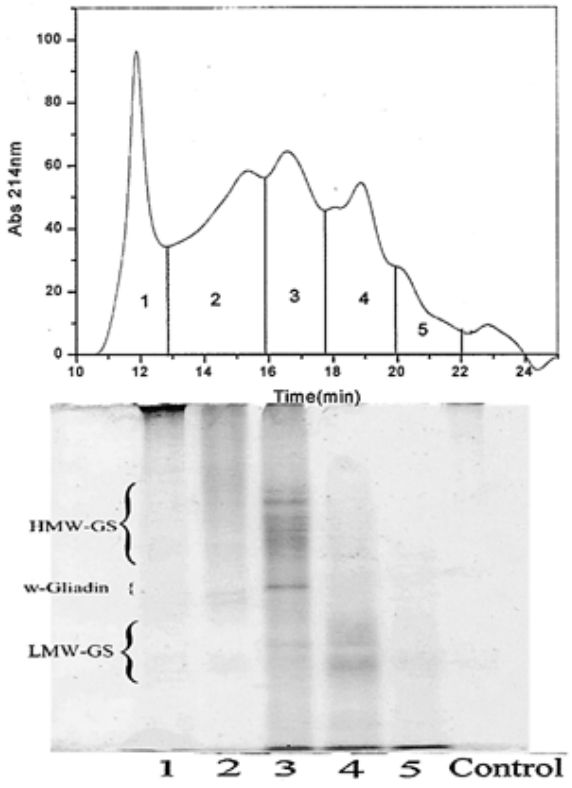

Fig. 8. SDS-PAGE of gluten protein fractions separated by size exclusion HPLC fractionation, unreduced conditions. Sonicated at 15W output for 2 min. Numbers correspond to SE-HPLC fractions. Absorbance units × 10^3.

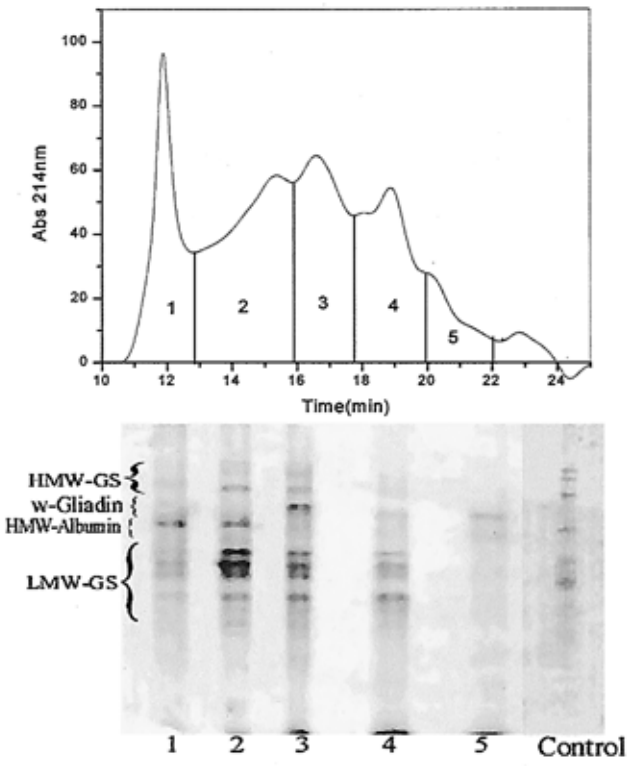

Fig. 9. SDS-PAGE of gluten protein fractions separated by size exclusion HPLC fractionation, reduced conditions. Sonicated at 15W output for 2 min. Numbers correspond to SE-HPLC fractions. Absorbance units × 10^3.

size but also by the conformation. The longer elution times means that the subunits formed by sonication are more retarded than those produced by chemical reduction. This implies a smaller hydrodynamic diameter, consistent with the evidence of folding from SH measurements and CD.

SDS-PAGE of SE-HPLC Fractions

A suspension of Ike glutenin was sonicated at high power (15W output) for 2 min and run on SE-HPLC. Five fractions were collected in five elution ranges as depicted in Figs. 8 and 9 and run on SDS-PAGE under unreduced (Fig. 8) and reduced (Fig. 9) conditions. In Fig. 8, unreduced polymers gave smears that penetrated further into the gel with increasing fraction number, as expected on the basis of decreasing molecular size. Fractions 3 and 4 show bands at mobilities expected for HMW-GS (fraction 3) and LMW-GS (fraction 4) on a diffuse background. In the reduced gel (Fig. 9), LMW-GS are evident in fraction 3. This indicates that, although discrete HMW-GS appear in this fraction, as shown by Fig. 8, smaller polymers responsible for the background staining in this fraction are made up of both HMW-GS and LMW-GS. A notable feature (Fig. 7) is that, although molecular weights are displaced to lower values with formation of glutenin subunits, the amount of material eluting in the void volume is only partially decreased. The composition of fraction 1 eluting in the void volume is of great interest in view of its apparent resistance to breakdown. This fraction does not appear to have a high concentration of HMW-GS but is relatively concentrated in HMW-albumins (β-amylases) as seen in Fig. 9.

CONCLUSIONS

Controlled sonication has potential for investigating the structure of glutenin in terms of how the different subunits are assembled in the glutenin polymers. The present work showed that, as molecular breakdown proceeded, the SH content of the protein decreased. A decrease in SH content has been observed previously during dough mixing (Tanaka and Bushuk 1973) but has not been fully explained. Sonication, like dough mixing, is believed to split SS bonds. The most obvious explanation for the behavior is that the cysteine residues, freed by SS scission, react with other free cysteine residues to form new SS bonds. It seems probable that the new bonds are on the same molecular fragment (i.e., they are intramolecular). Evidence was obtained from CD that formation of the new bonds occurs with an increase in molecular folding.

Polymer theory predicts that macromolecules subjected to high stress break preferentially at their centers (Bueche 1960). Previous studies of sonication support this. At relatively low power, the new material brought into solution elutes at the void volume in column chromatography. However, the present study shows that glutenin subunits are degradation products, even though large polymers (according to elution times) are still present. This might be interpreted to mean that there are differences in the ease of degradation of different glutenin polymers and, therefore, the interchain SS bonds. It should prove informative in relation to glutenin structure to follow up this work with more detailed analysis of the fragments produced at different times and intensities of sonication.

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