Solid-State, Cross-Polarization Magic-Angle Spinning Carbon-13 Nuclear Magnetic Resonance and Biochemical Characterization of Wheat Proteins

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ABSTRACT

Gluten and glutenin-enriched and gliadin-enriched subfractions of gluten, prepared by 1 *M* urea extraction, were examined by high-resolution, cross-polarization magic-angle spinning carbon-13 nuclear magnetic resonance (NMR) spectroscopy in the solid state to investigate the applicability of this technique in the physical characterization of gluten proteins. These fractions were also characterized biochemically to aid interpretation of the NMR data. An outstanding feature of the NMR spectra was that the glutenin-enriched fraction gave sharp resonances corresponding to aliphatic and aromatic amino acid side chains, whereas the gliadin-enriched fraction gave broad peaks in these regions. This suggests that the polypeptides in the gliadin-enriched fraction are much more tightly folded than those in the glutenin-enriched fraction and that, in the former, a much greater level of interaction exists among the hydrophobic side chains than in the latter. This implies that hydrophobic interactions may be less important in glutenin quaternary structure than some current models would suggest. The finding that a weighted summation of the spectra for the gliadin-enriched and the glutenin-enriched fractions cannot give a spectrum equivalent to that for the parent gluten suggests, however, that hydrophobic interactions between glutenins and gliadins may be important in the gluten complex.

Despite the considerable information concerning the biochemical characteristics of wheat proteins (Kasarda et al 1977, Wall 1979), much less is known about their physical properties, such as conformation, structure and structural dynamics, and the ways in which these properties are related to their physicochemical properties. This lack of information on the physical nature of these proteins has also delayed their detailed chemical characterization. The main reasons why progress has been hampered include the heterogeneity of gluten proteins (molecular size polydispersity, polypeptide subunit heterogeneity, and compositional variations among different wheat varieties) and the fact that most of the proteins are soluble only under relatively severe conditions (high alcohol concentrations or conditions of high or low pH) that may have significant but undetermined effects on protein structure.

Residual heterogeneity of isolated wheat protein fractions may partially account for the fact that these proteins have not so far been crystallized and characterized in the solid state by techniques such as X-ray diffraction. Studies of some wheat protein preparations in solution have been undertaken using techniques such as optical rotatory dispersion and circular dichrosim spectroscopy (Cluskey and Wu 1966, Kasarda 1970, Kasarda et al 1968, Wu and Cluskey 1965, Wu and Dimler 1964, Wu et al 1967), but whether these proteins exhibit the same properties in solution as in the solid state is a question that has not yet been answered.

The technique of high-resolution nuclear magnetic resonance spectroscopy (NMR) has been developed in recent years and is now proving to be of considerable value in the physicochemical characterization of organic solids (Pines et al 1973). Unlike that of X-ray diffraction, the application of high-resolution NMR of solids is not limited to crystallized proteins. Although a global view of the three-dimensional structure of proteins is not yet possible from NMR data, as it is from X-ray diffraction studies of crystallized proteins, high-resolution NMR does have the advantage that it can be used directly to identify specific chemical groups in proteins and generally to determine their mobilities, degree of ordering, and dynamics. At present, such properties cannot be studied to the same extent by X-ray diffraction techniques.

We have recently begun to study the conformations of wheat proteins in solution using high-resolution NMR (Baianu 1981, Baianu et al 1982). In the present work, these studies were further developed by applying the technique of high-resolution, cross-polarization magic-angle spinning (CP-MAS) carbon-13 NMR to examine conformational differences between gluten and gluten subfractions in the solid state. These protein preparations have also been characterized using biochemical techniques—gel filtration chromatography and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) (Bottomley et al 1982)—to aid in the interpretation of our NMR data.

MATERIALS AND METHODS

Gluten Preparation

The wheat used in these experiments was a high-yielding, hard-milling, European winter variety (*Triticum aestivum* cv. Maris Huntsman). Flour (unbleached, untreated) and gluten were prepared from 1978 crop wheat as described by Booth and Melvin (1979).

Fractionation of Gluten

Freshly washed, wet gluten (125 g) was cut into small pieces (approximately 0.5 cm in diameter) using scissors, and the gluten

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was suspended in distilled water (0.75 L) at 4° C. Solid urea (60 g) was added, the volume was made up to 1 L, and the suspension was stirred magnetically for 18 hr at 4° C. After centrifugation at 10,000 $\times g$ for 1 hr (4° C), the supernatant was dialyzed against 10 volumes of distilled water at 4° C for a total of nine changes. The pellet was resuspended in 1 L of distilled water (4° C) and, after stirring for 2.5 hr, the suspension was recentrifuged, and the pellet was again washed with water. The water washings were discarded. Both urea-soluble proteins in the supernatant and urea-insoluble proteins in the water-washed pellet were freeze-dried, and the dry products were ground in a Retsch Turbo Mill (0.5-mm sieve). Untreated gluten was freeze-dried immediately after preparation and ground in the Turbo Mill.

SDS-PAGE

Electrophoresis on polyacrylamide gel slabs was performed in the presence of SDS according to the method of Laemmli (1970), with the exception that the reservoir buffer composition was 0.384M glycine/0.025M Tris/3.47 $\times$ $10^{-3}M$ SDS, pH 8.3 (Bottomley et al 1982). The separating gel (150 $\times$ 150 $\times$ 1.5 mm) contained 154 g/L of total acrylamide monomer and 4 g/L of cross-linker, and the stacking gel (150 $\times$ 10 $\times$ 1.5 mm) contained 77 g/L of monomer and 2 g/L of cross-linker. After electrophoresis, done at 7.5 mA for approximately 18 hr, the gels were stained with Coomassie brilliant blue and subsequently scanned densitometrically using a Joyce-Loebl Chromoscan 201 system at 520 nm.

Gel Filtration Chromatography

Gel filtration chromatography was performed on columns of Sepharose CL-4B in the presence of SDS (Bottomley et al 1982). Samples were prepared for chromatography by extraction into 0.069M SDS/0.1M Tris/HCl, pH 8.0, in "Oakridge"-type centrifuge tubes for 17 hr under gentle rotation (28 rotations per min). After extraction, hexane was added, and the mixture was centrifuged for 1 hr at 50,000 $\times g$. The clear SDS extracts were carefully removed from the tubes and dialyzed against 3.47 $\times$ 10^{-3} M SDA/0.1 M Tris/HCl, pH 8.0, for 4 hr before application to the Sepharose column. The column (16$\times$850 mm) was eluted with 3.47 $\times$ $10^{-3}M$ SDS/0.1M Tris/HCl, pH 8.0, at a flow rate of 12 cm^3/h^{-1}. Both extraction and chromatogrphy were done at room temperature (20° C).

NMR Measurements

Proton decoupling and enhancement of carbon-13 resonances were achieved by the cross-polarization method (Pines et al 1973, Schaeffer et al 1979). This was combined with magic-angle spinning as described previously (Baianu and Forster 1980). Carbon-13 NMR spectra for dry wheat gluten powder and gluten subfractions were recorded at 75 MHz (with proton irradiation at 300 MHz) on a Bruker CXP 300 spectrometer. About 300 mg of sample powder was densely packed into a perdeuterated polymethacrylate rotor (6.3 mm i.d.) and spun at approximately 3.5 kHz at the "magic angle" (54° 44′) to the static magnetic field.

RESULTS

Extraction of Gluten Proteins Using 1M Urea

Fractionation of flour and gluten proteins using aqueous urea solutions was done previously (Lee 1968, Lee and MacRitchie 1971, MacRitchie 1973, Pomeranz 1965). In the present work, we used a urea concentration of 1M because preliminary measurements of Rayleigh light scattering by isolated gliadins in solution had indicated that scattering was minimal at this concentration.[1] At urea concentrations of 1.5M and 2M, Rayleigh scattering increased markedly, suggesting that gliadins unfold at such urea concentrations. Cluskey and Wu (1966) also reported helix loss by gluten proteins in solutions containing 3M urea. We found that a single extraction using 1M urea extracted 20% of the protein from Maris Huntsman gluten, similar to the 25% value obtained by Lee (1968) for the amount of protein extracted from flour using one extraction with 2M urea. Total protein recovery in the urea-soluble and urea-insoluble fractions was 95%. The proportion of the gluten protein in each fraction and the recovery of protein were measured by Kjeldahl nitrogen determinations.

[1] I. C. Baianu. Unpublished data.

Molecular Size Distribution of Proteins in Gluten and Gluten Subfractions

Proteins from Maris Huntsman wheat flour, gluten, and gluten subfractions were solubilized in SDS buffer and chromatographed on Sepharose CL-4B to determine the molecular size distribution of the proteins (Fig. 1). Our previous work (Bottomley et al 1982) showed that approximately 95% of flour and gluten proteins are extracted by SDS. By contrast, under our extraction conditions the more commonly used acetic acid/urea/cetyltrimethylammonium bromide solvent (AUC) extracts only 85% of flour proteins.

The glutenins extracted from flour or gluten have extremely polydisperse molecular size and are eluted over a large area of the chromatogram from the void volume (75 cm^3) to an elution volume of approximately 155 cm^3. Assessing absolute molecular weights for these components is difficult because of the lack of adequate standards with which to calibrate Sepharose gels, but they apparently range from about 10^5 to values in excess of 5 $\times$ 10^6. The peak between 155 and 175 cm^3 comprises the gliadin fraction, with molecular weights of 3–7 $\times$ 10^4, whereas the peak between 175 and 185 cm^3 comprises albumins and globulins of low molecular weight (approximately 1.5 $\times$ 10^4). These different fractions are distinct from each other not only in terms of their molecular sizes but also in terms of their polypeptide subunit distributions as determined by SDS-PAGE.

Examination of Maris Huntsman gluten (Fig. 1b) indicated that glutenins accounted for approximately 40% of the protein, and gliadins accounted for approximately 37%, as determined by measuring the areas beneath the respective regions of the chromatogram and expressing these as a percentage of the total area (based on absorbance at 280 nm of the column effluent). For the urea-insoluble fraction (Fig. 1c), glutenin comprised about 52% of the total protein, and gliadin comprised about 28%; for the urea-soluble fraction (Fig. 1d), gliadins were the predominant species, accounting for about 70% of the protein, with glutenins comprising only 17%. The relatively small proportion of glutenins

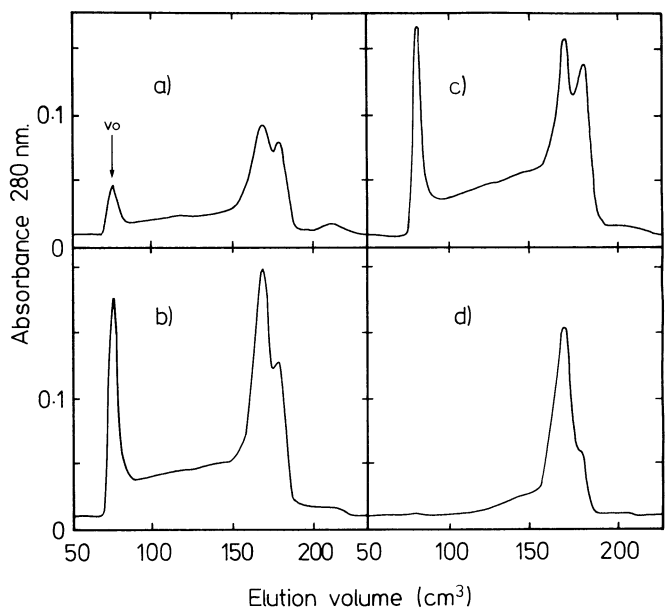

Fig. 1. Gel filtration chromatography of proteins in Maris Huntsman flour, gluten, and gluten subfractions. Flour (0.75 g) and gluten fractions (0.15 g) were extracted in 15 cm^3 of extraction buffer, and 3 cm^3 of the extract was applied to the column. The column effluent was monitored continuously at 280 nm. **a**, flour; **b**, gluten; **c**, urea-insoluble fraction; **d**, urea-soluble fraction.

in the urea-soluble fraction had a much lower average molecular weight than did the glutenins in the other samples.

The accuracy of quantification by this method depends on the assumption that the contents of aromatic amino acids in the different fractions are equivalent. Thus, the area values given above may not accurately reflect the actual amounts of protein in the different regions of the chromatograms. Nevertheless, such measurements do indicate the comparative compositions of the different samples. They also indicate that the urea-soluble fraction of gluten is considerably enriched in gliadins, and that the urea-insoluble fraction is enriched to a lesser extent in relatively high molecular weight glutenin species.

Polypeptide Subunit Distributions in Gluten and Gluten Subfractions

Polypeptide subunit distributions in the various samples were compared by SDS-PAGE of the reduced proteins. Previous work has shown that the subunit compositions of the glutenin and gliadin fractions prepared by gel filtration chromatography are quite distinct (Payne and Corfield 1979, Bottomley et al 1982). Subunits of molecular weights between 8×10^4 and 1.4×10^5 (migration distances 1.35–0.70 cm, Figs. 2 and 3) were found only in the glutenin fractions, and these are referred to here as high molecular weight glutenin subunits (HGS). Those subunits having molecular weights between 3.9 and 4.6×10^4 (migration distances 2.95–2.30 cm, Figs. 2 and 3), were distributed similarly, and these are referred to as low molecular weight glutenin subunits (LGS). Components with molecular weights between 2.9 and 3.6×10^4 (migration distances 4.50–3.35 cm) were largely α-, β-, and γ-gliadin (GL) subunits; some overlap occurred between LGS and GL subunits in this range. Subunits having molecular weights of about 6.0 and 6.6×10^4 (migration distances 1.95 and 1.58 cm, respectively) appear to be ω-gliadin subunits (WGL).

Measurement of the areas beneath the densitometer peaks indicated that the relative proportions of the different types of subunits were similar for flour and gluten (Figs. 2, 3a and b; Table I). The urea-insoluble fraction, on the other hand, was enriched in HGS components and somewhat depleted in GL subunits, compared with the parent gluten (Figs. 2, 3a and b; Table I), but the LGS and WGL components were present in the same proportions as in the gluten. Thus, the ratios of HGS to LGS, HGS to GL, and LGS to GL were all higher for the insoluble fraction than for the parent gluten. The urea-soluble fraction differed markedly from

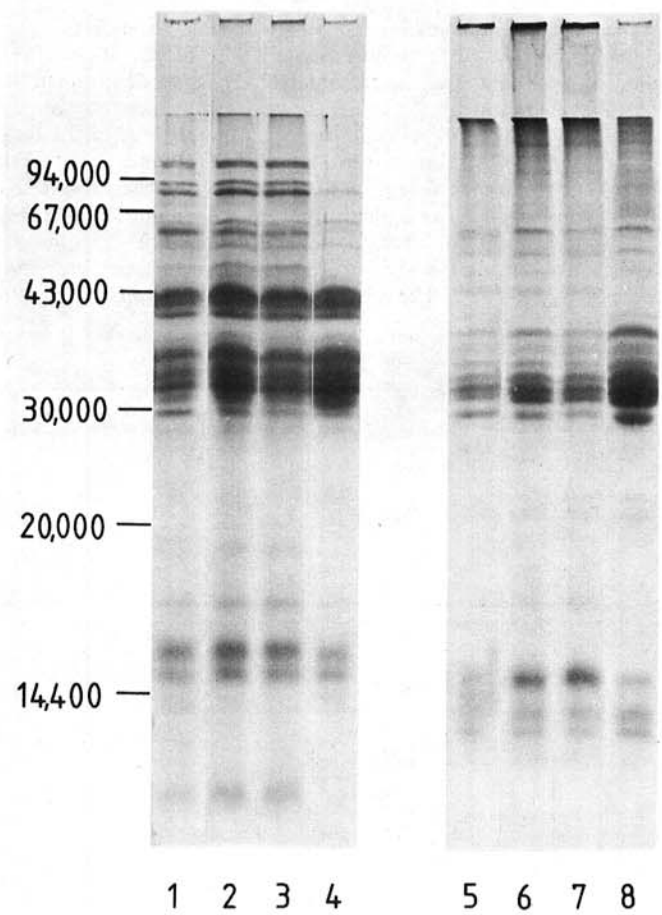

Fig. 2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of reduced and unreduced proteins from flour, gluten, and gluten subfractions. Flour (50 mg) and gluten fractions (5 mg) were solubilized in 1 cm^3 of sample buffer. When the samples were examined in the reduced state, 0.05 *M* dithioerythritol was included in the sample buffer. The samples were prepared for electrophoresis by heating at 100° C for 5 min. 1, flour, reduced; 2, gluten, reduced; 3, urea-insoluble fraction, reduced; 4, urea-soluble fraction, reduced; 5, flour, unreduced; 6, gluten, unreduced; 7, urea-insoluble fraction, unreduced; 8, urea-soluble fraction, unreduced.

TABLE I
Distribution of Polypeptide Subunits as Determined by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis of Reduced Samples[a]

	Glutenin Subunits		Gliadin Subunits				
	High Molecular Weight (HGS)	Low Molecular Weight (LGS)	ω-Gliadin (WGL)	Other (GL)	HGS/ LGS	HGS/ GL	LGS/ GL
Flour	16	26	12	46	0.62	0.35	0.45
Gluten	16	27	10	47	0.59	0.34	0.47
Urea-insoluble fraction	19	27	11	43	0.70	0.44	0.50
Urea-soluble fraction	4	27	8	61	0.15	0.07	0.39

[a] Values are expressed as a percentage of the total area beneath the densitometer peaks for all glutenin and gliadin subunits.

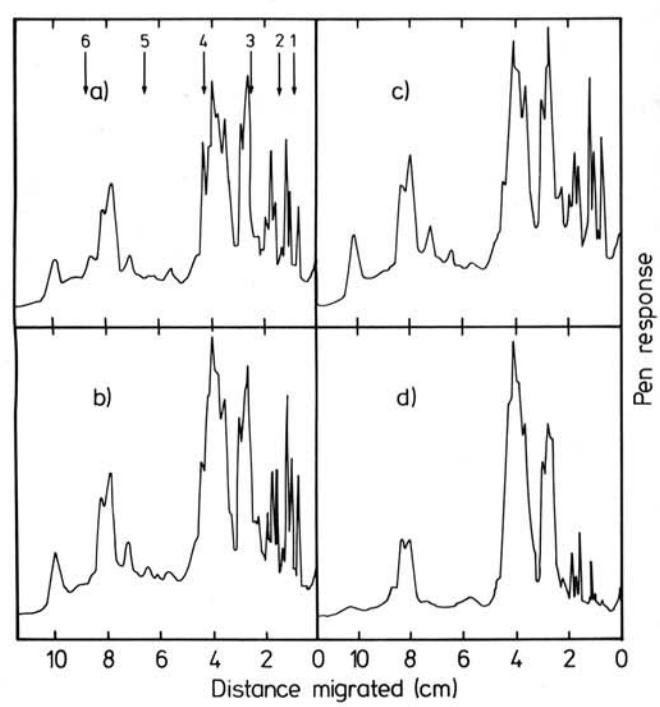

Fig. 3. Densitometer traces of sodium dodecyl sulfate polyacrylamide gel electrophorograms of reduced proteins from flour, gluten, and gluten subfractions. Flour (50 mg) and gluten fractions (5 mg) were solubilized in 1 cm^3 of sample buffer; 0.5 *M* dithioerythritol was included in the sample buffer. The samples were prepared for electrophoresis by heating at 100° C for 5 min. Migration distances are from the top of the separating gel. **a,** flour; **b,** gluten; **c,** urea-insoluble fraction; **d,** urea-soluble fraction. Arrows indicate the positions of molecular weight markers: 1, phosphorylase b, 94,000; 2, bovine serum albumin, 67,000; 3, ovalbumin, 43,000; 4, carbonic anhydrase, 30,000; 5, soybean trypsin inhibitor, 20,000; 6, α-lactalbumin, 14,400.

the insoluble fraction in that it was very much depleted in HGS subunits and considerably enriched in GL subunits compared with the gluten (Figs. 2, 3a and b; Table I). The glutenins in the urea-soluble fraction were atypical of most of the glutenins in the parent gluten in that the ratio of HGS to LGS was much lower for this fraction than for the gluten.

When the relative levels of total glutenin subunits (HGS + LGS) and total gliadin subunits (GL + WGL) were calculated by adding the relevant areas beneath densitometer peaks, the ratio of glutenins to gliadins was similar for flour and gluten (0.72 and 0.75, respectively); it was somewhat higher for the urea-insoluble fraction (0.85) but much lower for the urea-soluble fraction (0.45). The accuracy of this quantification of subunit distributions from SDS-PAGE depends on the basic amino acids, particularly lysine, contained in each polypeptide type. Because the content of such residues in different types of subunits varies (Kasarda et al 1977), the values quoted cannot be regarded as absolute, but they do give useful information when comparing different fractions. For the same reason, the values derived by SDS-PAGE and by gel filtration chromatography for the relative proportions of glutenins and gliadins cannot be expected to yield the same values; quantification from gel filtration chromatographs depends on the aromatic amino acid contents of the different polypeptide types.

The various samples were also examined by SDS-PAGE in the unreduced state (ie, dithioerythritol was omitted from the sample preparation buffer), and the results are presented in Figs. 2 and 4. HGS and LGS components were no longer observed, but a considerable proportion of the protein was present as a trailing smear from the top of the gel. This observation indicates that the glutenin subunits are linked by disulfide bonds originally and that the unreduced disulfide-linked glutenins are highly polydisperse with respect to molecular size. The patterns for flour, gluten, and the urea-insoluble fraction were quite similar, with protein staining being observed at the tops of both the separating and stacking gels (Fig. 4a, b, and c). However, the urea-soluble fraction was different in that essentially all of the protein penetrated the stacking gel (Fig. 4d); the densitometer peak at the stacking gel top was caused by the gel/destaining solution interface and not by protein staining. This again indicates that the glutenins in this fraction have a lower average molecular weight than those in the other fractions.

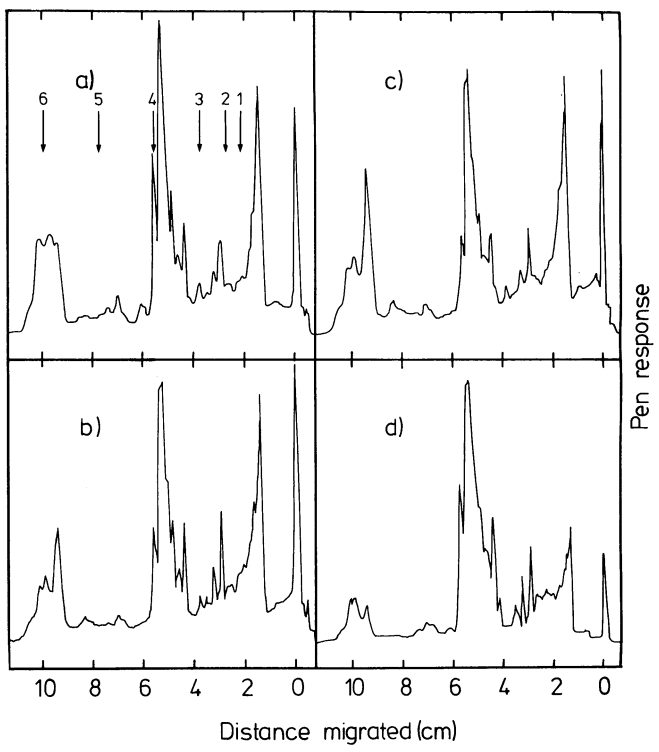

Fig. 4. Densitometer traces of sodium dodecyl sulfate polyacrylamide gel electrophorograms of unreduced proteins from flour, gluten, and gluten subfractions. Flour (50 mg) and gluten fractions (5 mg) were solubilized in 1 cm^3 of sample buffer; dithioerythritol was excluded from the sample buffer. The samples were prepared for electrophoresis by heating at 100° C for 5 min. Migration distances are from the top of the stacking gel. **a**, flour; **b**, gluten; **c**, urea-insoluble fraction; **d**, urea-soluble fraction. Molecular weight markers as in Fig. 3.

Cross-Polarization Magic-Angle Spinning Carbon-13 NMR of Gluten and Gluten Subfractions

The Maris Huntsman parent gluten, the gliadin-enriched, urea-soluble gluten fraction, and the glutenin-enriched, urea-insoluble fraction were examined in the dry state by CP-MAS carbon-13 NMR. The 75 MHz CP-MAS carbon-13 NMR spectra of these samples are shown in Figs. 5 and 6. In Fig. 6 the region from 0 to 80

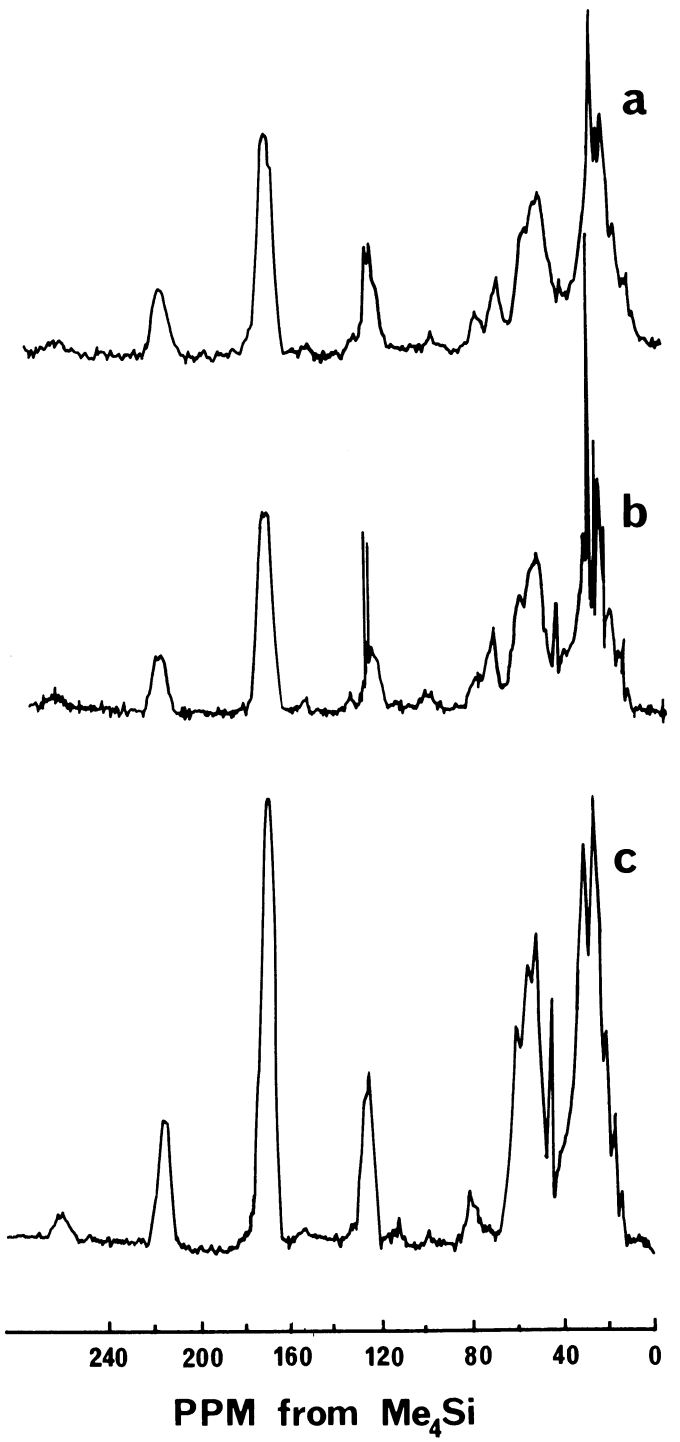

Fig. 5. Solid-state, cross-polarization magic-angle spinning carbon-13 nuclear magnetic resonance of Maris Huntsman gluten and gluten subfractions. **a**, gluten; **b**, urea-insoluble fraction; **c**, urea-soluble fraction.

ppm from tetramethylsilane (Me_4Si), is expanded to facilitate the comparison between the aliphatic side chain regions of the spectra for the urea-soluble and urea-insoluble fractions.

Owing to the high dispersion of the 7.0 T static field of the CXP 300 superconducting magnet and to the presence of molecular motion in these protein samples, many resolved carbon-13 resonances exist in these spectra. Those resonances that are 15–55 ppm from Me_4Si are mainly caused by aliphatic side chain carbons of the nonpolar amino acids, whereas the resonances that are 110 and 160 ppm from Me_4Si are caused by nonprotonated carbons of aromatic amino acid side chains (Table I in Baianu and Forster 1980). The most striking feature of the spectra is the presence of sharp resonances in these regions of the spectrum for the glutenin-enriched, urea-insoluble fraction.

The glutenin subunits are linked together through disulfide bonds, and these complexes have extremely polydisperse molecular size; molecular weights range from about 10^5 to more than 5×10^6. Thus, the presence of resolved sharp carbon-13 resonances must be attributed to averaging of the chemical shift anisotropy (Pines et al 1973), due to the high mobility of certain amino acid side chains, which are highly mobile. On the other hand, the broad CP-MAS spectrum of the gliadin-enriched, urea-soluble fraction suggests that this much less heterogeneous population has incompletely averaged chemical shift anisotropy under the conditions of these NMR experiments (7.0 T static field and 3.5 kHz spinning rate at 54° 44′). Since the proton-decoupling H_1 field used was 2.3 mT, insufficient decoupling cannot be the cause of the limited resolution illustrated in Figs. 5 and 6. The presence of residual chemical shift anisotropy indicates that the structure of the gliadins is much more rigid and that the 3.5 kHz spinning rates are insufficient at 7.0 T static field to eliminate this source of line broadening. This makes the radio frequency power-matching condition (Pines et al 1973) much less strict than in the case of glutenins.

Another feature of the spectra in Fig. 5 is that a weighted summation of the spectra for the urea-insoluble and urea-soluble gluten fractions cannot give a spectrum similar to that for the gluten (using weighting factors based on the amounts of protein recovered in each urea fraction or calculated from either the gel filtration chromatography data or the electrophoresis data). Two related explanations are possible: either the glutenins and gliadins are held together by noncovalent interactions that restrict the side chain motions in gluten, or the conformation of the urea-insoluble, glutenin-enriched fraction has been altered by the urea treatment. Solvent-induced conformational changes in gliadins are unlikely for two reasons: the carbon-13 NMR spectra of gliadins in the solid state (Baianu and Forster 1980) and in solutions (Baianu 1981, Baianu et al 1982) are broadly similar, as are the CP-MAS carbon-13 NMR spectra of gliadins extracted using different solvents (Baianu and Forster 1980).

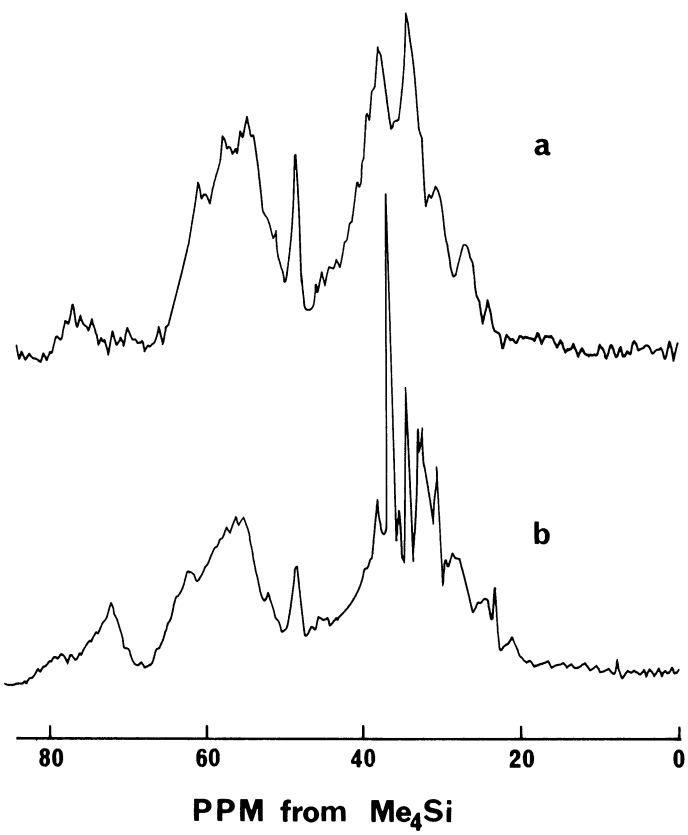

Fig. 6. Cross-polarization magic-angle spinning carbon-13 nuclear magnetic resonance spectra for the urea-soluble and urea-insoluble gluten fractions expanded in the region 0-80 ppm from tetramethylsilane. **a,** urea-soluble fraction; **b,** urea-insoluble fraction.

DISCUSSION

Examination of the CP-MAS carbon-13 spectra for gluten and gluten subfractions indicated that several resolved resonances were present due to amino acid side chain motions. The outstanding feature of these spectra was the presence of sharp resonances corresponding to aliphatic and aromatic residues in the glutenin-enriched, 1*M* urea-insoluble fraction and, in marked contrast, the presence of very broad peaks in the spectrum for the gliadin-enriched, 1*M* urea-soluble fraction. These observations indicate a high degree of mobility and a low level of interaction amongst the hydrophobic side chains in the glutenin-enriched fraction but a low degree of mobility and a high level of interaction between hydrophobic groups in the gliadin-enriched fraction. The results for the gliadin-enriched fraction are consistent with the concept that proteins in this fraction have tightly folded conformations.

The observation that the glutenin-enriched fraction gave spectra indicative of a high degree of mobility and a low level of interaction among the hydrophobic side chains may have certain implications for glutenin structure. The results suggest that glutenin subunits are not tightly folded, as the gliadins appear to be, and that the association of glutenin subunits through hydrophobic interactions involving aromatic and aliphatic amino acid side chains occurs only to a limited extent. This seems to suggest different glutenin conformations from those suggested recently (Kasarda et al 1977).

Noncovalent interactions between glutenins and gliadins involving hydrophobic amino acid side chains may be important in gluten itself, as suggested by the observation that a weighted summation of the NMR spectra for the gliadin-enriched and glutenin-enriched fractions cannot give a calculated spectrum equivalent to the observed spectrum for the parent gluten. This implies that interactions do occur in gluten between the gliadins and the glutenins, such that the CP-MAS NMR spectral characteristics of the hydrophobic amino acid side chains are modified in a major way (Figs. 5 and 6). However, spinning sidebands prevent a more specific interpretation.

Clearly, much more detailed studies are required before definitive answers can be obtained about the physical nature of wheat proteins. Nevertheless, the results of this work indicate that high-resolution CP-MAS NMR studies of gluten proteins are feasible and that this technique is potentially capable of providing valuable information regarding the conformations of gluten proteins and the nature of the noncovalent, interprotein interactions in gluten. Interpretation of the CP-MAS NMR data will be greatly aided if more discrete gluten subfractions that have been characterized by biochemical methods are available. In principle, further increases in the NMR spectral resolution and much more detailed information could be obtained by a combination of specific labeling and multiple resonance measurements (Baianu and Forster 1980).

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