Improved Chromatographic Separation and Characterization of Ethanol-Soluble Wheat Proteins

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ABSTRACT

A fraction of oligomeric wheat proteins, ethanol-soluble glutenin (ESG), formerly referred to as high molecular weight (HMW) gliadin, is coextracted with low molecular weight (LMW) monomeric gliadins using 70% ethanol. ESG has several subunits that are apparently identical to LMW-glutenin subunits (40–55 kDa). The cysteine residues of these subunits may be in different locations from those of LMW-monomeric gliadins, and these locations may favor intramolecular cross-linking. This distribution of cysteine residues enables these subunits to form oligomers of two to six subunits, with molecular masses of 80–250 ± 50 kDa. Variation of these polypeptides between wheat cultivars could significantly affect mixing and baking quality. To examine such quantitative and qualitative variation, we extracted defatted flours with 70% ethanol. Solubilized proteins were separated by size-exclusion liquid chromatography into ESG and LMW monomeric gliadins. Amounts of each fraction were determined gravimetrically after lyophilization. Fractions were then analyzed by reversed-phase high-performance liquid chromatography, before and after reduction of disulfide bonds, to compare cultivars and seek relationships to flour-quality parameters or wheat class. Carbohydrate and amino acid analyses of fractions were also done. ESG consisted of up to 37% of the total ethanol-soluble protein extracted, but results varied significantly among cultivars, possibly because of structural differences among proteins.

Wheat proteins consist of at least four major solubility groups: albumins, globulins, gliadins, and glutenins. Gliadins and glutenins are the major storage proteins. Each class is complex and contains many similar polypeptides.

Molecular weights of glutenins vary considerably: the molecular weight of native glutenin polymers ranges up to 10–20 million (Huebner and Wall 1976, 1980). Glutenin molecules contain both high molecular weight (HMW) and low molecular weight (LMW) subunits (Bietz and Wall 1972). LMW glutenin subunits (LMW-GS) are somewhat similar to gliadins in composition, molecular size, and solubility. For many years this has caused considerable confusion concerning the identity of gliadin and glutenin.

Gliadins consist primarily of chemically similar, 30–40 kDa monomers that are soluble in 70% ethanol. However, another fraction, which we refer to as ethanol-soluble glutenin (ESG), is also extracted under these conditions. This fraction (80–250 ± 50 kDa) was first recognized and isolated over 25 years ago (Beckwith et al 1966). Since then, it has received relatively little research attention. Nielsen et al (1968) showed that, like glutenin, ESG contains subunits joined by disulfide bonds. Bietz and Wall (1973) presented evidence that these subunits were equivalent to ethanol-soluble subunits of large glutenin molecules. They later supported this identity by amino-terminal sequence analyses (Bietz and Wall 1980). ESG sequences differed from those of most LMW-monomeric gliadins. Subsequent DNA sequencing of genes that code LMW-GS confirmed these results (Colot et al 1989). Results from another study (Tao and Kasarda 1989), using purified components for more definitive identifications, do not completely agree with all earlier findings.

Many studies have identified specific relationships between HMW glutenin subunits (HMW-GS) and wheat quality characteristics (Payne et al 1987, Ng et al 1989). Other studies have proposed relationships of LMW-monomeric gliadins to breadmaking quality (Huebner 1989, Metakovsky et al 1990). However, few attempts have been made to relate ESG to quality, possibly because of the apparent similarity of this fraction to monomeric gliadins. Also, because ESG seemed to be present in small quantities in wheat, it may have been thought to have little effect on mixing and baking quality.

Nevertheless, a few studies have explored relationships of LMW-GS to quality. Autran et al (1987) showed qualitative and quantitative variation of this fraction in good- and poor-quality durum wheats. Gamma gliadins are easily recognized markers of quality characters, but LMW-GS (which appear identical with

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subunits of ESG in this work) directly influence durum gluten viscoelasticity (du Cros 1987, Pogna et al 1988). Similarly in bread wheats, LMW-GS were significantly associated with dough resistance, extensibility, and strength (Gupta et al 1989). Other recent studies have dealt only with total LMW-GS of wheat flour (Gupta et al 1991 a,b) or N-terminal amino acid sequences of various subunits (Lew et al 1992).

In recent studies (Huebner et al 1991), size-exclusion high-performance liquid chromatography (SE-HPLC) was used to analyze proteins of many winter and spring wheats. There were significant differences in ESG among cultivars. To isolate sufficient quantities of proteins for accurate quantitation and further analyses, an improved gel-filtration material (Sephacryl S-200) was used. This provided faster separations than the Sephadex media used previously (Beckwith et al 1966). Fractions from gel filtration were analyzed by reversed-phase high-performance liquid chromatography (RP-HPLC), before and after disulfide-bond cleavage (reduction), to characterize components, especially ESG. This approach was applied to several wheat cultivars in an attempt to relate results to quality characteristics and wheat classification.

MATERIALS AND METHODS

Wheat Samples

Wheats were obtained from grain elevators and from the U.S. Grain Marketing Research Laboratory, Manhattan, KS, by the Federal Grain Inspection Service. Wheats were milled either at the USDA-ARS Hard Red Spring and Durum Wheat Quality Laboratory, Fargo, ND, or at the Manhattan, KS, laboratory. These samples were part of a cooperative project aimed at determining differences between winter and spring wheats. The spring wheats examined included Len, Newana, and Telemark; winter wheats examined were Concho, Newton, KS 501097, and Yecoro Rojo.

Flour samples (500 mg) were defatted using 3 ml of butanol with vortex shaking (dual-action shaker, model 3508, Lab-Line, Melrose Park, IL) for 15 min in 10-ml centrifuge tubes, followed by centrifugation for 10 min at 20,000 × *g*. Samples were then washed with 2 ml of petroleum ether and centrifuged. These initial extracts were discarded.

The defatted flour was extracted using 3 ml of 0.05*M* NaCl with vortex shaking for 20 min at 5°C, followed by centrifugation (15,000 × *g*) at 5°C. This extract was saved for further analysis.

The residue was extracted for 30 min using 3 ml of 70% ethanol with vortex shaking and centrifuged at 15,000 × *g* for 12 min. This extract was saved. The residue was then reextracted with 2 ml of 70% ethanol and centrifuged. This extract was combined with the 70% ethanol extract and concentrated in a Speed Vac concentrator (Savant Instruments, Farmingdale, NY) to ~2.5 ml before fractionation by gel filtration on a Sephacryl S-200 (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) 2.5- × 97-cm column. Samples were stored at 4°C until used. Entire extracts were applied to the column to compare equal amounts of flour from different wheats.

Low-Pressure SE-HPLC

Solvents used on the S-200 column contained 0.1–1.0*N* acetic acid, 0–12% ethanol, and 0–300 μl of trifluoroacetic acid (TFA) per liter. The eluate was monitored at 214 nm with a UA-5 detector (ISCO Lincoln, NE) at 0.5 absorbance units full scale. Solvent flow was maintained by gravity (overnight separation) or with a polystaltic pump (Buchler Instruments, Kansas City, MO) (7-hr separation). Eluate was collected (140 drops per tube; ~4 ml) with an automatic fraction collector (Gilson Medical Electronics, Middleton, WI).

Fractions were concentrated in the Speed Vac concentrator to approximately one-half their original volume. This avoided any temperature increase that could deamidate samples at low pH (due to the presence of TFA) (Huebner and Bietz 1984). Concentrated samples were lyophilized and weighed.

Protein standards were ferritin (450 kDa), catalase (240 kDa), aldolase (158 kDa), and chymotrypsinogen A (25 kDa) (Pierce Chemical Co., Rockford, IL) and soybean trypsin inhibitor (20.1 kDa) and alpha-lactalbumin (14.4 kDa) (Pharmacia LKB).

Analyses

Carbohydrates were analyzed spectrophotometrically at 490 nm using the phenol-sulfuric acid method (Dubois et al 1956). Soluble starch (Difco Lab, Detroit, MI) was used as standard. Amino acid analyses after digestion for 24 hr at 110°C were performed at the College of Agriculture, University of Missouri, Columbia, MO, using an automated cation-exchange procedure.

RP-HPLC

Samples were analyzed by RP-HPLC using a Spectra-Physics (San Jose, CA) apparatus including a SP8700 solvent delivery system and a SP8780XR autosampler (Huebner and Bietz 1987). Proteins were detected at 210 nm (0.1 absorbance units full scale/10 mV) with a SF770 Spectroflow monitor (Kratos, Ramsey, NJ). A Vydac (Separations Group, Hesperia, CA) C4 column (250 × 4.1 mm, particle size 5 μm, 300 Å pore size) was used. It was preceded by a 20- × 2-mm guard column (C-130B, Upchurch, Oak Harbor, WA) containing 0.5-μm end filters and filled with RSC (SynChrom, Lafayette, IN) packing material. The column was maintained at 60°C with a CH-460 column heater (FIAtron Laboratory Systems, Oconomowoc, WI).

Acetonitrile (ACN) and TFA were HPLC grade. Distilled water was further purified with a Barnstead NANOpure system. Solvents A (water containing 0.11% TFA) and B (90% ACN containing 0.09% TFA) were filtered through a 0.45-μm HVLP Millipore filter. Solvents were then deaerated under vacuum and initially sparged rapidly with helium for 5–10 min (Huebner and Bietz 1987). Slow purging with helium continued during analyses. Samples of 10–30 μl were generally analyzed at 1.0 ml/min. Proteins were applied to the column equilibrated with 25% solvent B and eluted with a gradient that increased to 31% B at 3 min and 47% B at 50 min. The column was held at 47% B for 5 min and then reequilibrated for 10 min for the next sample. Samples were reduced for 2 hr with 0.2% dithiothreitol (DTT) (Aldrich Chemical Co., Milwaukee, WI) in 0.05*N* sodium phosphate, pH 7.8, containing 2% sodium dodecyl sulfate. Alkylation was then performed using ~20% molar excess (over DTT) of 30% 4-vinylpyridine in isopropanol.

Data Analysis

Data were recorded on an Omniscribe recorder (Houston Instruments, Austin, TX) and stored in a ModComp (Fort Lauderdale, FL) computer system. Data could be plotted to any convenient scale and automatically integrated between specified times or by Gaussian deconvolution after correction for baseline shifts due to the gradient.

RESULTS

Column Conditions and Methods Used

Monomeric gliadins and LMW-GS are generally separated on Sephadex columns using a simple solvent, such as 0.05*N* acetic acid (Beckwith et al 1966). In such separations, lower or higher acetic acid concentrations have little effect resolution. Optimal SE-HPLC separations on an S-200 column, however, require different solvents, apparently because of interactions between proteins and the support media. Figure 1 shows typical separations of ethanol-soluble proteins using different solvents. Acetic acid alone (0.3*N*, Fig. 1a; also 1.0*N*, data not shown) did not resolve HMW and LMW proteins. Increasing the concentration of acetic acid or ethanol was also unsatisfactory (Fig. 1 b,c). Adding sodium acetate, glycine, or HCl (0.01*N*) improved resolution (results not shown), but we wished to use volatile solvents to prevent the need for dialysis, to save time, and to prevent sample loss. TFA (which is volatile) was tested (Fig. 1d) and found to promote good separations of LMW-GS and later-eluting monomeric gliadins. Batey et al (1991) also found that TFA significantly improved SE-HPLC separations of proteins. Ultimately, after

testing many combinations of reagents, we found that the solvent 0.1*N* acetic acid plus 12% ethanol plus 0.03% TFA best separated LMW-GS from monomeric gliadins on an S-200 column (Fig. 1d).

For varietal identification, a single extraction with 70% ethanol satisfactorily solubilized monomeric gliadins (Bietz et al 1984). However, the low solvent-to-flour ratio prevents complete recovery of soluble protein. In this study, a second extraction was performed to maximize recovery of all ethanol-soluble proteins. In the two combined extracts, recovery of LMW-GS and monomeric gliadins is nearly quantitative (~95%). As noted in Beckwith et al (1966), ESG extracted with 70% ethanol characteristically appears milky due to light scattering after SE-HPLC. Therefore, gravimetric analysis, rather than absorbance, must be used to accurately quantify LMW-GS recovery.

Preparative Sephadex G-100 gel filtration requires approximately two days to separate gliadins because slow flow rates must be used to avoid compressing the soft gel. With the more rigid S-200 material, however, flow rates can be up to 10 times faster. There is little difference in resolution on an S-200 between 7-hr separations (flow rate maintained with a peristaltic pump) and those taking 20 hr (gravity flow; not shown). Analysis of recoveries for various wheats (Table I) indicates that 25–37%, by weight, of whole gliadin is LMW-GS plus *x*-gliadin (fractions 1–3 of Fig. 2); 54–62% are monomeric gliadins (fraction 4, Fig. 2); and 8–17% are albumins not previously extracted with salt (fraction 5, Fig. 2). Albumins are poorly resolved from monomeric gliadins, so the amounts reported could be excessive. The total amount of protein extracted varied from 26 to 43 mg per 500 mg of flour. Recovery from the column appeared quantitative based on sample weight recovered. Higher concentrations of acetonitrile, sodium dodecyl sulfate, urea, and guanidine hydrochloride did not extract additional protein.

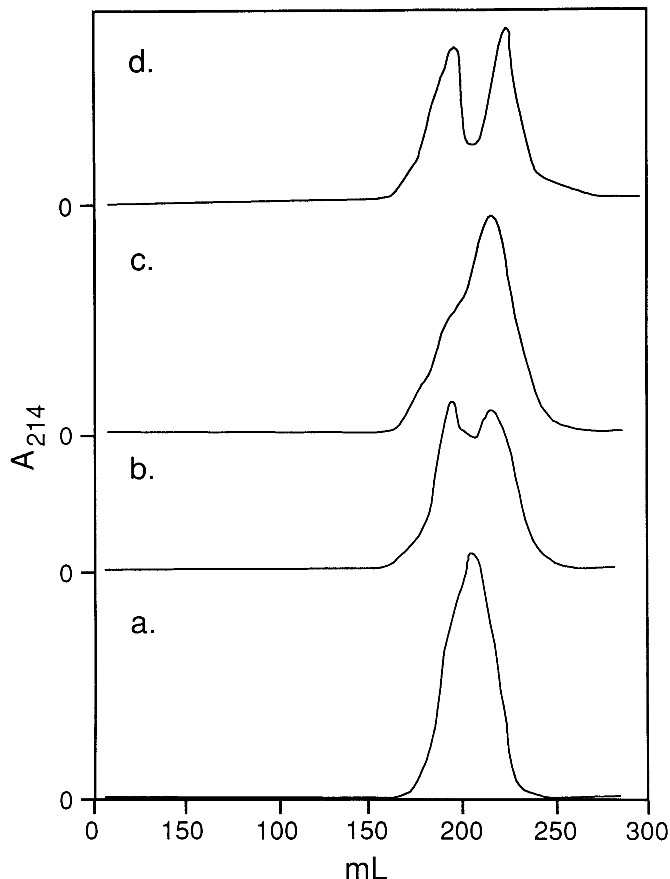

Fig. 1. Size-exclusion chromatography of whole gliadin on a Sephacryl S-200 column (2.1 × 90 cm) using different solvents. **a**, 0.3*N* acetic acid; **b**, 1.0*N* acetic acid plus 8% ethanol; **c**, 0.1*N* acetic acid plus 12% ethanol; **d**, 0.1*N* acetic acid plus 12% ethanol plus 0.03% trifluoroacetic acid.

TABLE I
Protein Recovery from Sephacryl S-200 Chromatography of Whole Gliadin

Wheat Cultivar	Protein Recovered[a] (mg)	Percent in Fraction[b]		
		LMWG[c]	Gliadins	Albumins
Newana	37	24	61	15
Telemark	43	37	55	8
Len	39	30	62	8
KS 501097	38	32	54	14
Yecoro Rojo	26	31	61	8
Newton	26	34	54	12

[a] ±1.5 mg.
[b] ±2%.
[c] Low-molecular-weight glutenins.

Fractionation of Gliadins

In many early studies, gliadin was prepared from gluten washed from a dough ball (Beckwith et al 1966). This procedure also extracts most water- and salt-soluble proteins. When proteins are extracted directly from flour, however, albumins and globulins must be preextracted to obtain purified gliadin. Figure 2 is a comparison of an S-200 separation of salt-extracted albumins plus globulins with separations of ethanol extracts, before and after NaCl extraction. Salt-soluble proteins (Fig. 2A) elute over a wide range of elution volumes, ranging from the elution position of LMW-GS to beyond the elution position of monomeric gliadins. However, little protein in the salt extract is similar in size to monomeric gliadins that elute from 215 to 250 ml. Up to 8.4 mg of protein was recovered in fractions 1–6 (Fig. 2A) of the salt extract from 500 mg of flour after S-200 fractionation. Comparison of ethanol extracts, with and without NaCl preextraction (Fig. 2 B,C), shows less HMW protein in the ethanol extract of flour preextracted with NaCl (Fig. 2B). Preextraction with 0.05*N* NaCl solubilizes an appreciable amount of protein that may otherwise be extracted by ethanol.

Figure 3 shows typical S-200 separations of gliadins of six wheat

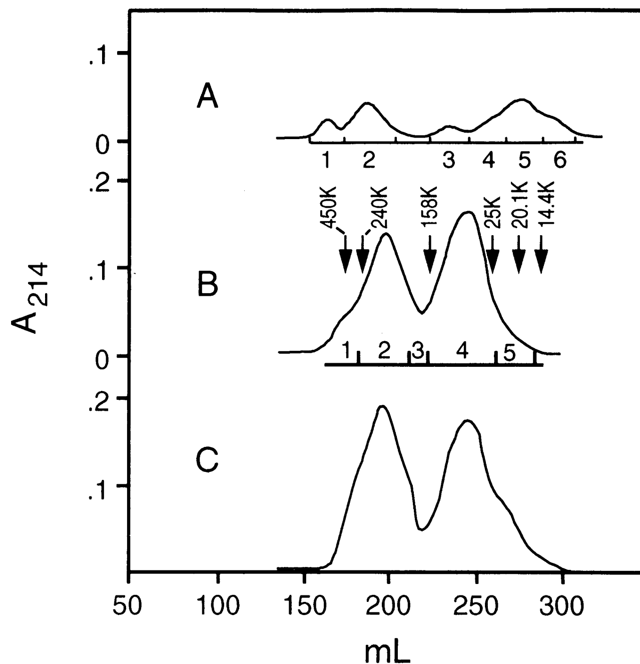

Fig. 2. Sephacryl S-200 separations of protein extracts from Newana flour including: 0.05*N* NaCl extract (**A**); ethanol extract after NaCl extraction (**B**); and direct ethanol extract of flour without NaCl extraction (**C**). **A**, fractions pooled and lyophilized. **B**, molecular weights of protein calibration standards. Flow rate: 12 ml/hr. Solvent: 0.1*N* acetic acid plus 12% ethanol plus 0.03% trifluoroacetic acid.

samples. Resolution and relative amounts of HWM and LMW fractions vary considerably, showing many quantitative, intervarietal differences in gliadin compositions.

RP-HPLC of S-200 Fractions

Fractions from the NaCl extract of Newana flour (Fig. 2A, 1–6) were further analyzed by RP-HPLC (Fig. 4). At least 40–50 components resolve. There is remarkably little overlap between adjacent fractions, showing the excellent resolving power of the S-200 column. Most resolved proteins elute considerably earlier than do major monomeric gliadins (compare to Fig. 5, fraction 4) because of the lower hydrophobicity of albumins and globulins. Some small peaks may be common to NaCl and ethanol extracts, but these are not usually evident in the latter because of their small amount.

Figure 5 shows RP-HPLC analyses of S-200 fractions from the ethanol extract. Proteins in ESG fractions 1 and 2 (Fig. 5) elute over a broad area from 30–60 min. Some ω-gliadins also occur in fraction 2 (Fig. 5) at 13–15 and 24 min. Monomeric (α, β, and γ) gliadins occur primarily in fraction 4 (Fig. 5) at 25–58 min. Fraction 5 (Fig. 5) contains a small amount of LMW and relatively hydrophilic albumins or globulins (12–20 min) not solubilized by NaCl extraction. These peaks appear identical with fractions 5 and 6 in Figure 4 and elute at the same time from the S-200 column (Fig. 2A). Thus, compositions of each ethanol-soluble protein fraction are distinctly different, again showing the good resolution of S-200.

Figure 6 shows unreduced ESG fractions 1 (Fig. 6A) and 2 (Fig. 6B) of several wheat cultivars. Some sharp peaks occur at ~8–10 min, particularly for fraction 2 (Fig. 6B). These are ω-gliadins not separated from ESG under these conditions. The major features of these chromatograms are two broad, poorly resolved peaks between 33–60 min. These are the predominant HMW proteins extracted by ethanol. Such results are not unexpected: native ESG are oligomeric proteins that contain many subunits joined by disulfide bonds (Bietz and Wall 1973). Exact elution times and shapes of these broad peaks in fractions 1 and 2 differ among cultivars, suggesting different ESG compositions. These results also demonstrate that elevated baselines and apparent low resolution of monomeric gliadins in some RP-HPLC studies may be due to the presence of LMW-GS coextracted with ethanol.

RP-HPLC of Reduced ESG

To investigate ESG further and to test the hypothesis that compositional differences affect baking or mixing quality, ESG fractions 1 and 2 from various wheat cultivars were combined, reduced with DTT, and analyzed by RP-HPLC (Fig. 7). This treatment eliminates the broad 33–60 min peaks seen in Figure 6, suggesting quantitative reduction of disulfide bonds in ESG. The chromatograms in Figure 7 have many sharp, well-resolved peaks, most with elution times comparable to monomeric gliadins (Fig. 5, fraction 4). Burnouf and Bietz (1985) showed that LMW-GS (equivalent to these subunits of ESG) have such elution characteristics. Although a few minor peaks between 15–25 min were found to be HMW-GS (Burnouf and Bietz 1985), their amount is small. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis also confirmed the presence of some HMW-GS (results not shown). Different gliadin extraction conditions, such as use of 60, 70, and 80% ethanol, all extracted a small amount of this material; 70% ethanol was judged to be the best solvent.

Subunit compositions of ESG fractions 1 and 2 differ among wheat cultivars (Fig. 7), confirming that unreduced proteins have different elution characteristics (Fig. 6). These differences, as well as quantitative differences (Fig. 3), support other studies (Autran et al 1987, du Cros 1987, Pogna et al 1988, Gupta et al 1989)

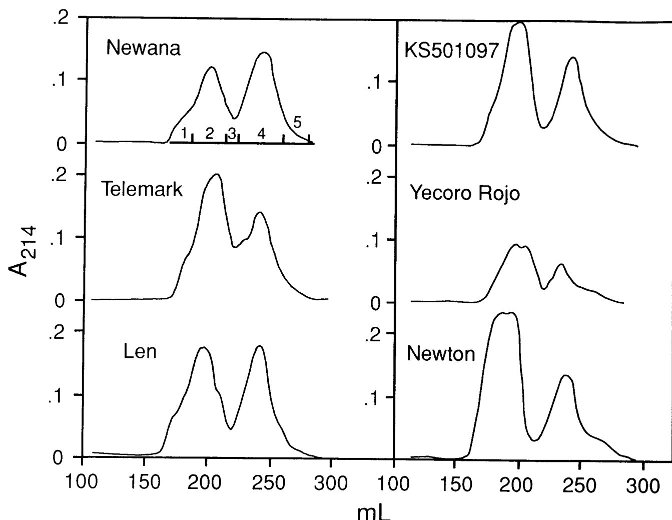

Fig. 3. Sephacryl S-200 separations of ethanol extracts from hard red spring wheats (Newana, Telemark, Len) and winter wheats (KS 501097, Yecoro Rojo, Newton). Flow rate: 12 ml/hr. Solvent: 0.1*N* acetic acid plus 12% ethanol plus 0.03% trifluoroacetic acid. Fractions 1–5 divided and pooled as in Fig. 2B.

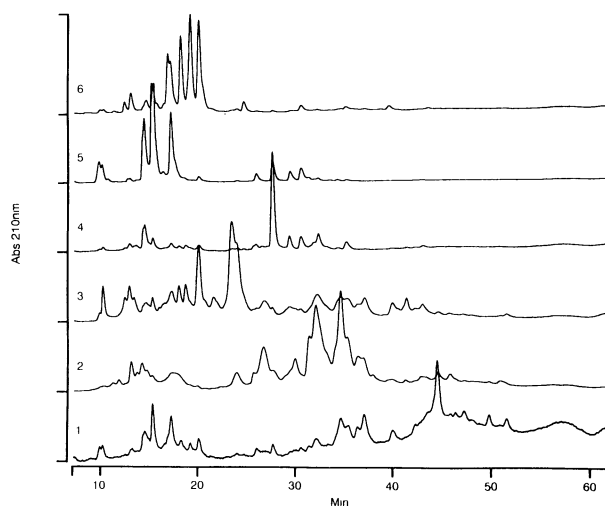

Fig. 4. Reversed-phase high-performance liquid chromatography analyses of NaCl-soluble flour protein fractions from a Sephacryl S-200 column (Fig. 2A). Samples dissolved in 20% ethanol plus 0.1*N* acetic acid; 15–30 μl injections. Chromatograms normalized to the highest peak. Relative amounts of protein in fractions of different chromatograms are not to scale.

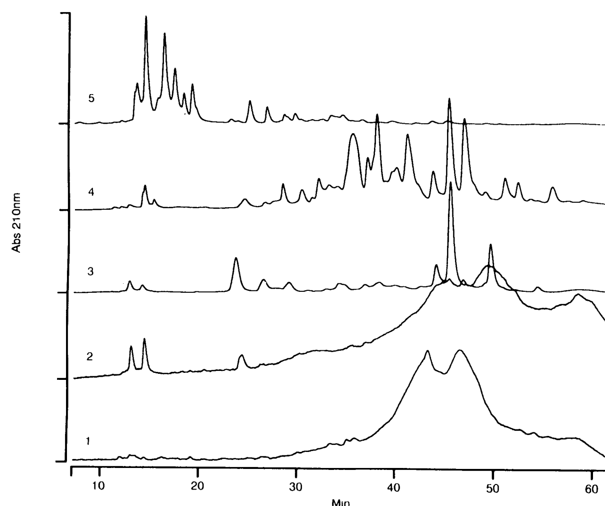

Fig. 5. Reversed-phase high-performance liquid chromatography separations of fractions 1–5 for the ethanol extract from Newton wheat flour on a Sephacryl S-200 column. Fractions pooled as in Fig. 2B.

showing that ESG compositional differences influence functional characteristics. Additional data (not shown) also suggest that fractions 1 and 2 have similar compositions in some wheat cultivars but differ slightly in others.

Chemical Analyses of S-200 Fractions

There has been some indication that carbohydrate may be associated with ESG. When concentrated (e.g., eluted from a gel-filtration column), ESG has reduced solubility and a milky appearance (Bietz and Wall 1973). Therefore, we tested protein fractions for carbohydrate using the phenol-sulfuric acid test. We found little carbohydrate in the protein fractions (Fig. 2B): 2–8% in ESG fraction 1, 0.9 ± 0.5% in ESG fraction 2, and <0.5% in monomeric gliadins (fraction 4). Bernardin et al (1976) also showed that LMW monomeric gliadins are not glycoproteins. However, analyses of various extracted albumin or globulin fractions (fractions 1 and 2, Fig. 2A) revealed 15–25% carbohydrate.

Amino acid analyses were also done on ESG fractions 1 and 2 (Fig. 2B); on the monomeric gliadin fraction (fraction 4, Fig. 2B); and on albumins or globulins coextracted with gliadins (fraction 5, Fig. 2B). Results (not shown) revealed fractions 1 and 2 (ESG) were similar to LMW-GS (Bietz and Wall 1973, Huebner et al 1974). The composition of fraction 4 was typical of monomeric gliadins, and the composition of fraction 5 was typical of albumins or globulins.

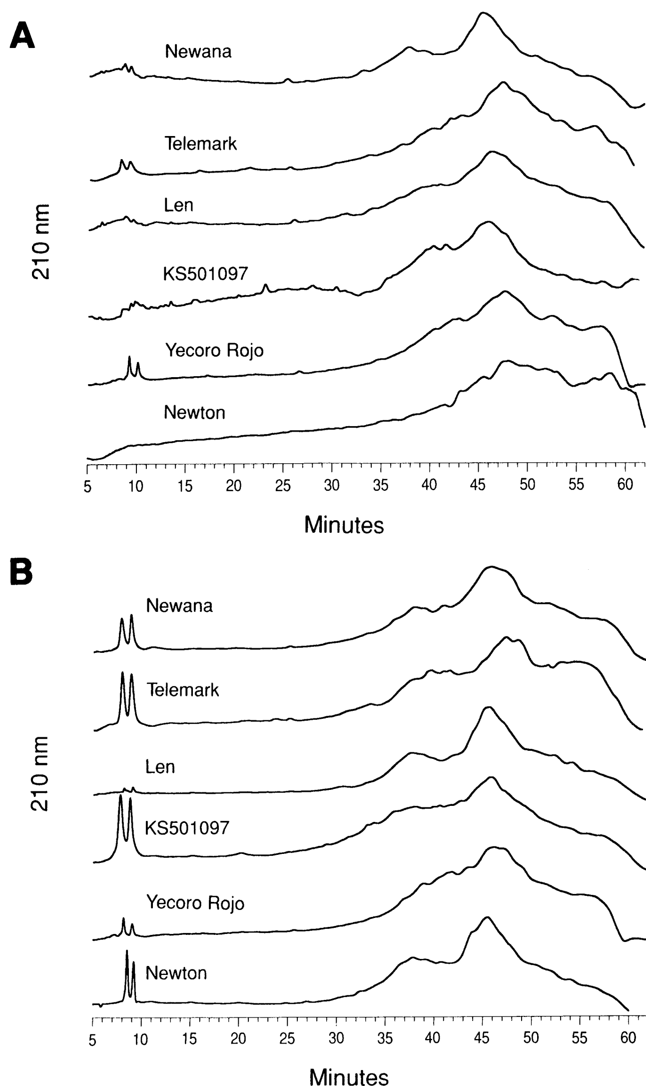

Fig. 6. Reversed-phase high-performance liquid chromatography separations of unreduced low molecular weight glutenin subunits from hard red spring and winter wheat cultivars. **A,** fraction 1. **B,** fraction 2. Chromatograms were normalized so the highest peak in each had the same absorbance. Relative amounts of protein in fractions are not to scale.

DISCUSSION

SE-HPLC on an S-200 column provides excellent separations of ESG from monomeric gliadins. It is much faster than preparative size-exclusion media used previously. Columns can be scaled to separate amounts of sample as needed. With S-200, however, solvent conditions are more specific and critical, presumably because ionic or hydrophobic sites must be masked for the column to operate entirely in a size-exclusion mode. The volatile solvent used with S-200 also greatly simplifies recovery of fractions for further analyses.

The ESG (first) peak to elute from S-200 (Fig. 2) is large and varies in size among samples. It often appears to consist of two fractions (Fig. 3). Nevertheless, the amount of ESG recovered (20–37% of total protein extracted with ethanol) is less than the amount of monomeric gliadins. However, many of our earlier studies, using conventional gel filtration, suggested much less ESG in gliadin extracts. Determining the actual amount of ESG is also complicated by its characteristic turbidity, leading to anomalously high UV absorbance (Bietz and Wall 1973). In the present study, recovery of ESG may have been enhanced by the second ethanol extraction and the different preparative method.

This study does confirm the quantitative significance of ESG. Many previous investigations concentrated on HMW-GS and monomeric gliadins, while ignoring this fraction. We now need to consider the contributions of ESG to quality and functionality.

In this study, we examined both hard red spring and winter wheats. Quantitative differences among gliadin fractions occurred among wheat cultivars, but no consistent relationship was noted between the two wheat classes. Results for Yecoro Rojo appeared particularly atypical. Yecoro Rojo is a spring wheat, grown in California and Arizona as a winter wheat. Thus, location or climatic effects, as well as genotype, could influence its composition. In any case, our results suggest the desirability of examining more samples with known mixing and baking characteristics to find correlations between LMW-GS and flour or dough parameters.

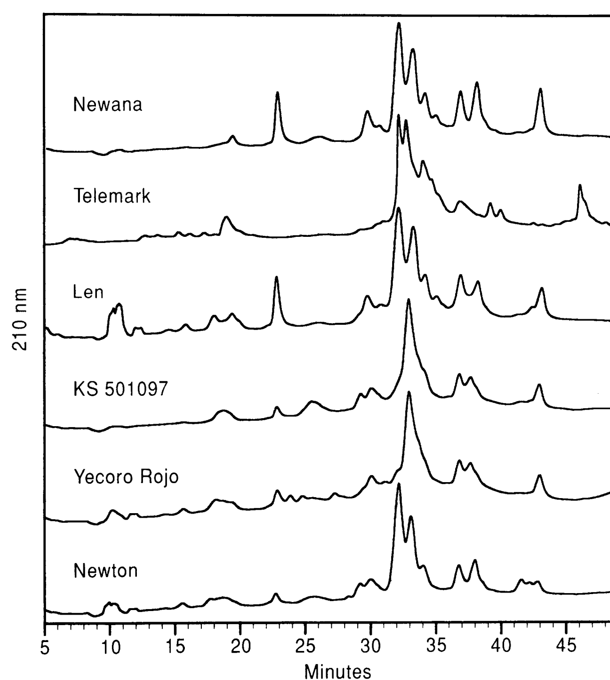

Fig. 7. Reversed-phase high-performance liquid chromatography separations of reduced high molecular weight gliadin from hard red spring and winter wheat cultivars. Fractions 1 and 2 combined. Proteins reduced with 0.2% dithiothreitol in 0.05*N* sodium phosphate, pH 7.7, and alkylated with 4-vinylpyridine.

After reduction, ESG subunits elute during RP-HPLC in the same area as monomeric gliadins. Relatively little is known about these subunits and how they may affect mixing or baking quality characteristics. Statistical evaluation of more well-characterized samples (work is currently in progress) should help answer that question.

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