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Extraction of up to 95% of Wheat (*Triticum aestivum*) Flour Protein Using Warm Sodium Dodecyl Sulfate (SDS) without Reduction or Sonication

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Extraction of glutenin polymers without sonication is an essential prerequisite for accurate determination of their composition and molecular size distribution. Sequential fractionation of wheat flour with 0.1 M KCl and 0.25% sodium dodecyl sulfate (SDS) at 21 °C and 2% SDS at 60 °C extracted up to 95% of total protein. We propose that 2% SDS at 60 °C disrupts hydrogen bonds in glutenin and gliadin aggregates, reduces hydrophobic interactions, and facilitates solubilization. Analysis by size-exclusion high-performance liquid chromatography (SE-HPLC), reverse-phase (RP)-HPLC, and SDS−polyacrylamide gel electrophoresis (PAGE) revealed that partitioning of gliadins and glutenins among the extracts differed for two flours with good baking quality (Butte 86 and Jagger) and one with poor baking quality (Chinese Spring). More gliadin was associated with the 0.25% SDS extract for Chinese Spring, whereas more gliadin was associated with the 2% SDS extract for Butte 86 and Jagger. Unextractable glutenin polymer was only 4−5% of total protein for Butte 86 and Chinese Spring and 14% for Jagger.

KEYWORDS: Flour quality; fractionation; glutenin; gliadin; HPLC; polymer

INTRODUCTION

Flour quality in large part depends upon the monomeric gliadins and polymeric glutenins that together produce the viscoelastic properties that are unique to wheat doughs. Glutenin subunit type and amount, polymer amount, and polymer size-distribution profiles have been hypothesized to account for major differences in flour-baking quality (*1*–*5*). However, measuring glutenin polymer size distribution is difficult. It is also challenging to study interactions between gliadins and glutenins. Therefore, most reports that assess the relationship between protein and dough strength simply measure differences in amount and extractability of the gliadins and glutenins.

Under most circumstances, gliadins and glutenins are not soluble in water. Typical solvents, such as 0.5% sodium dodecyl sulfate (SDS), 0.1 N acetic acid, 40% ethanol, or 50% propanol, that interrupt hydrogen bonds and hydrophobic interactions between the proteins are used to extract gliadins and glutenins (*6, 7*). Acetic acid is commonly used as a solvent because is tolerated by high-performance liquid chromatography (HPLC) columns and is easily removed. High concentrations of urea and guanidine-HCl (*8*) or SDS are effective but are more difficult to work with. These commonly used solvents solubilize only part of the glutenin polymer. For example, SDS concentrations between 0.5 and 1.5% solubilized only 57% of the glutenin polymer from weak flours and as little as 22% from very strong flours (*1, 4*). Therefore, insoluble protein is often solubilized by sonication, which is likely to alter polymer size distribution or is extracted after use of a reducing agent, such as dithiothreitol (DTT), to disrupt the intermolecular disulfide bonds that link the high-molecular-weight glutenin subunits (HMW-GSs) and low-molecular-weight glutenin subunits (LMW-GSs).

Although complete solubilization is desirable for physical studies of the glutenin polymer, aggregative properties of glutenin are also of interest. For evaluation of glutenin polymer extractability, one commonly used term is unextractable polymeric protein (UPP). Although the definition of UPP varies somewhat among reports, it is generally that of Gupta et al. (*1*), in which flour protein is extracted with 0.5% SDS in 0.1 M NaP_i (extractable protein). The remaining protein is then extracted in the same buffer with sonication (unextractable protein). The polymer amount is then determined for both fractions by size-exclusion (SE)-HPLC, and percent UPP is calculated as the ratio of unextractable polymer to total polymer. Unlike total polymer, the percent UPP correlated well with measures of dough strength for *Triticum aestivum* and *Triticum*

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durum flours (*1, 9, 10*). Protein remaining after extraction with 50% propanol also correlated with measures of dough strength (*11*).

SE-HPLC is used to estimate polymer and monomer proportions but not to determine polymer size distribution because most glutenin polymers are so large that they emerge in the void volume. Analyses by SE-HPLC have generally used 0.1% SDS or acetonitrile and trifluoroacetic acid (TFA). Some improvement may be possible using Superdex size-exclusion columns that have a size range up to 660 000 and tolerate up to 1.0% SDS (*12*). Size distributions of larger polymers have been estimated by the combination of flow field flow fractionation and multiple-angle laser light scattering (MALLS) (*2, 4, 6, 7, 13, 14*). The unextractable polymers that are most correlated with dough strength have only been analyzed after sonication. A recent paper (*2*) estimated the size range of sonicated glutenin polymers to be between 2×10^5 and 2×10^7. Although it may be impossible to solubilize and measure the entire size range of undenatured, nonsonicated, unreduced glutenin polymers, it is desirable to optimize the amount of glutenin polymer that can be analyzed, to determine the physical properties of these unique large molecules.

Studies of wheat flour protein composition in our laboratory have used a single hard red spring wheat Butte 86 to ensure consistency between measures of gene expression, protein accumulation, and protein composition during grain development and under different environmental conditions (*15–17*). Methods for protein extraction included one designed to maximize separation of gliadins from glutenins, for quantification of gluten protein fractions (*18*), and one designed to separate nongluten from gluten proteins, for identification of the nonstorage proteins (*16, 19*). In this paper, a three-step extraction is described in which up to 95% of flour protein is solubilized without sonication or reduction, to maximize extraction of intact glutenin polymer. Albumins and globulins were extracted with KCl prior to extracting the gliadins and glutenins with SDS. When the three fractions were analyzed by SE-HPLC on a Superdex column, differences among flours from three wheat varieties in gliadin/glutenin interactions were also revealed. This method will be useful for studies of the polymer composition and structure as well as examining effects of genes and environment on gliadin/glutenin interactions.

MATERIALS AND METHODS

Plant Materials. Plants of the U.S. hard red spring wheat *T. aestivum* "Butte86" and hard red winter wheat "Jagger" were grown at 24 °C days and 17 °C nights with drip irrigation as described in Altenbach et al. (*15*). Plants were watered by drip irrigation with 0.6 g/L Plantex fertilizer (NPK, 20:20:20). Samples of 100 g each were milled to flour and were analyzed for flour quality at the Hard Winter Wheat Quality Laboratory (USDA, Agricultural Research Service, Manhattan, KS). Three separate samples of flour from each variety were extracted and analyzed. Break flour from field-grown Chinese spring, of poor baking quality (*20*), was obtained from Dr. D. Kasarda, USDA, Albany, CA.

Protein Extractions. Albumins and globulins were extracted from 400 mg of flour with 2 mL of 100 mM KCl and 5 mM ethylenediaminetetraacetic acid (EDTA) in 50 mM Tris-HCl at pH 7.8 by stirring on ice for 5 min. The mixture was centrifuged at 14500*g* for 20 min. The albumin and globulin-rich supernatant solution is referred to as the KCl extract (*16*). Gliadins were then extracted from the pellet by stirring for 1 h with 6 mL of 0.25% SDS in borate buffer consisting of 0.1% glycine in 50 mM $Na_2BO_4 \cdot 10\ H_2O$ and adjusted to pH 8.5 with HCl. The mixture was centrifuged at 40000*g* for 30 min, using a Sorval High Speed RC2B centrifuge and an SS34 rotor at 18 200 rpm. The gliadin-rich supernatant solution is referred to as the 0.25% SDS extract. The remaining gliadins and most polymeric glutenin were extracted by stirring at 60 °C for 2 h with 2.0% SDS in 6 mL of borate buffer. The mixture was centrifuged at 40000*g* for 30 min. The glutenin-rich supernatant solution is referred to as the 2% SDS extract. All remaining protein in the pellet was then extracted by stirring at room temperature for 1 h with 2% SDS in 6 mL of borate buffer with 50 mM DTT, followed by centrifugation at 40000*g* for 30 min. The supernatant is referred to as the 2% SDS/DTT extract.

Size-Exclusion HPLC. Each extract was run in triplicate. KCl, 0.25% SDS, and 2% SDS extracts were applied to a SE-HPLC column, Superdex 200 10/300 GL, 10.300 mm (GE Healthcare/Amersham/Pharmacia). The injection volume was 15 μL, equivalent to 3 mg of flour for the KCl extract or 1 mg of flour for the SDS extracts. A Hewlet Packard Series 1100 HPLC (Wilmington, DE) was used to elute the proteins. Running buffer was 0.1% SDS in 0.1 M Na phosphate buffer at pH 6.8 at 0.5 mL min, with the detector set at 210 nm. To obtain reproducible SE-HPLC profiles, it was essential to pre-equilibrate the SE-HPLC column for 4 h. If the three replicate runs were not identical, a new extract was prepared and three new chromatographs were performed. The column resolved standard proteins in a size range from 12 000 to 660 000 Da (Figure S1 in the Supporting Information). Elution times for gliadins were somewhat retarded compared to those of the standard proteins. Fractions were collected with a Gilson fraction collector at 1 min intervals and precipitated with 4 volumes of acetone at −20 °C.

Reverse-Phase HPLC. Freeze-dried proteins were dissolved at a concentration of 1 mg mL^{-1} in 6 M guanidine HCL adjusted to pH 8.0 with TRIS plus 50 mM DTT and alkylated with vinylpyridine prior to HPLC. Proteins were analyzed using a Hewlet Packard Series 1100 HPLC (Wilmington, DE). A volume of 0.5 mL (0.5 mg) of the protein solution was applied to a Nucleosil (Ansys, Lake Forest, CA) C8 analytical column. Proteins were eluted using a gradient with a 10 min delay followed by an increase from 10 to 65% acetonitrile and 0.5% TFA at 0.8 mL min^{-1} for 60 min. Peptide bond absorbance was detected at 210 nm. Each analysis was performed in triplicate. Peaks were collected by hand and freeze-dried (*17*).

SDS−Polyacrylamide Gel Electrophoresis (PAGE). Freeze-dried or acetone-precipitated protein samples were suspended in Novex NuPage sample buffer (Invitrogen, Carlsbad, CA) with 50 mM DTT by vortexing for 1 h at 22 °C in a TOMY MT-360 Microtube Mixer (Tomy Seiko, Tokyo, Japan) and then centrifuged for 10 min at 14 000 rpm in a microfuge. To achieve the amounts of sample indicated in the figures, 5−20 μL was loaded onto a Novex NuPAGE 4−12% acrylamide, Bis-Tris gel and separated using the Novex MES SDS running buffer. The Mark12TM protein standard (Invitrogen) was used. Gels were stained overnight using Brilliant Blue G (Sigma, St. Louis, MO) and destained with water for less than 8 h or stored in 20% ammonium sulfate to ensure retention of the ω-gliadins in the gels.

Protein Identification. Abundant, well-characterized, and easily recognized storage proteins dominate the patterns obtained by SDS−PAGE, reverse-phase (RP)-HPLC, or SE-HPLC of wheat flour protein extracts (*3, 18, 21–23*). Protein patterns for the wheat variety Butte 86 and Chinese Spring were previously described (*23*). Identifications in this paper were also confirmed by mass spectrometry of digested gel lanes (unpublished data).

Protein Quantification. To determine the relationship between the area under the SE-HPLC trace and the area under the RP-HPLC trace for the same amount of protein, the areas for the 0.25 and 2% SDS extracts were compared by both methods. An equivalence of 1.5:1 was determined for the total area under the SE-HPLC trace in 0.1% SDS to the total area under the RP-HPLC trace of vinyl-alkylated subunits in acetonitrile (Table S1 in the Supporting Information). The optical density at 210 for protein dissolved in acetonitrile is determined mainly by the amide bond and has a close relationship to the protein amount (*24*). This may not be the case for SE-HPLC in an aqueous buffer with SDS, where light scattering and/or absorbance may also contribute to the measure of optical density. SE-HPLC may also overestimate the proportion of protein in the void volume, where absorbance or light scattering may be increased by the presence of nonprotein compounds or be affected by polymer size. Therefore, the HPLC areas are used to

estimate the relative protein amounts and detect differences between treatments or varieties, with the caveat that systematic errors in actual protein amount may occur. Alternative methods would also have problems, because glycine would interfere with determining nitrogen by combustion, and the Lowry method underestimates gliadins and glutenins. In contrast, the absorbance at 210 allows estimation of the size distribution of proteins when analyzed by SE-HPLC. The percentage of individual flour components was calculated from the HPLC areas as (area per milligram of flour for each component) × 100/(total area per milligram of flour).

Statistical Analysis. Extracts were obtained from three samples of flour for each wheat variety. Each extract was analyzed in triplicate, giving a total of nine analyses for each extract type for each variety. Data were analyzed by single-factor analysis of variation (ANOVA).

RESULTS

Flour Quality. On a mixing tolerance scale of 0−6, where 0 is poor and 6 is excellent, the flour sample from Butte 86 was scored at 3 and that from Jagger was scored at 4. Flour from field-grown Chinese spring is generally known to be of poor baking quality (*20*).

Fractionation and Analysis of Flour Proteins. Several methods for serial extraction with SDS were tested to increase extraction of glutenin polymers while reducing cross-contamination of protein types. The three-step extraction with buffered solutions of 0.1 M KCl and 0.25% SDS (at 22 °C) and 2% SDS (at 60 °C) was as effective as methods that included additional extractions with intermediate increments of SDS concentration. The three-step extraction was compared for flours from Butte 86, for which the laboratory has extensive data, and for two other flours, Chinese Spring and Jagger.

To evaluate the effectiveness of the fractionation procedure, the fractions were evaluated by SDS−PAGE under reducing and nonreducing conditions that distinguish polymeric glutenin from monomeric gliadins. The results were similar for all three varieties, as illustrated for Butte 86 and Chinese Spring (**Figure 1**). The unreduced fractions from all three varieties were evaluated by SE-HPLC on a Superdex column (parts **A**−**C** of **Figure 2**). The SE-HPLC fractions for Butte 86 were subsequently analyzed by SDS−PAGE under reducing and nonreducing conditions (parts **D**−**F** of **Figure 2**). The residual protein was extracted with SDS/DTT and analyzed by RP-HPLC for all three varieties (**Figure 3**).

KCl Extract. Proteins with a wide range of molecular weights were detected in the KCl extract, many of which were previously identified in a two-dimensional gel/proteomic study for Butte 86 (*16*). The majority of the albumins/globulins are in the broad size range shown in lanes 1 and 2 of **Figure 1** and parts **A** and **D** of **Figure 2** and are ignored by the classic method of Gupta et al. (*1*). SE-HPLC profiles for the KCl extract obtained from the three varieties differed somewhat (**Figure 2A**). Varietal differences in albumins and globulins are discussed in ref *25*.

There was little polymeric glutenin in the KCl extracts (lanes 1 and 2 of **Figure 1** and parts **A** and **D** of **Figure 2**). Note that the amount of protein in lanes 1 and 2 of **Figure 1** was equivalent to that extracted from 5 times as much flour as that in lanes 3−6, and the protein in parts **A** and **D** of **Figure 2** was equivalent to that from 3 times as much flour as that in parts **B**, **C**, **E**, and **F** of **Figure 2**. Reduction of intramolecular disulfide bonds with DTT altered the mobility of several protein bands, including the prominent α-amylase trypsin inhibitor band of approximately 12 000 Da (lanes 1 and 2 of **Figure 1**). Additional low-molecular-weight bands appear in lane 2 that may be reduced components of nonglutenin polymers or oligomers.

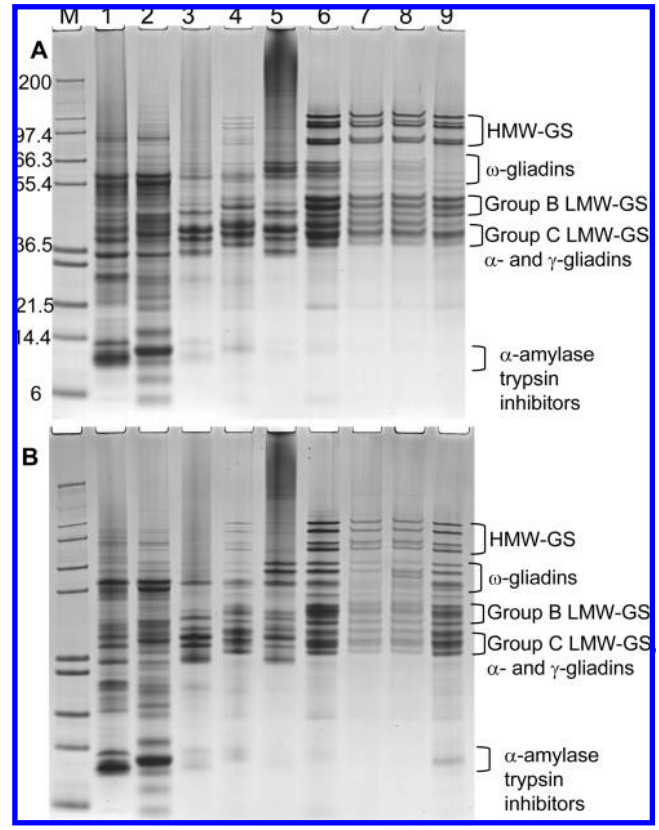

Figure 1. Flour protein extracts analyzed by SDS−PAGE of protein extracts from (A) Butte 86 and (B) Chinese Spring. Unreduced protein is in lanes 1, 3, and 5. Reduced protein is in lanes 2, 4, and 6−9. M, molecular weight standards; lanes 1 and 2, KCl extracts equivalent to 160 μg of flour; lanes 3 and 4, 0.25% SDS extracts equivalent to 33 μg of flour; lanes 5 and 6, 2% SDS extracts equivalent to 33 μg of flour; lanes 7 and 8, 2% SDS/DTT extracts equivalent to 100 μg of flour. (A) Lane 9, 5 μg of a glutenin extract from Butte 86 (*18*). (B) Lane 9, total flour extract equivalent to 50 μg of Chinese Spring flour.

SE-HPLC on other column types is reported to resolve a peak or area enriched in the 12 000 to 14 000 Da α-amylase-trypsin inhibitors. When total flour extracts are analyzed by SE-HPLC, that peak is generally referred to as the albumin/globulin section of the SE-HPLC profile (*1*). Although SDS−PAGE of the KCl extract showed the dark band of α-amylase-trypsin inhibitor at approximately 12 000 (lanes 1 and 2 of **Figure 1**), only small amounts of protein in that size range emerged from the Superdex column. The separate peak at 43 min did not contain protein (parts **A** and **D** of **Figure 2**). Gel electrophoresis of the KCl extract before and after prefiltration demonstrated some loss of the 12 000 Da proteins on the filter. However, omitting the prefiltration step did not improve recovery from the column. After testing a number of column buffers and washes, we concluded that the α-amylase-trypsin inhibitors were selectively bound to the Superdex column and were not eluted. It is difficult to compare these results with those of other reports that use SE-HPLC but did not show gels or used gels that did not resolve proteins under 20 000 Da.

0.25% SDS Extract. SE-HPLC profiles for the 0.25% SDS extracts from all three varieties were nearly identical (**Figure 2**B). The 0.25% SDS extracts contained mostly monomeric proteins in the size range of 30 000 to 40 000 Da that decreased in mobility upon reduction of disulfide bonds (lanes 3 and 4 in **Figure 1** and parts **B** and **E** of **Figure 2**). The predominant flour proteins in this size range are α- and γ-gliadins. There were few ω-gliadins in the 0.25% SDS extract. **Figure 1** shows

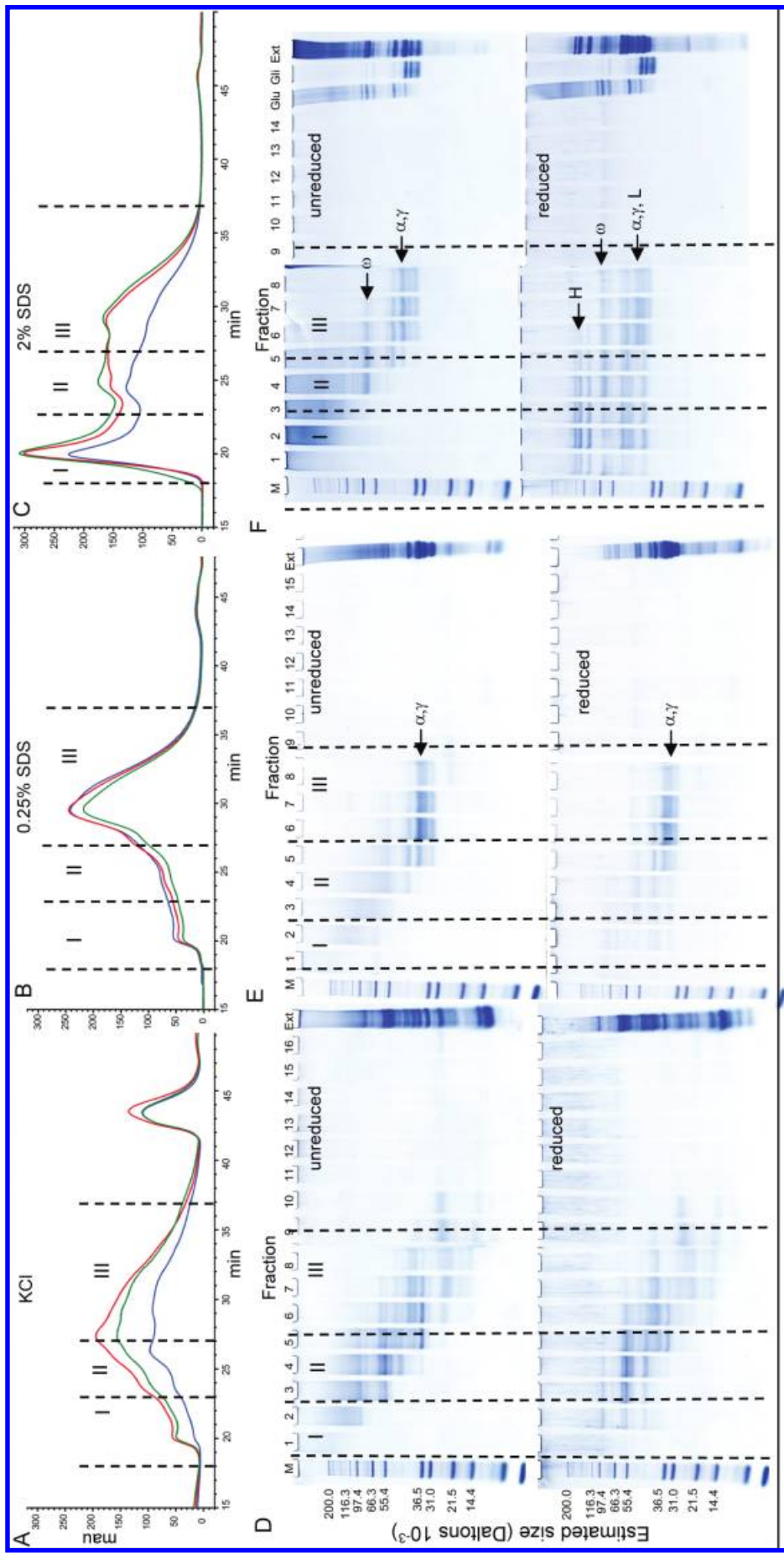

Figure 2. Flour protein extracts analyzed by SE-HPLC and SDS−PAGE. (A−C) Typical SE-HPLC traces for the KCl, 0.25% SDS, and 2% SDS extracts. Flour is from Butte 86 (green), Jagger (red), and Chinese Spring (blue). (A) KCl extracts equivalent to 3 mg of flour, (B) 0.25% SDS extracts equivalent to 1 mg of flour, and (C) 2% SDS extracts equivalent to 1 mg of flour. Vertical dashed lines divide the HPLC trace into regions I, II, and III. (D−F) SDS−PAGE of the unreduced and reduced SE-HPLC fractions from Butte 86. (D) KCl extract, (E) 0.25% SDS extract, and (F) 2% SDS extract. Fractions were collected at 1 min intervals. Two or more fractions were then pooled, freeze-dried, and resuspended in gel buffer, and $^1/_8$ of the pooled fraction was loaded per lane. Numbered lanes indicate pooled SE-HPLC fractions. M, molecular size markers; Ext, total protein in extract; Glu, an ω-gliadin-rich fraction; and Gli, an ω-gliadin-rich fraction, both obtained from Chinese Spring. Abbreviations: α, α-gliadin; γ, γ-gliadin; ω, ω-gliadin; H, HMW-GS; L, LMW-GS.

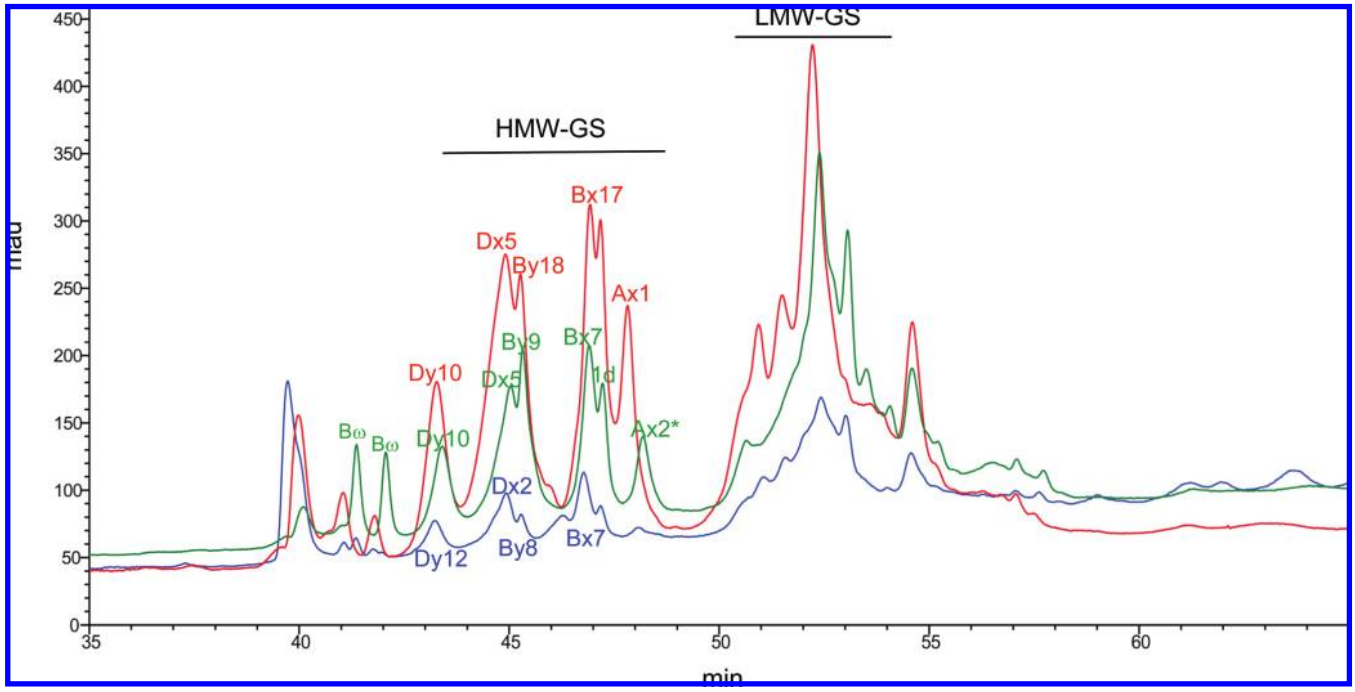

Figure 3. Residual protein extracts analyzed by RP-HPLC. RP-HPLC traces for the 2% SDS/DTT extracts for flour from Butte 86 (green, equivalent to 5.4 mg of flour), Jagger (red, equivalent to 3.5 mg of flour), and Chinese Spring (blue, equivalent to 3.7 mg of flour). HMW-GS are indicated. The HMW-GS assignments for Jagger are based on Pike and MacRitchie (*34*). 1d is a putative ω-gliadin-like subunit.

that a small amount of glutenin polymer was present in the 0.25% SDS extracts from Butte 86 and Chinese Spring, visualized as unresolved smears of protein in lane 3 and lightly stained HMW-GS bands in lane 4. Any LMW-GS bands in lane 4 cannot be distinguished from the abundant α- and γ-gliadin monomers.

SE-HPLC of the 0.25% SDS extract revealed little protein in the void volume (parts **B** and **E** of **Figure 2**). SDS−PAGE of the unreduced proteins revealed a small amount of polymer in the fractions that eluted between 18 and 29 min (lanes 1−4 in **Figure 2E**). The size range of these polymers gradually decreased. When reduced, lightly stained bands of HMW-GS and LMW-GS were revealed in these fractions, as well as possible ω-gliadins. The bulk of the protein eluted in a broad peak with a maximum optical density at 30 min. Those proteins were resolved in the size range of the α- and γ-gliadins in SDS−PAGE.

2% SDS Extract. The three varieties clearly differed in the proportion and distribution of protein in the 2% SDS extract (**Figure 2C**). The 2% SDS extract contained a mixture of glutenin polymer, ω-gliadins, and α- and γ-gliadins (lanes 5 and 6 in **Figure 1** and parts **C** and **F** of **Figure 2**). The unreduced polymer was evident as a dark smear from the loading well to around 97 000 Da as well as smaller oligomers between 150 000 and 97 000 Da (lane 5 in **Figure 1**). The ω-, α-, and γ-gliadin monomers were also evident in the unreduced fractions. The prominent ω-gliadin bands did not change in mobility upon reduction, because they have no disulfide bonds. Upon reduction, the four or five HMW-GS were resolved between 70 000 and 112 000 Da (lane 6 in **Figure 1**). The reduced LMW-GS were resolved between 30 000 to 45 000 Da and overlap the monomeric α- and γ-gliadins. Few proteins smaller in size than 30 000 Da were present in the 2% SDS extract.

SE-HPLC of the 2% SDS extract revealed proteins in a broad size range. The large void volume peak at 20 min was followed by smaller, broad peaks with maximum optical density at 25 and 30 min (**Figure 2C**). SDS−PAGE of the fractions revealed considerable amounts of unreduced proteins in the void volume peak from 18 to 22 min and trailing into fractions collected from 22 to 28 min (lanes 1−5 in **Figure 2F**). Monomeric proteins in the size range of the ω-gliadins were in fractions that eluted between 24 and 32 min (lanes 4−6 in **Figure 2F**), and monomeric proteins in the size range of the α- and γ-gliadins in fractions that eluted from 26 to 35 min (lanes 5−8 in **Figure 2F**). SDS−PAGE of the reduced fractions shows the HMW-GS and LMW-GS patterns (lanes 1−6 in **Figure 2F**, 18−30 min). Additional proteins in the size range of the chromosome 1B encoded ω-gliadins were also revealed (lanes 1−3 in **Figure 2F**, 18−24 min) only after reduction, suggesting that these proteins were incorporated into the glutenin polymer.

SDS/DTT Extract. The remaining protein that was extracted from Butte 86 with 2% SDS/DTT consisted mainly of HMW-GS and LMW-GS (lanes 7 and 8 in parts **A** and **B** of **Figure 1** and **Figure 3**). The biggest differences between the varieties were in the amount of this unextractable polymer (**Figure 3**). Note that the protein in lanes 7 and 8 in parts **A** and **B** of **Figure 1** was equivalent to that extracted from 3 times as much flour as in lanes 3−6. The SDS/DTT extract was analyzed by RP-HPLC for purposes of quantification (**Figure 3**).

Protein Quantities. Areas under the HPLC traces were calculated and used to estimate protein distribution among the extracts (**Table 1**). Total flour protein was highest for Jagger and lowest for Chinese Spring. Total protein extracted, including that with SDS/DTT, was correlated with flour protein content ($r^2 = 0.999$), as expected. The intercept for a total HPLC area of zero was 4.8% protein, however. The most likely explanation was that not all protein was recovered from the column, as discussed for the α-amylase-trypsin inhibitors above.

The KCl extract, considered to be mainly albumins/globulins, was 12% of total extractable protein for Butte 86 and Jagger but only 9% for Chinese Spring. The 0.25% SDS extract, considered to be mainly α- and γ-gliadins, was only 33% of total extractable protein for Butte 86 and 31% for Jagger but

Table 1. Area under HPLC Traces for Sequential Extracts of Protein from Flour from Chinese Spring, Butte 86, and Jagger

	Chinese Spring		Butte 86		Jagger		Chinese Spring	Butte 86	Jagger
extract	area[a]	SD	area[a]	SD	area[a]	SD	%	%	%
KCl	20 506	750	34 000	*1044*	37 930	*1711*	8.7	12.2	11.5
0.25% SDS	107 925	3455	90 961	*2219*	103 087	*5496*	45.7	32.5	31.4
2% SDS	96 155	1976	143 383	*4915*	141 864	*6485*	40.7	51.3	43.3
2% SDS/DTT	11 590	1355	11 114	*470*	44 424	*4478*	4.9	4.0	13.6
total	236 176		279 458		327 305		100.0	100.0	100.0

[a] Area (mau) normalized for 1 mg of flour. Average of data for four replicate extractions, as depicted in **Figures 2** and **3**. Flour protein content was 12.7% for Chinese Spring, 14.0% for Butte 86, and 15.6% for Jagger. The area of the SDS/DTT RP-HPLC trace was multiplied by the 1.5 RP-HPLC/SE-HPLC factor.

Table 2. Proportional Areas under the SE-HPLC Traces[a]

			I 18−23 min[b]	II 23−28 min[b]	III 28−37 min[b]	
extract	variety		alb/glob	alb/glob	alb/glob	total
KCl	Chinese Spring	area	8.68	35.86	55.46	100
		variance	*1.09*	*0.04*	*0.91*	
	Butte 86	area	11.20	36.58	51.89	100
		variance	*0.89*	*0.32*	*0.36*	
	Jagger	area	11.97	38.20	49.83	100
		variance	*0.05*	*0.005*	*0.08*	

			large polymer	smaller polymer and ω-gliadins	α- and γ-gliadins	
0.25% SDS	Chinese Spring	area	10.10	25.19	64.72	100
		variance	*0.82*	*0.02*	*0.60*	
	Butte 86	area	9.02	23.65	67.34	100
		variance	*0.05*	*0.06*	*0.16*	
	Jagger	area	9.72	25.83	64.45	100
		variance	*0.04*	*0.25*	*0.48*	
2% SDS	Chinese Spring	area	36.70	35.92	27.38	100
		variance	*0.42*	*0.01*	*0.45*	
	Butte 86	area	33.90	33.08	33.02	100
		variance	*1.39*	*0.13*	*0.67*	
	Jagger	area	33.66	33.70	32.64	100
		variance	*0.42*	*0.09*	*0.13*	

[a] Average of data for the three replicate extractions in **Table 1**. [b] The areas were subdivided as shown in **Figure 2**.

46% for Chinese Spring. The 2% SDS extract, considered to be a mixture of glutenin polymer plus α-, γ-, and ω-gliadins, was 51% for Butte 86, 43% for Jagger, and only 40% for Chinese Spring. The 2% SDS/DTT extract, considered to be mainly glutenin, was only 4.9% for Chinese Spring and 4.5% for Butte 86 but 13.6% for Jagger.

Proportional Distribution of Protein Types Based on SE-HPLC and RP-HPLC of the Four Protein Extracts. The size-distribution of the proteins within the extracts was evaluated by calculating the SE-HPLC areas from regions I, II, and III of parts **A**−**C** of **Figure 2** (**Table 2**) based on the divisions made in ref *1*. Regions I−III contain a wide variety of proteins for the KCl extract. For the 0.25 and 2% SDS extracts, region I represents mainly larger glutenin polymer, region II represents smaller polymers and ω-gliadins, and region III represents mainly α- and γ-gliadins. The size distributions within the 0.25% SDS extracts were similar for the three varieties. The largest proportion of regions I and II polymer for the 2% SDS extract was for Chinese Spring, and the largest proportions of region III α- and γ-gliadin in the 2% extract were for Butte 86 and Jagger.

The data from **Tables 1** and **2** were used to estimate total extractable glutenin and gliadin (**Table 3**). Region III α- and γ-gliadins in the 0.25% SDS extract were estimated to be 30% of total protein for Chinese Spring, 21% for Butte 86, and 20% for Jagger. Region III α- and γ-gliadins in the 2% SDS extract were estimated to be 11% of total protein for Chinese Spring, 17% for Butte 86, and 14% for Jagger. The sum of the α- and γ-gliadins was 41% of total extractable protein for Chinese Spring, 39% for Butte 86, and only 34% for Jagger. Total extractable polymer was calculated as region I plus region II to include the same range of proteins as in ref *1*, even though this number also encompasses the ω-gliadins. Polymer in the 0.25% SDS extract was estimated to be 16% of total protein for Chinese Spring, 11% for Butte 86, and 11% for Jagger. Polymer in the 2% SDS extract was estimated to be 29% of total protein for Chinese Spring, 34% for Butte 86, and 29% for Jagger. Unextractable protein in the 2% SDS/DTT extract was 5% for Chinese Spring, 4% for Butte 86, and 14% for Jagger. Total polymers were 50% of the total protein for Chinese Spring, 49% for Butte 86, and 54% for Jagger.

Table 3. Estimates of Extractable Gliadins and Glutenins and Insoluble Glutenin Polymers as a Percentage of Total Protein[a]

	Chinese Spring	Butte 86	Jagger
α- and γ-gliadins			
0.25% SDS region III	29.6	21.1	20.2
2% SDS region III	11.1	16.9	14.1
total α- and γ-gliadin	40.7	38.0	34.3
soluble polymeric protein			
O.25% SDS			
region I	4.6	2.9	3.1
region II[b]	11.5	7.7	8.1
total	16.1	10.6	11.2
2% SDS			
region I	14.9	17.3	14.6
region II[b]	14.6	17.0	14.6
total	29.5	34.3	28.6
total soluble polymeric protein	45.6	44.9	39.8
unextractable polymer			
2% SDS/DTT	4.9	4.0	13.6
total polymer, with ω-gliadins	50.5	48.9	54.0

[a] Calculated using data from **Tables 1** and **2**. [b] Includes ω-gliadins.

DISCUSSION

It is difficult to optimize both extraction and separation of wheat flour protein types. A method that used NaI to cleanly separate gliadins from glutenins (*5, 18*) was not suitable for extraction of glutenin polymer. The method in this paper optimized solubilization of the glutenin polymer but not separation of gliadins from glutenins. Unlike other reports, however, the method extracted from 74 to 92% of the intact polymer. In addition, the method separated the polymers from albumins/globulins and α- and γ-gliadins to a greater extent than the standard method of ref *1*.

There are advantages and disadvantages to the initial extraction with 0.1 M KCl. Because many albumins and globulins are in the same size range as gliadins (*16, 19, 25*), it is advantageous to remove them before quantifying gliadins and

glutenins by SE-HPLC. However, it is possible that pre-extraction of albumins/globulins with a salt solution made it more difficult to separate gliadins from glutenins (*21*). This method could be simplified by omitting the extraction with KCl and/or 0.25% SDS and then collecting total soluble polymer by SE-HPLC of the 2% SDS extract for analysis of size distribution by field flow fractionation and MALLS.

The three-step extraction revealed several trends that may be associated with flour strength, although this must be tested using samples from more varieties and environments. For the two strong flours, Butte 86 and Jagger, there was increased protein in the albumin/globulin, 2.0% SDS, and SDS/DTT extracts but decreased protein in the 0.25% SDS extract compared to Chinese Spring. There was more monomeric α- and γ-gliadin in the 0.25% SDS extract from Chinese Spring but more monomeric α- and γ-gliadin in the 2% SDS extract for Butte and Jagger. Total polymer was higher for Jagger but not for Butte 86. The biggest differences were for the proportion of unextractable polymer for Jagger and α- and γ-gliadin monomers in the 2.0% SDS extract for Jagger and Butte 86. The ratio of total glutenin polymer to total α- and γ-gliadin monomers was only 1.24 for Chinese Spring and 1.29 for Butte 86 but 1.57 for Jagger.

Southan and MacRitchie (*4*) proposed that there is a higher molecular-weight distribution for polymers in stronger flours that is responsible for the greater degree of percent UPP. Gupta et al. (*1*) reported that total polymer did not correlate well with quality, but percent UPP did. Because the method in this paper solubilized more polymer, it is more closely measures differences in total polymer than in the type of aggregation measured as percent UPP. However, more unextractable protein for Jagger and more polymer in region I of the 2% SDS extract for Butte 86 may also indicate a higher molecular-weight distribution for polymers from these varieties than for Chinese Spring.

An unexpected difference between flour from Jagger and Butte 86 compared to Chinese Spring was in the SE-HPLC profile for α- and γ-gliadins in the 2% SDS extract. If the glutenin polymer of Butte 86 and Jagger is more abundant, more aggregated and/or has a larger size-distribution profile than that of Chinese Spring, the glutenin aggregates may have trapped more α- and γ-gliadin in a form that was not easily released until the aggregates were solubilized in warm 2% SDS.

We suggest that aggregation of the glutenins and gliadins is mainly due to hydrogen bonds that are sensitive to temperature. Glutamine-rich repeat sequences of gliadins and glutenins offer ample opportunities for hydrogen bonds between the amide side chains. Holme and Briggs (*26*) first commented on the role of hydrogen bonds in the tendency of gliadins to aggregate. The combination of 2% SDS at 60 °C may have disrupted hydrogen bonds that were responsible for the aggregates of glutenin polymers and gliadins that resisted extraction with 0.1 N KCl and 0.25% SDS. Hydrophobic interactions may also play a role in forming and maintaining gliadin and glutenin aggregates and may also have been reduced by the treatment with 2% SDS at 60 °C.

The ability to hydrate and mix a flour dough is influenced by protein amount and composition and the solvent system. Extractability differs among gliadin types, with α- and γ-gliadins being more readily separated from glutenins than ω-gliadins, and solvents may increase or decrease gliadin/glutenin interactions. Exposure to salt greatly affects solubility and extractability of gliadins (*27–29*), and gliadin extractability may be related to flour quality (*28, 30, 31*). The methods, solvents, and results in this paper differ from those of Fu and Sapirstein (*28*) or Dupuis et al. (*30*). In this paper, more α- and γ-gliadin was extracted from the weak flour with 0.25% SDS and more was associated with the glutenin polymer extracted with 2% SDS from the stronger flours. We suggest that this method of sequential fractionation detects stronger gliadin−glutenin interactions in protein aggregates formed by the stronger flours.

One of the main characteristics used to distinguish glutenin subunits from gliadins is release of the subunits after addition of a reducing agent. These glutenin subunits include gliadin-like proteins with an odd number of Cys residues. They are incorporated into the glutenin polymer but are otherwise similar in overall sequence to gliadins. Some of the ω-gliadins in regions I and II of **Figure 2F** were evident only after the polymer was reduced. The protein bands are in the size range of the chromosome 1B ω-gliadins. These may be ω-gliadins with one Cys that are covalently bound to the polymer that have been referred to as "D-type" glutenin subunits (*32, 33*). However, it is also possible that these are ω-gliadin monomers that were not covalently linked but were so tightly associated with the polymer by hydrogen bonds and hydrophobic interactions that they were not released until the polymeric subunits were reduced by DTT.

Sequential fractionation and analysis by SE-HPLC revealed differences in gliadin/glutenin interactions that may be related to flour quality. The effect of temperature on the ability to extract monomers and polymers suggests that hydrogen bonds and hydrophobic interactions among glutamine-rich areas of glutenins and gliadins, especially the long repeats of HMW-GS and of ω-gliadins, strongly influence the solubility of gluten aggregates. Use of warm 2% SDS in a three-step procedure permitted extraction of up to 95% of total flour protein. Polymer sizes range from approximately 70 000 Da dimers of γ-gliadins (Vensel et al., unpublished data) to glutenin complexes of 1 000 000 Da or higher (*2, 6, 13, 14*). The ability to extract up to 90% of total polymer using the three-step extraction may be valuable for studies of the composition and size distribution of the glutenin polymers. The method is currently being used to detect differences in flour protein aggregation that are related to the effects of a high-temperature growth regimen on flour quality.

ABBREVIATIONS USED

2DE, Two-dimensional gel electrophoresis; HMW-GS, high-molecular-weight glutenin subunit; LMW-GS, low-molecular-weight glutenin subunit; RP-HPLC, reverse-phase high-performance liquid chromatography; UPP, unextractable polymeric protein.

Supporting Information Available: Total and relative areas under SE-HPLC and RP-HPLC peaks for the 2% SDS extracts (Table S1) and protein standards resolved by SE-HPLC (Figure S1). This material is available free of charge via the Internet at http://pubs.acs.org.

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