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Sequential Extraction and Quantitative Recovery of Gliadins, Glutenins, and Other Proteins from Small Samples of Wheat Flour

FRANCES M. DUPONT,* RONALD CHAN, ROCIO LOPEZ, AND WILLIAM H. VENSEL

Western Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, 800 Buchanan Street, Albany, California 94710

Methods to sequentially extract and fractionate wheat flour proteins were evaluated to reliably quantify gliadins, glutenins, and albumins/globulins in single flour samples. Compositions of the resulting protein fractions were analyzed by RP-HPLC combined with SDS−PAGE. Unknown proteins were identified by mass spectrometry or N-terminal sequencing. The best separation and recovery of discrete albumin/globulin, gliadin, and glutenin fractions from the same flour sample was achieved by extraction with 0.3 M NaI in 7.5% 1-propanol followed by 2% SDS, 25 mM DTT in 25 mM TRIS, pH 8.0, and precipitation of the solubilized proteins with ammonium acetate/methanol followed by acetone. Average flour composition for the variety Butte86 was 10% albumin/globulin, 40% gliadin, and 48% glutenin. This method should be useful for determining flour composition in diverse samples and evaluating relationships between proteins and end-use functionality.

KEYWORDS: Albumin; flour; fractionation; glutenin; gliadin; HPLC; protein; quality; *Triticum aestivum*; wheat

INTRODUCTION

After more than 250 years of research on the roles of proteins in flour breadmaking quality, there is still a critical need for rapid and effective methods to separate and quantify the protein components (*1*−*3*). Breadmaking quality and nutritional value are strongly influenced by protein content and composition, but the complexity of the flour proteins and difficulty of separating and quantifying them make it challenging to evaluate their roles in flour quality and to compare different flour samples. The major flour protein types are the water-soluble albumins, the salt-soluble globulins, the alcohol-soluble gliadin monomers, and the high and low molecular mass glutenin subunits (HMM-GS and LMM-GS). The HMM- and LMM-GS are soluble in alcohol solutions as monomers, dimers, or small polymers but are mainly present in flour as large, insoluble polymeric aggregates that surround the starch granules. The glutenin polymers have the largest effect on flour breadmaking quality, but the other storage proteins also contribute to the unique functional characteristics of flour (*4*−*11*). The proteins are encoded by dozens of genes. Four to five members of the HMM-GS gene family and larger numbers of the LMM-GS, α- γ-, and ω-gliadin gene family members are expressed in a single wheat variety (*10*−*15*). The albumins and globulins represent heterogeneous gene families; abundant low molecular mass albumins/globulins (LMM-albumins) include a variety of alpha-amylase inhibitors, and abundant high molecular mass albumins/globulins (HMM-albumins) include enzymes such as beta-amylase (*16*). Both genes and environment influence the quantity of total flour protein, amount and size range of glutenin polymer, and proportions of glutenin subunits, gliadins, albumins, and globulins (*8*, *11*, *17*−*22*).

To accurately determine flour protein composition, it is desirable to separate and quantify the proteins by type. Ideally, the fractionation procedure should be simple, suitable for small flour samples, maximize recovery of each protein type, and minimize cross-contamination. This might be perceived as a problem that is already solved, since the original definitions of the albumin, globulin, gliadin, and glutenin protein types were based on their differential solubility in water, salt, and alcohol solutions (*2*). However, the protein types overlap in solubility and extractability, making quantitative separation difficult (*3*, *23*, *24*). Most methods, whether large or small scale, do not adequately solve problems of cross-contamination, and many studies used only one-dimensional methods to separate and evaluate these highly complex protein groups. Also, several reports describe methods to solubilize flour proteins and then directly analyze them by SDS−PAGE, acid PAGE, or HPLC but do not discuss how to quantitatively recover the proteins from the solvent (*7*, *25*, *26*). While it may be trivial to recover flour proteins from commonly used volatile solvents such as 0.1 M acetic acid, 40−70% ethanol, or acetonitrile and trifluoroacetic acid (TFA), the question of how to quantitatively recover flour proteins from SDS, KI, or propanol solutions has not been addressed.

We used a combination of RP-HPLC and SDS PAGE to test and compare the effectiveness of several procedures for separating, recovering, and quantifying the different categories of flour protein from 100 mg to 2 g flour samples. Methods used to

* Corresponding author. E-mail: fmd@pw.usda.gov; phone: 510-559-5702; fax: 510-559-5818.

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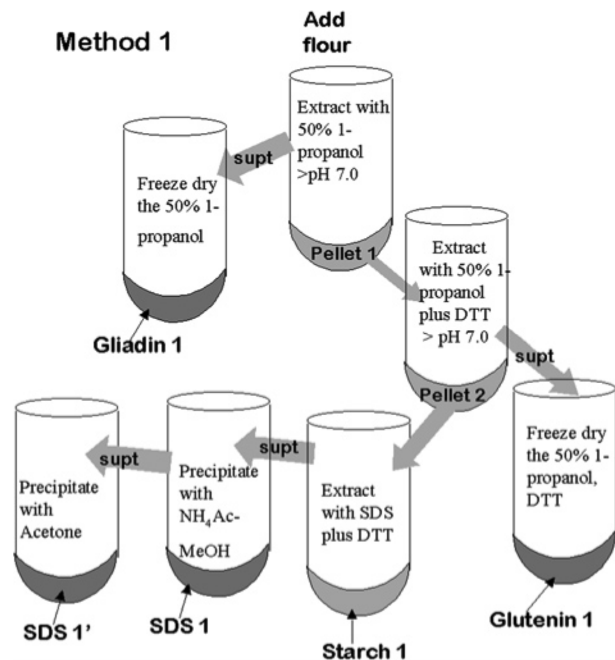

Figure 1. Extraction and recovery of flour proteins by method 1.

solubilize gliadins also extracted some albumins/globulins and glutenin; methods for extracting albumins/globulins extracted some gliadin. Two procedures are contrasted and compared in this paper. The procedures were expanded from the methods of Fu and Sapirstein (*3*), who used 1-propanol solutions to separate gliadins, small glutenin polymers, and large glutenin polymers; Bean et al. (*27*), who extracted gliadins from glutenins using 1-propanol; and Fu and Kovacks (*26*), who used sodium iodide in dilute 1-propanol to separate gliadins, albumins, and globulins from glutenin. Extracted proteins were recovered by freeze-drying or differential precipitation with ammonium acetate/methanol and acetone. By modifications of the method of Fu and Kovacks (*26*), we developed a successful method for quantitative separation and recovery of albumins/globulins, gliadins, and glutenins that minimizes cross-contamination and maximizes recovery.

METHODS

Plant Materials and Treatments. Hard red spring wheat plants, *Triticum aestivum* L. "Butte86", were grown in the greenhouse as previously described, under various regimens of fertilizer and temperature (*28*). To test method 2, a set of eight flour samples was from plants that were grown under half-strength fertilizer from anthesis to maturity, under a moderate temperature regimen, 24/17 days/nights from anthesis to maturity, or a hot temperature regimen of 37/28 days/nights from anthesis, 10 days or 20 days to maturity (*28*). Grain was tempered to 15% moisture and milled to flour in a Brabender Quadrumat Junior mill (South Hackensack, NJ) equipped with a sifter reel.

Sequential Solubilization and Recovery of Flour Proteins. Method 1 was modified from Fu and Sapirstein (*3*) and was used to prepare gliadin 1, glutenin 1, and SDS 1 protein fractions (**Figure 1**). Flour was extracted two times with 1 mL of 50% 1-propanol per 100 mg of flour and centrifuged in an Eppendorf 5810 R centrifuge for 10 min at 4500*g*. The two 1-propanol extracts were pooled and dried in a freeze-dry system (FreeZone, Labconco, Kansas City, MO) with a −80 °C collector coil to obtain the gliadin 1 fraction. The residue (pellet 1) was freeze-dried, ground to a powder in a mortar and pestle, extracted with 0.4 mL 50% 1-propanol, 25 mM DTT, 25 mM TRIS HCl, pH 8.0 per 100 mg of original flour amount, and centrifuged as above. After three sequential extractions, the supernatant fractions were pooled and freeze-dried to obtain the glutenin 1 fraction. The residue (pellet 2) was freeze-dried, powdered, extracted with 0.4 mL 2% SDS and 25 mM DTT in 25 mM TRIS, pH 8.0 (SDS−DTT−TRIS), and centrifuged as above. After two sequential extractions, the supernatant fractions were pooled and the proteins were precipitated by adding four volumes of cold (4 °C) 0.1 M ammonium acetate in 100% methanol (NH_4Ac-MeOH) (*29*), maintained at 4 °C overnight, and centrifuged as above to obtain the SDS 1 fraction. The SDS 1 supernatant solution was then precipitated with four volumes of acetone to precipitate any remaining protein (SDS 1′). All protein fractions were freeze-dried and stored at −80 °C. The remaining starch 1 pellet also was freeze-dried.

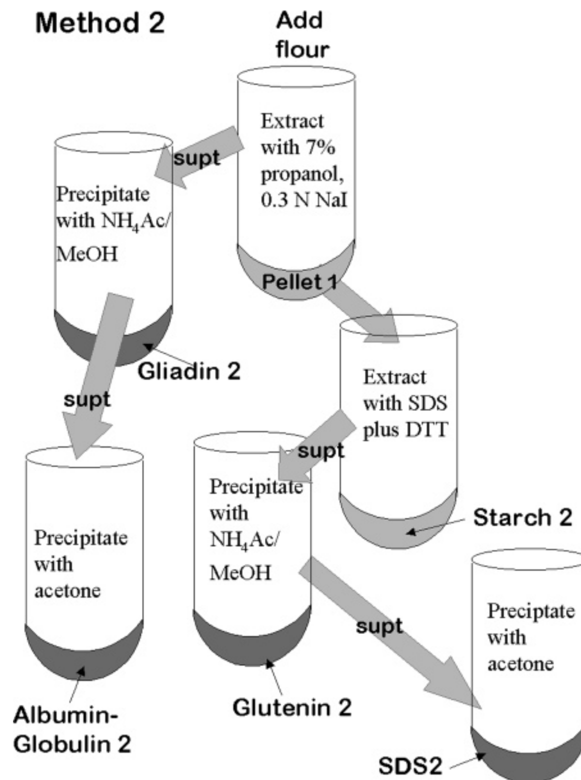

Figure 2. Extraction and recovery of flour proteins by method 2.

Several variations of method 1 were also used. To simplify extraction of total glutenin, the gliadin 1 fraction was prepared by extracting flour with 50% 1-propanol. The remaining glutenin was then extracted from pellet 1 with SDS−DTT−TRIS and precipitated with acetone (glutenin/SDS1). An even simpler method to measure total glutenin was to extract the gliadin 1 fraction with 50% 1-propanol and then freeze-dry method 1 pellet 1 for N analysis of the unextracted glutenin 1 plus starch (*27*). A final variation was to first extract the flour with 1 mL of 0.1 M KCl, 50 mM TRIS HCl, and 5 mM EDTA, pH 7.8 (KCl−TRIS−EDTA) per 100 mg of flour, to remove salt-soluble albumins and globulins (*27*) prior to extracting gliadins with 50% 1-propanol. The original method of Fu and Sapirstein did not include adjusting the pH of the 50% propanol, 25 mM DTT solution, but we found that extraction of glutenin was poor unless the pH was made alkaline to improve reduction of disulfide bonds by DTT.

Method 2 (**Figure 2**) was used to prepare the gliadin 2, albumin/globulin 2, and glutenin 2 fractions. Flour was extracted with 1 mL of 0.3 M NaI, 7.5% 1-propanol (NaI-propanol) per 100 mg of flour (*26*), and centrifuged as above. After two extractions, the supernatant fractions were pooled, precipitated with four volumes of cold (−20 °C) NH_4-Ac-MeOH, stored at −20 °C for at least 48 h, and centrifuged as above to obtain the gliadin 2 fraction. The residue (pellet 1) was saved. After precipitation of gliadin 2 with NH_4Ac-MeOH, the remaining proteins were recovered from the NaI supernatant fluid by precipitating the proteins with four volumes of acetone, storing at −20 °C overnight, and centrifuging as above to produce the albumin/globulin 2 fraction. To prepare the glutenin 2 fraction, pellet 1 was freeze-dried, powdered, and then extracted with 0.4 mL SDS−DTT−TRIS per 100 mg of flour and centrifuged as above. After two extractions, the solubilized proteins were pooled and then sequentially precipitated with NH_4Ac-MeOH to yield the glutenin 2 fraction and with acetone to yield the SDS 2

fraction, as above. Alternatively, the proteins solubilized in SDS were all precipitated with acetone to prepare the glutenin 2′ fraction (not shown). A variation of method 2 was to extract the gliadins and then simply freeze-dry the remaining method 2 pellet 1, which contained starch plus the glutenin 2 protein, for N analysis. All protein fractions were immediately freeze-dried and stored at −80 °C.

To obtain larger quantities of protein, 2 g of flour was extracted twice with 20 mL of NaI-propanol, necessitating the use of large centrifuge tubes for the precipitation step. The NaI-propanol supernatant solution was transferred to glass round-bottom centrifuge tubes. Four volumes of NH_4Ac-MeOH were added, the mixture was stored at −20 °C overnight or longer, and then centrifuged in a Sorval GSA rotor at 3600*g* in a RC5C high-speed centrifuge (Kendro Laboratory Products Newtown, CT). The precipitate was dissolved and washed from the sides of the glass tube with 0.1 M acetic acid, transferred to small tubes in measured portions, and freeze-dried (gliadin 2). Glass tubes were used for the GSA rotor because it was difficult to recover the precipitated protein from the sides of large plastic tubes. The proteins that remained in the NH_4Ac-MeOH supernatant solution were recovered by dividing the solution among smaller tubes before adding four volumes of acetone. They were then stored at −20 °C overnight and centrifuged to produce the albumin/globulin 2 fraction.

Methods 1 and 2 were also tried with only 20 mg of flour, approximately the amount obtained from a single seed. However, NH_4-Ac-MeOH did not precipitate the gliadins from the NaI solution when such a small amount of flour was used. Therefore, this method may not be useful for flour samples smaller than 100 mg.

Some proteins in the albumin/globulin 2 fraction were not soluble in acetic acid or water. Therefore, the proteins were either solubilized with NaOH for the Lowry (*30*) protein assay, with guanidine HCl for RP-HPLC, or with SDS for SDS PAGE. To recover all of the albumin/globulin 2 proteins from large centrifuge tubes and to transfer them to smaller tubes for long-term storage, the proteins were dissolved in 1.0 M KCl, and stored, frozen, in the salt solution.

Protein Determination. Flour protein content was determined by NIR, using the NIR Systems 6500 (NIR Systems, Silver Springs, MD) (AACC Method 39-11) (*31*). Standard error of prediction for moisture was 0.06% and for protein was 0.13%. Protein content of the freeze-dried protein fractions was determined in triplicate by Dumas nitrogen combustion analysis of 15-mg samples, using a Model FP-428 LECO nitrogen analyzer with a 10-mL gas collection tube (LECO Corporation, St. Joseph, MI), an EDTA standard, and a protein to N ratio of 5.7 (AACC Method 46-30) (*31*). The albumin/globulin fractions generally contained insufficient protein for the combustion analysis procedure. Therefore, protein was measured in triplicate by a colorimetric procedure (*30*) using bovine serum albumin (BSA) as the standard. Protein content of all fractions also was determined by RP-HPLC of weighed samples, by comparing the total peak areas at 210 nm with BSA, glutenin, or gliadin calibration curves (*7*, *32*). The freeze-dried protein fractions ranged from 50 to 90% protein. The nonprotein material may consist of carbohydrate and lipid as well as buffer, salt, or detergent, depending upon the fraction. Precipitation with NH_4Ac-MeOH, followed by freeze-drying, did not contribute measurable N as tested by precipitation of known amounts of protein standards with NH_4Ac-MeOH and then measuring N by the Dumas method, as well as by estimating protein by RP-HPLC absorbance at 210. There also appeared to be little SDS in the protein fractions after precipitation by NH_4Ac-MeOH or acetone, judging by the lack of interference with RP-HPLC. Other flour components that contain N, such as lipids, make minor contributions to the protein estimates.

Sources of error in determining the total amount of protein in a flour sample included the initial margins of error in determining flour N, the original flour moisture determination, any changes in flour moisture prior to the protein extraction, water absorption by protein samples, and nonprotein N in lipids and metabolites.

RP-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 were alkylated with vinyl pyridine prior to HPLC (*33*). Five hundred microliters of protein was applied to a Jupiter (Phenomenex, Torrance, CA) C18 semipreparative RP-HPLC column or a Nucleosil (Ansys, Lake Forest, CA) C8 analytical column. Proteins were eluted using an Agilent Technologies Series 1100 HPLC (Palo Alto, CA). Peptide bond absorbance was detected at 210 nm (*32*). For method 1, proteins were eluted from the Jupiter column using gradient 1, which incorporated a 4-min delay for sample loading, followed by an increase from 10 to 50% acetonitrile in 0.5% TFA for 90 min, at a rate of 1.5 mL min^{-1}, followed by an increase from 50 to 65% acetonitrile in TFA for 18 min (*33*). For method 2, proteins were eluted from the Jupiter column using gradient 2, which incorporated a 10-min delay, followed by a gradient of 10−65% acetonitrile in TFA at 1.5 mL min^{-1} for 60 min, or from the Nucleosil column at 0.8 mL min^{-1} for 60 min.

SDS−PAGE. Protein samples were suspended in Novex NuPAGE sample buffer (San Diego, CA) with 50 mM DTT, mixed by rotation for 1 h at 22 °C, then centrifuged for 10 min at 14 000*g* in a microfuge. Samples were loaded onto a Novex NuPAGE 4−12% acrylamide, Bis-TRIS gel, and separated using the Novex MES SDS running buffer at 133 V hours (*34*, *35*). Proteins in HPLC fractions were dried in a Speed Vac (Savant Instruments, Farmindale, NY), then resuspended in 20 or 30 μL of sample buffer, and 5 or 10 μL of each sample was loaded onto a gel. Gels were stained overnight with Brilliant Blue (Sigma, St. Louis, MO) in 20% methanol and destained with water.

Protein Identification. Protein identification was based on previous studies that included SDS−PAGE, RP-HPLC, N-terminal sequencing, and mass spectrometry of the storage proteins and on prior characterization of the major classes of wheat storage proteins (*33*, *34*, *36*−*41*). The proteins in the albumin/globulin 2 fraction were identified by LC-ESI-MS/MS mass spectrometry using a QSTAR Pulsar I hybrid quadrupole-TOF instrument (Applied Biosystems/MDS Sciex, Toronto, Canada) configured with a nanospray source (Proxeon Biosystems, Odense, Denmark) for ESI analysis (*42*). Proteins were separated by HPLC followed by SDS−PAGE, and individual bands were cut from the gels and automatically digested with trypsin using a Digest Pro (INTAVIS Bioanalytical Instruments AG, Bergish Gladbach, Germany) (*43*). Spectra were acquired with Analyst QS software Spectra and analyzed using the KNEXUS automation client (Genomic Solutions, Ann Arbor, MI). Proteins were identified by searches of the NCBI Viridiplantae database, the NCBI *Triticum aestivum*: UniGene Build #37 and a wheat EST database (*44*) that can be accessed at HarvEST: Wheat (http://harvest.ucr.edu).

RESULTS AND DISCUSSION

Analysis of Flour Protein Composition by Sequential Fractionation. Procedures for sequentially extracting and recovering protein fractions from small flour samples, ranging from 100 mg to 2 g, were tested and compared (**Figures 1**−**3**). The protein patterns in an SDS−PAGE gel illustrate the outcome of the two methods (**Figure 3**). Total flour protein was extracted from flour with SDS gel loading buffer (T_1) or extracted with SDS-DTT, then precipitated with acetone (T_2) prior to dissolving it in gel-loading buffer. Comparison of T_1 and T_2 demonstrated that almost all protein in the SDS extract was recovered by precipitation with acetone. Major protein bands in T_1 and T_2 include all of the predominant flour storage proteins. The individual HMM-GS are clearly resolved at apparent mol mass 70 000−116 000 and consist of the chromosome 1A-encoded 1Ax2*, 1B-encoded 1Bx7 and 1By9, and 1D-encoded 1Dx5 and 1Dy10. Distinct bands of 1B-encoded ω-gliadins (1B ω-gliadins) were at apparent mol mass 55 000−65 000 and the 1D-encoded ω-gliadins (1D ω-gliadins) resolved as a single band at apparent mol mass of approximately 50 000. The many individual proteins in the region from apparent mol mass 36 000 to 48 000 were not resolved by SDS−PAGE, although there is a distinct banding pattern in this size range. Protein bands in this size range include the chromosome 1A-encoded ω-gliadins (1Aω-gliadins), the α- and γ-gliadins, and the LMM-GS. Protein bands between 6000 and 31 000 include LMM-albumins, such as members of the complex α-amylase inhibitor and α-amylase-trypsin inhibitor families that range in mass from 13 000 to 18 000.

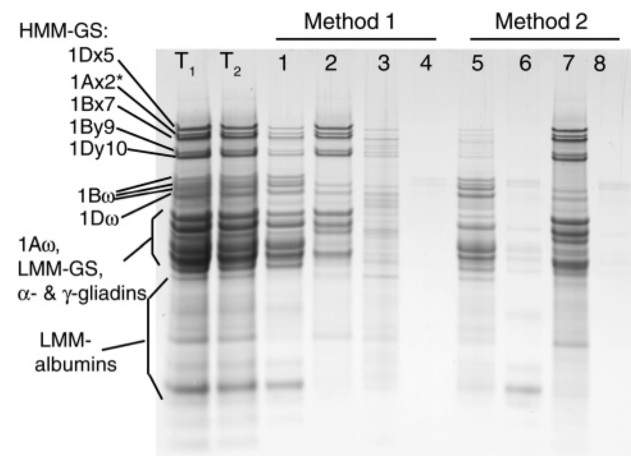

Figure 3. SDS–PAGE of flour proteins recovered by methods 1 and 2. Total flour protein was extracted with gel sample buffer (T_1) or extracted with SDS-DTT and recovered by precipitation with acetone (T_2). Sequential fractionation was by method 1 to produce 1, gliadin 1; 2, glutenin 1; 3, SDS1; and 4, SDS 1′; or method 2 to produce 5, gliadin 2; 6, albumin/globulin 2; 7, glutenin 2; and 8, SDS 2. The amount loaded in each lane was equivalent to the protein extracted from 100 μg of flour. Positions of the individual 1Ax2*, 1Bx7, 1By9, 1Dx5, and 1Dy10 HMM-GS and 1B and 1D ω-gliadins and groups of gliadins, LMM-GS, and LMM-albumins are indicated.

Lanes 1−4 illustrate the results of sequential fractionation with 50% propanol and SDS by method 1. As reported by Fu and Sapirstein (*3*), 50% 1-propanol extracted the majority of the gliadins but also extracted some HMM-GS and LMM-GS (**Figure 3**, lane 1, gliadin 1). This fraction also contained several major LMM-albumin bands. Most remaining HMM-GS and LMM-GS were extracted from pellet 1 with propanol-DTT-TRIS (**Figure 3**, lane 2, glutenin 1). Presumably, they had been incorporated into glutenin polymers but were solubilized upon reduction of the intermolecular disulfide bonds. Additional protein was extracted with 2% SDS, 50 mM DTT, and recovered by precipitation with NH_4Ac-MeOH (**Figure 3**, lane 3, SDS 1). Little protein was recovered by subsequent precipitation with acetone (**Figure 3**, lane 4, SDS 1′).

Lanes 5−8 for method 2 (**Figure 3**) illustrate the results of sequential extraction with NaI and SDS and sequential precipitation with NH_4Ac-MeOH and acetone. As reported by Fu and Kovacs (*26*), the NaI-propanol solution solubilized almost all the gliadins, albumins, and globulins along with traces of glutenin. The gliadins, and small amounts of albumins/globulins and glutenins, were recovered from the NaI-propanol solution by precipitating the proteins with NH_4Ac-MeOH (**Figure 3**, lane 5, gliadin 2). The remaining proteins were then precipitated with acetone (**Figure 3**, lane 6, albumin/globulin 2). The glutenins along with small amounts of other protein types were extracted from pellet 1 with SDS-DTT and subsequently recovered by precipitation with NH_4Ac-MeOH (**Figure 3**, lane 7, glutenin 2). A few additional proteins were recovered by precipitation with acetone (**Figure 3**, lane 8, SDS 2).

Analysis of the Protein Fractions by RP-HPLC Followed by SDS−PAGE. One-dimensional SDS−PAGE analysis indicated that method 1 gave considerable overlap of the protein types, whereas method 2 greatly improved the separation of the flour proteins into the gliadin, glutenin, and albumin/globulin types (**Figure 3**). However, wheat storage protein composition is too complex to be adequately evaluated by a one-dimensional method. Two-dimensional analysis by 2DE (two-dimensional gel electrophoresis) or a combination of RP-HPLC and SDS−PAGE, along with protein reference maps developed by use of N-terminal sequencing or mass spectrometry, makes it possible to separate and identify most of the abundant proteins (*11*, *33*, *34*, *36*, *37*, *41*−*43*).

To assess the detailed composition of each protein fraction, the fractions were separated by RP-HPLC and the peaks were collected and analyzed by SDS−PAGE (**Figures 4** and **5**). Analysis of the proteins obtained by method 1 further confirms the cross-contamination of flour protein fractions seen in **Figure 3**. The gliadin 1 fraction (**Figure 4A**,**B**) included 1B ω-gliadins that eluted in peaks 5 and 7, 1D ω-gliadins (1Dω) in peak 10, and 1A ω-gliadins (1Aω) in peaks 15−19. Nongliadin trace components include HMM-GS in peaks 7−20 and LMM-albumins in fractions 7−14. Over 30 major protein bands and 70 minor bands that ranged between apparent molecular mass 40 to 55 were revealed by SDS−PAGE of peaks 21−38. Analysis of the gliadin 1 preparation by 1D SDS−PAGE under reducing and nonreducing conditions (not shown) indicated that the majority of these proteins were monomers, but polymeric LMM-GS, which only enter the gel when reduced, were also present, as reported by Fu and Sapirstein (*3*). By comparison with purified A-gliadins obtained from Dr. D. Kasarda (not shown) and LMM-GS (*37*), peaks 21−29 most likely are mainly α-gliadins, peaks 24−30 also contain LMM-GS, and fractions 30−38 contain mainly γ-gliadins.

RP-HPLC followed by SDS−PAGE revealed that glutenin 1 was a highly purified glutenin fraction (**Figure 4C**,**D**). The main proteins were the five HMM-GS in peaks 7−12 and over 14 different LMM-GS protein bands in peaks 14−21. The main LMM-GS band in peak 15 is the abundant chromosome 1B-encoded LMM-GS with N-terminal sequence of SHIPGL (*10*, *37*). There was no evidence of LMM albumin bands in the glutenin 1 fraction. Subsequent extraction of the propanol insoluble residue with SDS-DTT, followed by precipitation with NH_4Ac-MeOH, produced a minor protein fraction highly enriched in nongluten proteins (SDS 1, **Figure 4E**,**F**). Some of these proteins may have been albumins or globulins not extracted by 50% 1-propanol, and some may have been insoluble structural or metabolic proteins, such as membrane proteins, that required SDS plus DTT for extraction. Glutenins also were present in the SDS 1 fraction. Each HMM-GS in **Figure 4E**,**F** eluted with the same retention time as in **Figure 4A**−**D** but subsequently trailed through additional HPLC fractions, so that peak 12, for example, contained all five of the HMM-GS. Peaks 17−22 included LMM-GS candidates. Peaks 23−44 contained many proteins not observed in the other protein fractions, including many with greater retention times than any gliadins and glutenins in **Figure 4B** or **4D**. Additional proteins that might have been precipitated by acetone were not examined.

One method to produce a gliadin and glutenin fraction free of albumins and globulins might be to first extract the albumins and globulins with a salt solution. Fu and Sapirstein (*3*) discussed the shortcomings of this method. We tried extracting the albumin/globulin 1 fraction from flour with KCl−TRIS−EDTA prior to extracting the gliadins with 50% 1-propanol. However, the salt solution also extracted α-, γ-, and ω-gliadins that were clearly detected when the sample was analyzed by RP-HPLC followed by SDS−PAGE. Gliadins were also extracted by water or 1.5 M NaCl. We estimated that gliadins composed up to 50% of the salt extracts. Subsequently, extractability of the remaining gliadins was altered. Although most α- and γ-gliadins were then extracted by 50% 1-propanol, many ω-gliadins were only extracted along with the glutenins. Freeze-drying the starchy residue and grinding it to a fine

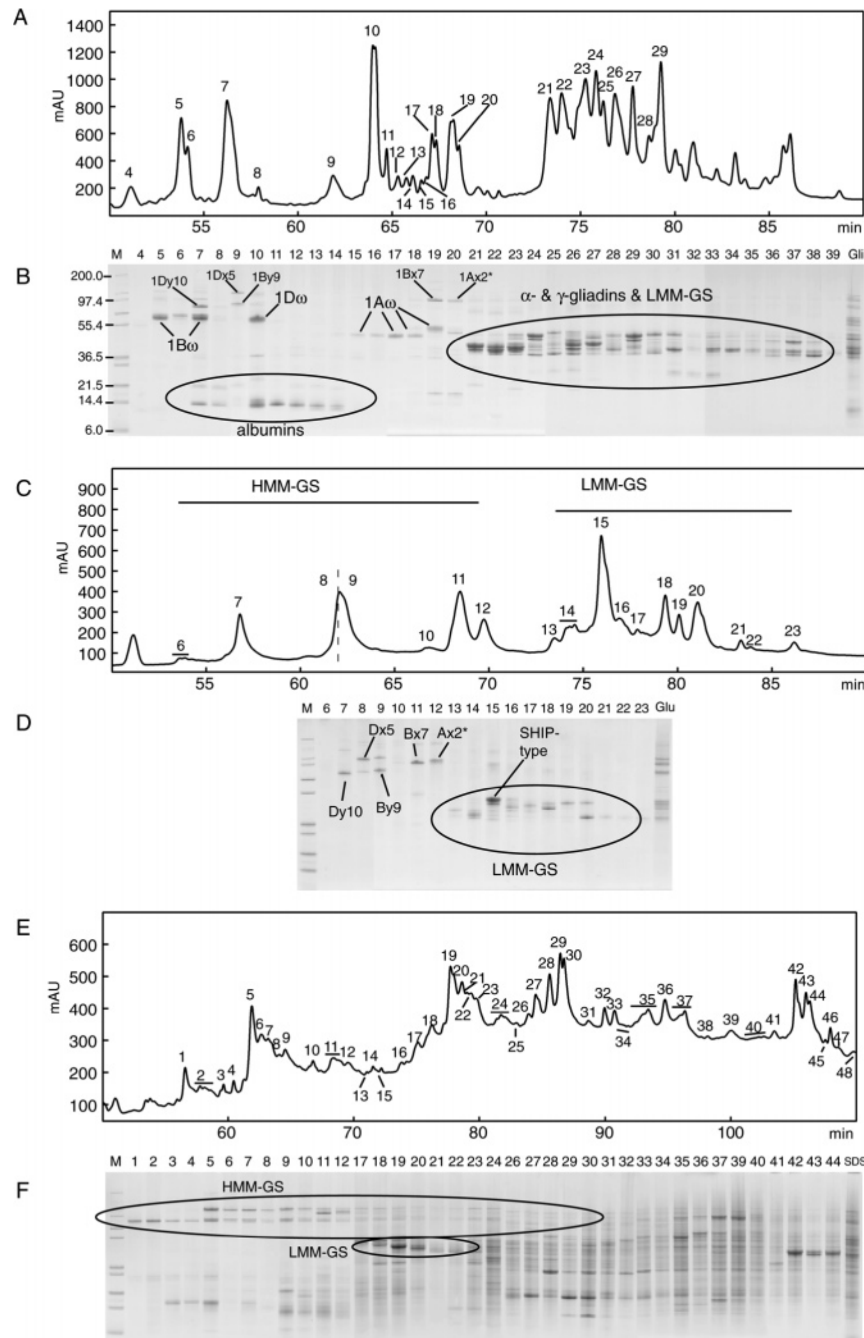

Figure 4. RP-HPLC elution profiles of method 1 fractions. Proteins were separated using gradient 1 on a Jupiter C18 column (**A**, **C**, **E**) and SDS–PAGE of the RP-HPLC peaks (**B**, **D**, **F**) for the gliadin 1 (**A**, **B**), glutenin 1 (**C**, **D**), and SDS 1 (**E**, **F**) protein fractions. Numbers above the HPLC peaks correspond to numbered lanes in SDS–PAGE; M indicates molecular size markers; Gli, Glu, and SDS indicate 8 μg of the total gliadin 1, glutenin 1, or SDS 1 fractions. Positions of the individual 1Ax2*, 1Bx7, 1By9, 1Dx5, and 1Dy10 HMM-GS, 1B, 1D, and 1A ω-gliadins and the major SHIP-type LMM-GS are indicated, along with groups of albumins, LMM-GS, and α- and γ-gliadins.

powder after extracting with KCl and then extracting gliadins with 50% 1-propanol did not improve extraction of the ω-gliadins. Therefore, pre-extraction of albumins and globulins was not effective for quantitative separation of the protein types. In summary, method 1 produced a highly purified glutenin fraction (glutenin 1, **Figure 4C**,**D**), but it did not give quantitative recovery of glutenin and did not produce pure gliadin or albumin/globulin fractions.

Method 2 improved separation of albumins and gliadins from glutenins, and differential precipitation by NH_4Ac-MeOH followed by acetone enabled separation of the most abundant LMM-albumins from the gliadins (**Figure 5**). The albumin/globulin 2 fraction contained little gliadin and encompassed approximately 10% of the total flour protein (**Figure 5A**,**B**). Purothionins eluted in peaks 3–6, with apparent molecular mass of approximately 6000. Proteins in peaks 11–12 were grain softness proteins, in peaks 14–16, alpha amylase-0.19 inhibitor proteins, and in peaks 17–19, CM3-type alpha-amylase trypsin inhibitors. Several small peaks were combined in lane 20, which contained α-gliadins, and peak 21 contained γ-gliadin. Peak 22 contained a protein related to an avenin storage protein from oats. The composition of peaks 9–19 of **Figure 5A**,**B** was similar to that of the LMM-albumins in peaks 7–14 of **Figure 4A**,**B**, though the latter was analyzed in less detail.

Analysis of the gliadin 2 fraction (**Figure 5C**,**D**) indicated that it was mainly composed of gliadin, although there was

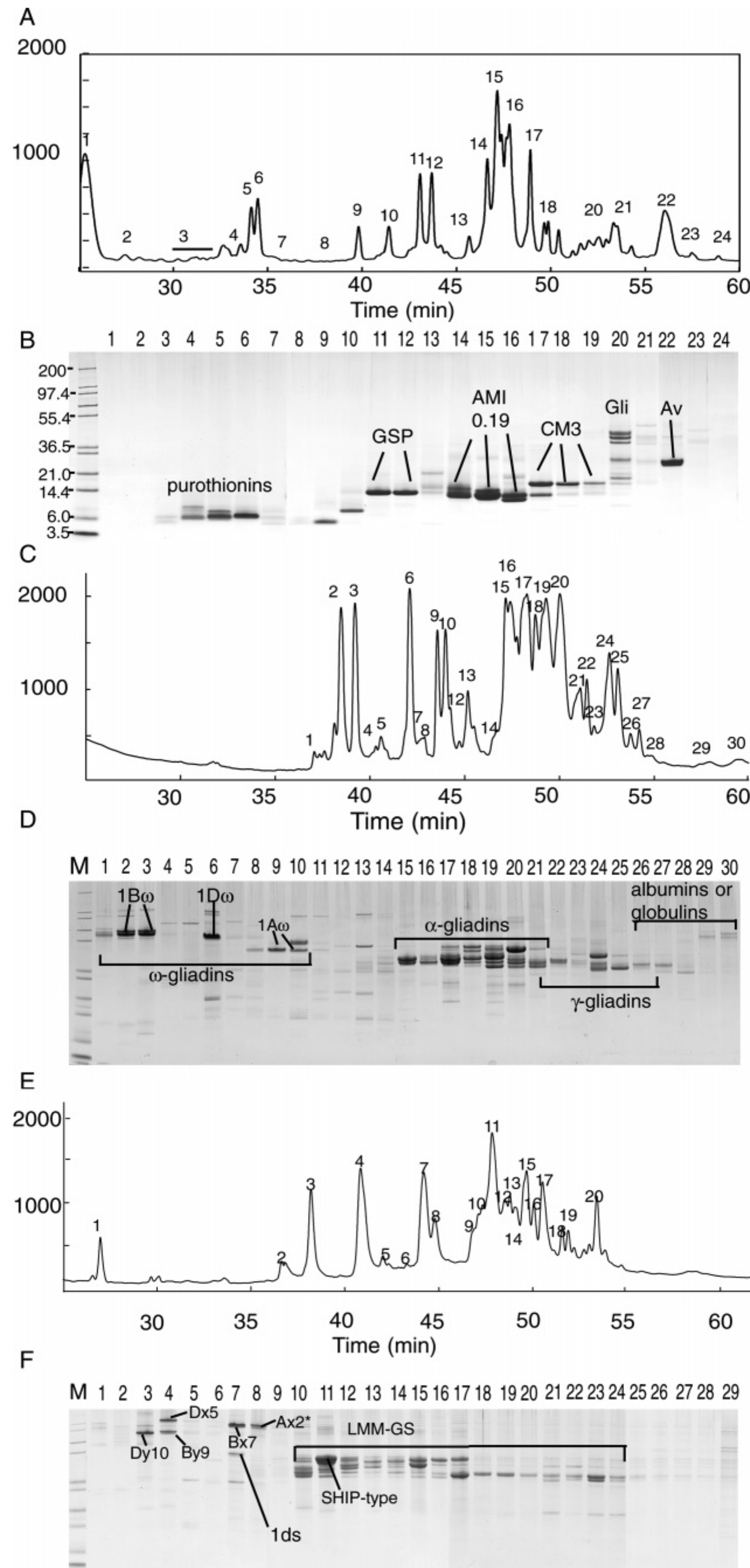

Figure 5. RP-HPLC elution profiles of method 2 fractions. Proteins were separated using gradient 2. The albumin/globulin 2 fraction (**A**, **B**) was from a different flour sample than used for **Figure 4** and was separated on the Jupiter C18 column. The gliadin 2 (**C**, **D**) and glutenin 2 (**E**, **F**) protein fractions were from the same flour sample as in **Figure 4** but were separated on a Nucleosil C8 column. Tandem MS identifications of major proteins in the albumin/globulin 2 fraction, with Genbank Accession numbers and expectation scores, were GSP, grain softness proteins (GSP1a, S51770, $e^{-14.4}$; GSP1b, S48187, $e^{-21.4}$), AMI19 (alpha-amylase inhibitor 0.19 (P01085, $e^{-19.6}$); CM3 (alpha-amylase trypsin inhibitor, P17314, $e^{-30.4}$); Gli (α-gliadin AJ133610, e^{-3}); and AV (aveninlike protein, oat B36433, e^{-3}). Purothionins were identified by N-terminal sequence. Other proteins are labeled as in **Figures 3** and **4**.

minor contamination from albumins/globulins of many sizes, as well as glutenins. The 1B1 and 1B2 ω-gliadins were prominent in peaks 2 and 3, the 1D ω-gliadins in peak 6, 1A ω-gliadins in peaks 8−10, and many α- and γ-gliadins in peaks 15−26. Minor traces of HMM-GS were present in peaks 6−13, and minor albumins or globulins were present throughout, including HMM-albumins in fractions 26−30. SDS−PAGE of the gliadin 2 fraction under reducing and nonreducing condition revealed trace amounts of contamination by LMM-GS (not shown).

RP-HPLC followed by SDS−PAGE of the glutenin 2 fraction demonstrated that it was highly enriched in HMM-GS and LMM-GS (**Figure 5E**,**F**). Identifications of the LMM-GS and gliadins are based on N-terminal sequences determined by ourselves and prior authors (*33*, *34*, *37*, *41*) and by comparison of patterns for highly purified gliadins and glutenins (not shown). The principal proteins were the HMM-GS in fractions 3−8 and the LMM-GS in fractions 10−24, including the major band in fraction 11 that is abundant SHIP-type LMM-GS. There is a striking difference between the gliadin pattern in **Figure 5D** and the LMM-GS pattern in **Figure 5F** because there was minimal cross-contamination between LMM-GS and gliadins. The LMM-GS pattern is similar to that of Lew et al. (*37*) and that in **Figure 4D**. Small amounts of LMM proteins were detected in HPLC fractions 10, 11, 21, 23, and 24 and HMM proteins in HPLC fraction 29 that may be traces of albumin and globulin. An omega gliadin-like protein (1ds) that may be a d-type glutenin subunit (*40*) was in fraction 7.

Final identification of all gliadin and glutenin bands awaits a thorough mass spectrometry analysis. Currently, attempts to identify α- and γ-gliadins and LMM-GS by mass spectrometry tends to give low expectation scores, probably because there are special problems in obtaining useful fragmentation patterns from proline-rich proteins. Also, trypsin digestion is the first step in the most common techniques for obtaining protein identifications by mass spectrometry, but gliadins and glutenins are somewhat resistant to trypsin digestion. For example, there is no trypsin site in the first half of known LMM-GS coding sequences, and most lys and arg residues in α-gliadins are adjacent to proline residues, making digestion unlikely.

Quantitative Recovery of Flour Proteins. Quantitative recovery by precipitation or freeze-drying makes it possible to estimate total protein in each fraction by N analysis and to store dried protein samples for future use. To test the usefulness of these fractions for quantitative analysis and estimate the proportions of albumins/globulins, gliadins, and glutenins in the flour samples, protein was extracted from 1 to 2 g samples to ensure sufficient protein for N analysis.

The gliadin 1 fractions, extracted from flour with 50% 1-propanol, contained 51−58% of total flour protein, depending upon the experiment. The amount of protein remaining in method 1 pellet 1 after extracting the gliadin 1 fraction was determined by N analysis. In one set of 33 samples, pellet 1 comprised 44.5 ± 2.9% of total flour protein. Bean et al. 1998 (*27*) reported that this protein residue correlated with flour baking quality and suggested that it was a good indicator of glutenin polymer amount. Total N recovered in the gliadin 1 plus pellet 1 fractions was equivalent to 95.4−102.5% of the amount of protein estimated to be present in the flour sample, on the basis of NIR.

It was difficult to account for protein in the subfractions of method 1 pellet 1. Protein in the glutenin 1 fraction, obtained by extracting pellet 1 with DTT and unbuffered 50% 1-propanol, ranged from 7 to 17% of the total flour protein. In contrast, Fu and Sapirstein (*3*) reported that this procedure extracted almost all glutenin from the 50% propanol residue. To improve extraction of the glutenins, the 50% propanol, 25 mM DTT solution was buffered to an alkaline pH to ensure effective reduction of disulfide bonds by DTT. The amount of glutenin that was extracted increased to as much as 21% of total flour protein. Nonetheless, total protein in gliadin 1 plus buffered glutenin 1 was 71% or less of total flour protein. Any remaining protein in the starch residue (pellet 2) was then extracted with SDS-DTT and precipitated with NH_4Ac-MeOH to obtain the SDS 1 fraction, which contained 5−21% of total flour protein, or with acetone to obtain up to 35% of total flour protein. An independent estimate of the amount of albumins and globulins in the original flour sample was obtained by extracting the protein with 0.15 M KCl and precipitating it with acetone (albumin/globulin 1). RP-HPLC and SDS−PAGE of the albumin/globulin 1 fraction demonstrated that it also contained gliadin (data not shown). For one set of 15 flour samples that varied greatly in protein content, albumin/globulin 1 averaged 18.1 ± 4.9% and ranged from 14 to 23% of total protein, depending on the flour sample.

Table 1. Protein Recovered by Method 2 for Sequential Fractionation of Wheat Flour, as a Percent of Total Flour Protein

fraction[a]	components	protein[b] (%)
gliadin 2	gliadins	40.2 ± 1.5 ($n = 24$)[c]
albumin/globulin 2	albumins and globulins	9.6 ± 2.0 ($n = 24$)[c]
glutenin 2	glutenins	48.3 ± 2.2 ($n = 24$)[d]
total		98.1
method 2, pellet 1	glutenins	48.1 ± 2.9 ($n = 30$)[c]

[a] Details are in Methods and Results. [b] Eight different flour samples were extracted and measured three times each. Standard deviation is indicated. Protein content for the original flour samples ranged from 12.2 to 18.7%. [c] Determined by N combustion analysis. [d] Determined by HPLC using a BSA standard curve.

Results for method 2 are summarized in **Table 1**. A set of eight flour samples from plants grown under several different temperature regimens was used. Three separate extractions were carried out for each flour sample, giving a total of 24 each of albumin/globulin 2, gliadin 2, and glutenin 2 fractions. Protein was determined in triplicate for the 24 fractions. The gliadin 2 fraction comprised approximately 40% of total flour protein. The albumin/globulin 2 fraction comprised approximately 10% of total flour protein. The glutenin 2 fraction comprised approximately 48% of total flour protein. A similar estimate of 48% was obtained by N analysis of method 2 pellet 1. The sum of the albumin/globulin 2, gliadin 2, and glutenin 2 fractions accounted for 98% of total flour protein. Duration of grain fill ranged from 30 to 45 days, and flour protein concentrations ranged from 12.2 to 18.7%. For this set of treatments, however, proportions of albumins/globulins, gliadins, and glutenins were similar in all seven experimental treatments.

All methods for measuring small amounts of protein have shortcomings that are exacerbated for gliadins and glutenins. Colorimetric procedures tend to underestimate gliadin and glutenin amounts, and extinction coefficients for the flour protein types differ greatly because of the low and variable amounts of tyrosine, tryptophan, and phenylalanine, the main contributors to absorbance at 250 to 288 nm (*45*). Absorption of the peptide amino bonds at 210 nm is a good method (*7*, *32*, *39*, *46*) when using a nonaqueous solvent, as in HPLC, but some proteins may be lost if the sample is filtered prior to HPLC, and this method also detects vinyl pyridine residues in alkylated protein preparations. Additional sources of N such as lipids and metabolites may contaminate partially purified proteins and add

to estimates of flour protein on the basis of N. The conversion factor of 5.7 that is commonly used to convert for %N to %flour protein is not an exact factor for each flour protein type. Controlling moisture is a problem for any method that requires determining an accurate sample weight of highly purified protein. With these caveats in mind, **Table 1** indicates that protein in the albumin/globulin 2, gliadin 2, and glutenin 2 fractions accounted for 98.1% of the original flour protein content estimated by NIR. This compares favorably with the original estimated error in extrapolating total protein per gram of flour on the basis of NIR values, which was ±0.98%.

Estimates of total albumin/globulin, gliadin, and glutenin vary with method and wheat variety and may vary with growing conditions. In particular, when plants are grown under conditions where availability of N varies, the proportion of albumins plus globulins is reported to vary in inverse proportion to the grain or flour protein content (*8, 19, 47*). However, there are surprisingly few published estimates of total flour protein composition, perhaps because making such estimates turns out to be more difficult that one would expect. Some estimates are 13% albumins, 2% globulins, 29% gliadins, and 57% glutenin residue (*12*) and 13−22% for albumins (*8*). In a careful, detailed study, Triboi et al. (*47*) obtained estimates of approximately 25% albumins and globulins, 25% gliadins, 40% glutenins, and 10% amphiphilic proteins for total grain protein. However, total grain contains significant amounts of albumins from the embryo and aleurone that are not present in white flour. Also, they are likely to have extracted gliadins along with the albumins and globulins. Thus, it is likely that their study significantly overestimated albumins and globulins and underestimated gliadins. In this paper, the results of method 2 for white flour from a single variety, Butte86, of 10% LMM-albumins, 40% gliadins, and 48% glutenin subunits somewhat underestimate albumins and globulins and overestimate gliadins, because methanol-insoluble HMM-albumins were present in the gliadin fraction.

Wheat flour contains hundreds of different proteins that can be detected by 2DE. Skylas et al. (*48*) detected over 1000 proteins in total extracts from flour, and Finnie and Svensson (*49*) detected at least 1000 proteins in barley seeds. Recent advances in proteomic techniques lend increasing precision to identification of these individual flour protein components in 2DE maps (*42*, *48*). Many are of low abundance and probably have metabolic functions in the living endosperm but are of minimal importance to flour quality. Only the highly abundant proteins are likely to be significant in terms of nutrition or flour functionality. Of these, the gliadins, glutenins, and the most abundant albumins/globulins still represent over a hundred different proteins. This complexity is an analytical challenge that must be overcome to determine effects of genes and environment on composition and functionality, to simplify breeding for protein functionality, and eventually to enhance the ability of grain inspectors, millers, and bakers to rapidly determine protein composition to predict flour performance.

Since this paper was submitted, a recent report by Hurkman and Tanaka (*50*) utilized a KCl extraction method to separate the soluble, nongluten proteins from the insoluble, gluten proteins. Similar to method 2, the KCl-soluble proteins were subsequently separated into two subclasses using differential precipitation by ammonium acetate/methanol followed by acetone. This procedure resulted in a KCl-soluble/methanol-insoluble protein fraction substantially free of gliadins and glutenins and permitted the identification of over 250 metabolic proteins by a 2DE/mass spectrometry approach (*51*). Unlike method 2, the KCl-soluble/methanol-soluble subfraction contained a considerable portion of gliadins along with LMM-albumins. In addition, the KCl-insoluble fraction was not separated into gliadin and glutenin subfractions, a separation that we found cannot be accomplished quantitatively. In contrast, method 2 in this paper permits quantitative separation and recovery of distinct albumin/globulin, gliadin, and glutenin fractions.

A recent paper by Suchy et al. (*52*) also elaborates on the method of Fu and Kovacks (*26*) by subdividing the glutenin residue on the basis of solubility in propanol and propanol plus DTT but does not separate and recover distinct albumin and globulin fractions nor a single total glutenin fraction.

Conclusions. To compare the protein composition of flour samples from different wheat varieties or from plants grown under different environmental conditions, it is essential to separate, recover, and quantify total proteins of each major storage protein gene family. Until recently, definitions of albumins, globulins, gliadins, and glutenin types were based more on solubility than on gene and protein sequence. However, large numbers of gene and protein sequences are now available in public databases, and methods to identify proteins are improving. Therefore, flour protein types now are based on sequence similarity rather than solubility, and cross-contamination between the types is both evident and a significant problem, as illustrated by method 1.

Eventually, it should be possible to use proteomics to predict quality and classify grain and flour samples by cultivar type or class. In the future, mass spectrometry also may be applied to obtain a quantitative analysis of the distribution of protein types in a flour sample. However, such instrumentation is not currently available, nor are mass spectrometry methods to accurately identify most gliadins and glutenins, because of their resistance to digestion by trypsin. Meanwhile, method 2 from this paper provides good quantitative recovery and estimation of total albumin/globulin, gliadin, and glutenin from flour samples.

ABBREVIATIONS USED

2DE, two-dimensional gel electrophoresis; BSA, bovine serum albumin; DTT, diothiothreitol; EDTA, ethylamine diamine tetraacetic acid; ESI, electrospray ionization; EST, expressed sequence tag; GS, glutenin subunit; HMM, high molecular mass; LC-ESI-MS/MS, liquid chromatograph electrospray tandem mass spectrometry; LMM, low molecular mass; MES (2-morpholinoethanesulfonic acid); NIR, near-infrared spectroscopy; NH_4Ac-MeOH, ammonium acetate/methanol; RP-HPLC, reverse-phase high-performance liquid chromatography; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; TOF, time-of-flight; TRIS, tris(hydroxymethyl)aminomethane.

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