

Simplified Dilute Acetic Acid Based Extraction Procedure for Fractionation and Analysis of Wheat Flour Protein by Size Exclusion HPLC and Flow Field-Flow Fractionation

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ABSTRACT

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A simple, highly efficient and reproducible two-step extraction procedure using dilute acetic acid without (AN) and then with sonication (AS) has been developed for the fractionation of wheat flour protein. Approximately 97% of total protein was extracted from a Canadian hard red spring wheat flour; an additional 1.2% protein could be recovered by further extraction with 1% DDT and 50% 1-propanol (AR). Size-exclusion HPLC (SE-HPLC) and flow field-flow fractionation (flow FFF) showed that the AN extract, which accounted for most of the total extractable protein (AN + AS + AR), consisted primarily of monomeric protein. The AS extract was composed primarily of polymeric proteins. Flow FFF showed that AN polymeric protein, including that eluting at the

SE-HPLC void volume, showed smaller Stokes diameters than AS polymeric protein. Flow FFF profiles of AS SE-HPLC subfractions showed that the void volume subfraction contained monomeric and small polymeric protein in addition to large polymeric protein, indicating formation of larger complexes through interaction between some or all of the components. AN and AS extracts, as well as SE-HPLC and flow FFF fractions thereof, showed a fairly wide range of values among 12 Canadian hard red and white spring wheat cultivars. The proportion of total protein in the AS extract and in the larger sized polymeric protein fractions from SE-HPLC and flow FFF were highly positively correlated to farinograph mixing time.

The amount and size distribution of polymeric wheat proteins (glutenins) play a major role in determining flour physical dough properties and baking performance (MacRitchie 1992). The proportion of very large polymeric proteins appears to be particularly important in determining these properties (Gupta et al 1993; Bean et al 1998). To date, most measurements of these polymeric proteins have been made using techniques such as size-exclusion HPLC (SE-HPLC), multilayer SDS polyacrylamide gel electrophoresis (MLGE) and gel filtration. With the exception of MLGE (Khan and Huckle 1992), poor resolution is normally obtained for the high molecular weight (HMW) material because the size of most components is above the exclusion limit at $\approx 0.5\text{--}1.5 \times 10^6$ Da. Flow field-flow fractionation (flow FFF) is not impeded by an exclusion limit (Giddings et al 1977) and has been used successfully to separate a number of these HMW fractions (Stevenson and Preston 1996; Wahlund et al 1996; Stevenson et al 1999).

SDS is the most widely used solvent for the extraction of polymeric proteins from flour or gluten (Dachkevitch and Autran 1989; Singh et al 1990; Gupta et al 1993). Initial extraction with SDS, followed by extraction with the same solvent using sonication, removes $\approx 98\%$ of the proteins (Singh et al 1990). The first extraction removes almost all the monomeric proteins (mainly gliadins) and some of the smaller polymeric glutenins. The second (sonicated) extraction removes most of the remaining larger polymeric glutenins. Although SDS extracts provide good profiles in SE-HPLC, their use for flow FFF can be a problem due to high back-pressures in the system during operation (Stevenson and Preston 1996). In addition, the chaotropic nature of SDS can disrupt native protein structure and is difficult to remove for further analysis (Waterhous and Johnston 1994).

Previous studies in our laboratory (Stevenson and Preston 1996; Stevenson et al 1999) have demonstrated the ability of dilute acetic acid combined with sonication to extract larger polymeric glutenins from the residue remaining after Osborne fractionation. This extract proved suitable for both SE-HPLC and flow FFF analysis, with

the latter showing good size resolution of these proteins. In this study, we report the optimization of a simple sequential extraction procedure using dilute acetic acid, followed by the same solvent with sonication, for fractionation of wheat flour proteins into primarily monomeric and polymeric fractions with high overall extraction rates. The properties of these protein fractions have been assessed using SE-HPLC and flow FFF and their relationship to protein quality has been investigated.

MATERIALS AND METHODS

Chemicals and Protein Standards

Commercially obtained chemicals used were of ACS purity unless otherwise noted and were made up to designated concentrations using deionized, distilled water. Acetic acid, FL-70 (mixed surfactant), dithiothreitol (DTT), sodium dodecyl sulphate (SDS), and chloroform were obtained from Fisher Scientific (Winnipeg, MB). Acetonitrile and 1-propanol were obtained from VWR (Winnipeg, MB). Trifluoroacetic acid (TFA) was purchased from Chromatographic Specialties (Brockville, ON). Standard molecular weight marker proteins for routine monitoring of SE-HPLC and flow FFF were purchased from ICN Biomedicals (Costa Mesa, CA). Proteins with their molecular weight (supplied by manufacturer) and Stokes diameters (nm, calculated from diffusion coefficients [Stevenson and Preston 1996]) were cytochrome C (12,500 and 3.3), myoglobin (18,000 and 3.8), chymotrypsinogen A (24,000 and 4.5), ovalbumin (45,000 and 5.5), bovine serum albumin (69,000 and 7.3), gamma-globulin (160,000 and 10.7) and ferritin (470,000 and 11.9).

Flour Samples

For optimization of the extraction process, a straight-grade flour milled from a sample of Katepwa No. 1 Canada Western Red Spring (CWRS) wheat with a protein content of 13.2% was used. A limited number of extractions were also performed using the same flour defatted with chloroform as described by MacRitchie and Gras (1973). For comparison of the acetic acid optimized procedure with 2% SDS-phosphate buffer, a different Katepwa straight-grade flour with a protein content of 13.8% was used. Straight-grade flour samples, milled from hard red and hard white spring wheat cultivars grown as checks in 1997 advanced plant breeder cooperative tests, were used to assess the relationship between farinograph dough development time and the extractability and size distribution of SE-HPLC and flow FFF fractions of the flour proteins. Cultivars included Glenlea, Wildcat, Roblin, Neepawa (two

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samples), AC Karma, AC Majestic, Columbus, AC Crystal, AC Vista, and AC Taber. Farinograph dough development time was determined by Approved Method 54-21 (AACC 2000) for constant flour weight using the 50-g bowl. Protein content ($N \times 5.7$) was determined by combustion nitrogen analysis (Leco model FP-428 CNA analyzer calibrated with EDTA) and reported on a 14.0% moisture basis as described by Williams et al (1998).

Protein Extraction

Extraction was performed sequentially in 50-mL Oak Ridge polypropylene copolymer centrifuge tubes, first with 0.05M acetic acid, then with 0.05M acetic acid with sonication, and finally with 50% 1-propanol containing 1.0% DTT. For the 0.05M acetic acid extract, 1.0 g of flour was mixed with 20 mL of extractant and rotated for 2.0 hr at 15 rpm (Roto-Torque rotator, Fisher Scientific, Winnipeg, MB) at room temperature followed by centrifugation at $28,000 \times g$ for 10 min. One to three additional extractions with 0.05M acetic acid were performed by suspending the pellet in 10 mL of extractant with vortexing, rotating for 1 hr at room temperature then centrifuging at $28,000 \times g$ for 10 min. Supernatants were heated at 60°C for 1 hr to stabilize (minimize protease activity) the extracts.

The pellet from the 0.05M acetic acid extract was suspended in 20 mL of 0.05M acetic acid with vortexing then sonicated at 15, 30, or 60 sec at an output power setting of 100 (Vibra Cell High Tech Ultrasonic Processor, model VC 60, Fisher Scientific, Winnipeg, MB) equipped with a standard 13-mm microtip. After sonication, the suspension was rotated at room temperature for 1 hr followed by centrifugation at $28,000 \times g$ for 10 min. The supernatant was heated at 60°C for 1 hr to stabilize the extract.

The pellet was then suspended with vortexing in 6 mL of 50% 1-propanol containing 1.0% DTT, rotated for 30 min at room temperature, and centrifuged as described above. The supernatant was heated at 60°C for 30 min to stabilize the extract. The pellet remaining after the propanol-DTT extraction step was analyzed for N₂ content using combustion nitrogen analysis as described by Williams et al (1998).

Supernatants were stored at room temperature and then filtered through a 1.0- μ m filter for flow FFF and 1.0 and 0.45- μ m filters for SE-HPLC. All samples were analyzed within 48 hr of extraction. Protein recoveries were determined by integration of SE-HPLC peak areas.

SE-HPLC

SE-HPLC of extracted wheat proteins was performed using a Waters HPLC system with software (Millennium 32, v. 3.2, Waters Corp., Mississauga, ON), a guard column (Ultraspherogel, Beckman Instruments, San Ramon, CA), and two Ultraspherogel SEC 4000 columns connected in series as described by Nightingale et al (1999). Isocratic elution of protein was achieved using 0.1% TFA in 50% acetonitrile. Column temperature was maintained at 30°C and column

eluent was monitored by measuring ultraviolet absorption at 210 nm. Aliquots (20 μ L) of undiluted sample were used for analysis in duplicate. Integration of peaks and size ranges were performed post-run using the Millennium software. Standard molecular weight (MW) marker proteins were used to aid in setting size ranges.

Flow FFF

Flow FFF was performed using an automated model 1000-FIFO combined frit inlet-frit outlet Universal fractionator channel (FFFractionation, Salt Lake City, UT) fitted with a YM-10 (10,000 Da cutoff) cellulose membrane (Amicon) as described by Stevenson et al (1999). The carrier fluid was 0.05M acetic acid with 0.002% FL-70, which had been filtered through an 0.8 μ m Metrical membrane filter and degassed by boiling. Flow rates of 0.2 mL/min for channel flow (V_s), 1.4 mL/min for frit flow (V_f), and 5 mL/min for cross flow (V_c) were used. Absorbance of channel outlet flow was monitored at 210 nm. Samples were diluted with 0.05M acetic acid buffer containing 0.002% FL-70 until loading samples contained $\approx 1 \mu$ g of protein. Integration of peaks and size ranges of fractograms was performed post-run using Class VP (v. 4.2) Shimadzu software (Man-Tech Associates, Inc., Guelph, ON). Elution time was calibrated to size (Stokes diameter) using standard proteins as outlined previously (Stevenson et al 1999). All runs were made at room temperature ($20 \pm 2^\circ\text{C}$) in duplicate.

Analysis of SE-HPLC Fraction Cuts by Flow FFF

Samples of sonicated 0.05M acetic acid extracts were fractionated by SE-HPLC using two preparative 300×21.2 mm BioSep SEC-S 4000 (Phenomenex, Torrance, CA) columns connected in series to a Phenomenex 60×21.2 mm guard column. Eluent was collected in 1-min intervals using a fraction collector. Selected fraction cuts were evaporated to dryness using a vacuum rotary concentrator (Jouan RC 10.10, Canberra Packard, Mississauga, ON). The dried samples were then resuspended in sufficient 0.05M acetic acid to produce a protein concentration giving an absorbance peak of ≈ 0.05 at 210 nm when analyzed by flow FFF.

Experimental Design

All extractions were replicated a minimum of two times on different days. SE-HPLC and flow FFF measurements on each replicate were performed in duplicate. For optimization of the extraction procedure, the effect on extractability of the number of 0.05M acetic acid extractions (one to three) before sonication, sonication time (15, 30, and 60 sec), stabilization temperature (room temperature and 60°C) and acetic acid concentration (0.05M and 0.20M) were tested. In addition, the effect of defatting the flour before extraction was assessed. Results from the optimized procedure were also compared against those obtained with 2% SDS-phosphate buffer (0.05M, pH 6.9) using the same protocol. For the optimized acetic acid procedure, 16 replicates were analyzed over several months.

TABLE I
Recoveries (%) of Extract and Fractions from Size-Exclusion HPLC (SE-HPLC) and Flow Field-Flow Fractionation (flow FFF)^a

Fraction ^b	AN		AS		AR
	SE-HPLC	FFF	SE-HPLC	FFF	SE-HPLC
f ₁	14.4 ± 0.83		67.3 ± 1.60		3.8 ± 1.54
f ₂	11.9 ± 0.30		19.5 ± 1.02		25.1 ± 3.19
f ₃	59.2 ± 0.48		9.7 ± 0.60		62.9 ± 3.26
f ₄	14.5 ± 0.39		3.5 ± 0.50		8.3 ± 2.08
f _a		81.0 ± 0.94		6.5 ± 0.65	
f _b		19.0 ± 0.94		60.2 ± 1.73	
f _c				24.2 ± 1.16	
f _d				9.1 ± 0.95	
Total extract recovery ^c	83.7 ± 1.72		15.2 ± 1.60		1.2 ± 0.52

^a Extracted using dilute acetic acid without (AN) then with sonication (AS) or further extraction with 1% DDT and 50% 1-propanol (AR). Mean \pm standard deviation for 13 replicates. Largest proteins elute first with SE-HPLC. Largest proteins elute last with FFF.

^b Area of fraction/total area of extract $\times 100$.

^c Extract area/area AN + AS + AR $\times 100$.

Results from three of the replicates were removed from the data set due to very low SE-HPLC and flow FFF area integration values.

RESULTS AND DISCUSSION

Straight-grade flour from a Canadian red spring wheat cultivar (Katepwa) was used to optimize the sequential extraction procedure. Three extracts were obtained during the sequential extraction procedure. The initial extract, AN (acetic acid, nonsonicated), contained proteins extractable in dilute acetic acid. The second extract, AS (acetic acid, sonicated), contained proteins that could not be extracted solely with dilute acetic acid but were extractable when sonication was used in combination with this solvent. The third extract, AR (acetic acid, sonicated, residue), contained proteins extracted with 1% DTT and 50% propanol from the pellet remaining after sonication with acetic acid. All three extracts were analyzed using SE-HPLC and integrated peak areas were compared to obtain extract recoveries as a percentage of total extractable protein normalized to 100%. CNA analysis of the residue after sequential extraction showed that the extraction procedure was highly efficient. In all cases, sequential extraction removed >98.5% of the total flour nitrogen.

Preliminary studies indicated that extracts required stabilization by mild heat treatment at 60°C for 1 hr to obtain consistent SE-HPLC and flow FFF results. When nonheated and heated extracts were compared after room temperature storage of up to four days, the heated samples showed no detectable change in elution profiles. These profiles were also very similar to fresh nonheated samples. In contrast, increasing the storage time of nonheated samples resulted in a decrease in the average size distribution of the AN, and in particular the AS extracts (data not shown). This decrease can probably be associated with proteolytic flour enzymes that are deactivated during heating (Larroque et al 2000).

Extraction and Properties of Acetic Acid Extractable AN Proteins

The effect of the number of extractions of the Katepwa flour with 0.05M acetic acid on total protein recovery in AN was examined. A second extraction removed an additional 3–4% of protein relative to a single extraction, while further extractions had no effect. Defatting the flour before extraction had no effect on extractability. There was also no effect on extractability when a higher concentration of acetic acid (0.2M) was used (data not shown). It should also be

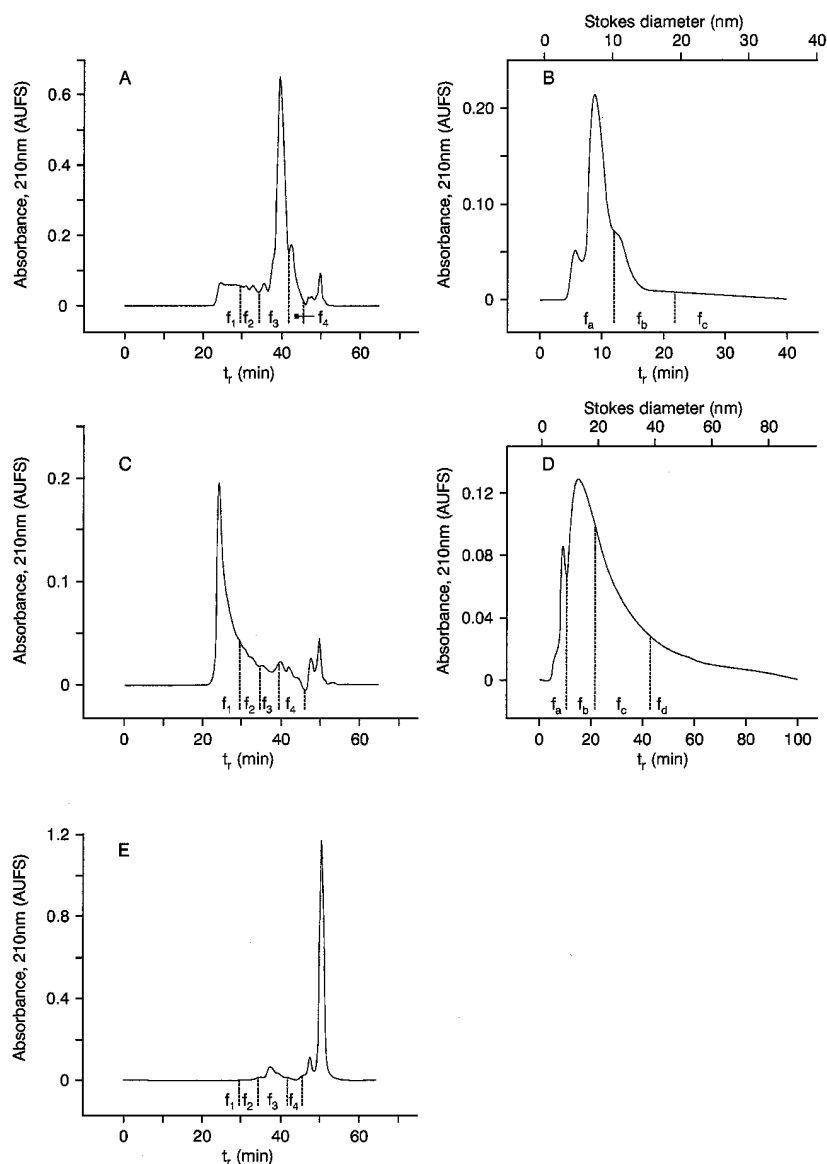


Fig. 1. Size-exclusion HPLC and flow field-flow fractionation (flow FFF) profiles of extracts from Katepwa flour using dilute acetic acid without (AN) and with sonication (AS) and further extraction with 1% DTT and 50% 1-propanol (AR). **A**, SE-HPLC of AN; **B**, flow FFF of AN; **C**, SE-HPLC of AS; **D**, flow FFF of AS; **E**, SE-HPLC of AR. SE-HPLC size fractions f_1 to f_4 ; flow FFF size fractions f_a to f_d .

noted that, based on these data, a double extraction with 0.05M acetic acid before sonication was adopted to optimize recovery of the AN fraction. For 13 replicates over a period of several months, 83.7% (Table I) of total extractable flour protein was accounted for in the AN extract. The standard deviation (SD 1.72%) indicates good reproducibility.

SE-HPLC and flow FFF profiles for the optimized flour AN extract are shown in Fig. 1A and B. Four fractions (f_1 to f_4) were used to quantify SE-HPLC size distribution ranges. These fractions were based on previous studies (Batey et al 1991; Gupta et al 1993; Nightingale et al 1999; Stevenson et al 1999) and the elution times of the molecular weight calibration protein standards. Fraction 1 (f_1) contains the larger polymeric proteins eluting at or near the void volume. Fraction 2 (f_2) consists of smaller polymeric proteins and probably small amounts of larger ω gliadins. Fraction 3 (f_3) includes the major gliadin peaks (α , β and γ gliadins) as well as ω gliadins (shoulder peak at shorter elution time). Fraction 4 (f_4) has been associated with low molecular weight gliadins, globulins, and albumins. Peaks occurring at the longest elution times (>44 min) were ignored because they represent nonprotein material (Batey et al 1991).

Table I shows the distribution of SE-HPLC size fractions obtained from the AN extract based on 13 replicates. The monomeric protein fractions (f_{3-4}) accounted for 73.7% of this extract; the remainder (26.3%) eluted in the polymeric protein range (f_{1-2}). For the monomeric proteins, the f_3 fraction containing the major gliadin peak predominated, representing 59.2% of the extract. The polymeric protein region showed a flat shape with no distinct peak (Fig. 1A), indicating an even distribution of proteins across the size range. The larger polymeric fraction (f_1) showed a slightly larger contribution (14.4%) than the smaller polymeric fraction (f_2) at 11.9%. Good reproducibility was obtained for all fractions as shown by the low SD values.

The flow FFF profile of the optimized flour AN extract is shown in Fig. 1B. The major peak eluting at 8.8 min shows a calculated

Stokes diameter (d_s) value of 7.4 nm, identical with the 7.4 nm value obtained previously by flow FFF for the major Osborne alcohol soluble gliadin peak (Stevenson et al 1999). The small peak eluting at 5.7 min (d_s 4.6 nm) corresponds to one of the major flow FFF peaks (d_s 4.5 nm) obtained for Osborne salt soluble albumin and globulin in the same study. A shoulder peak was also evident on the major (gliadin) peak starting at 12.0 min (d_s 10.3 nm) which compares closely in size to the major Osborne acetic acid extractable flow FFF peak (d_s 9.9 nm) previously attributed to small polymeric glutenin proteins (Stevenson et al 1999).

Integration of peak areas showed that monomeric proteins eluting at $d_s < 10.3$ nm (f_3) accounted for 81.0% of the AN extract; polymeric proteins (>10.3 nm) accounted for the remaining 19.0% (Table I). Standard deviations for both fractions were <1%, indicating good reproducibility. Almost all of the polymeric proteins (f_6) were eluted at ≈ 12 –22 min, corresponding to d_s 10.3–19 nm, with the majority appearing as shoulder peaks at d_s 10.3–14.5 nm. These d_s values strongly suggest that only smaller polymeric glutenins are extracted in AN, including those proteins eluting at the SE-HPLC void volume.

Extraction and Properties of Acetic Acid Sonicated Extractable AS proteins

The effect of sonication time with 0.05M acetic acid after removal of AN extractable proteins using the optimized double 0.05M acetic acid extraction procedure was assessed on the basis of protein extractability and size distribution. For two replicates, AS extractability showed an increase from 12.8 to 14.7% of total extractable protein when sonication time was increased from 15 to 30 sec. No further increase in extractability was obtained at the longest sonication time (60 sec). SE-HPLC analysis indicated that increasing sonication time from 15 to 30 sec slightly increased the amount of polymeric protein eluting at the SE-HPLC void volume, while a decrease was evident with 60 sec sonication (data not shown). Similar results were evident with flow FFF (data not shown) where

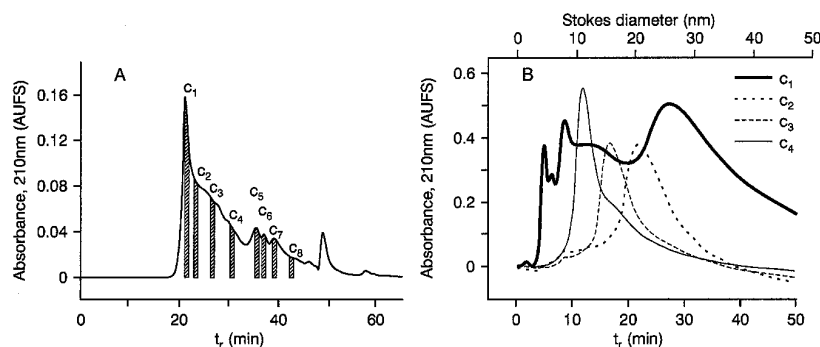


Fig. 2. Flow field-flow fractionation (flow FFF) analysis of size-exclusion HPLC subfractions from Katepwa wheat flour. **A**, SE-HPLC subfractions c_{1-8} ; **B**, Flow FFF profiles of SE-HPLC subfractions c_{1-4} .

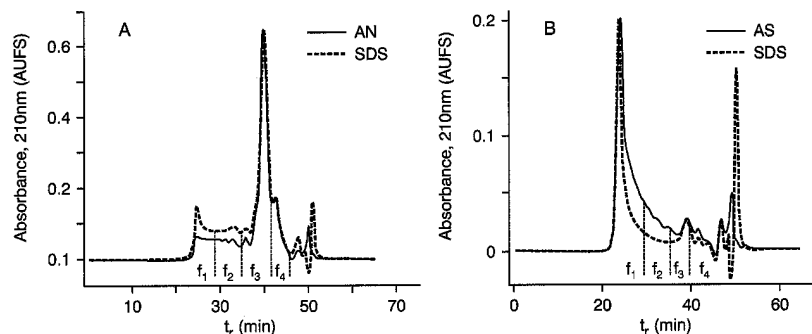


Fig. 3. Size-exclusion HPLC profiles of Katepwa wheat flour. **A**, Extracted with dilute acetic acid without sonication (AN) (solid line) and SDS-phosphate buffer (dotted line). **B**, Extracted with dilute acetic acid with sonication (AS) and SDS-phosphate buffer with sonication (dotted line).

the longest sonication time shifted the AS profile to shorter elution times (decreased d_s). These results are consistent with previous studies showing that increased sonication time can increase the extractability of polymeric wheat proteins in SDS based extractants but that overly long sonication can result in cleavage of the larger polymeric proteins resulting in a downward shift in the size distribution profile (Singh et al 1990; Larroque et al 1999). Based on these results, a sonication time of 30 sec was adopted for optimizing AS extraction. For 13 replicates, AS accounted for an average of 15.2% of total extractable flour protein (SD 1.60%) (Table I).

SE-HPLC and flow FFF profiles for the optimized flour AS extract are shown in Fig. 1C and D; the distribution of size fractions is shown in Table I. The polymeric fractions (f_{1-2}) dominated the AS SE-HPLC profile, accounting for an average of 86.8% of the total AS protein. Only 13.2% of the extract eluted in the monomeric range (f_{3-4}). In contrast to AN, most of the polymeric proteins (67.3%) eluted at or near the void volume in f_1 , indicating a much larger average protein size. Low SD values for all fractions indicate good reproducibility.

Flow FFF analysis of the AS extract (Table I) showed 93.5% of the protein eluted in the polymeric region (f_{b-d}) while only 6.5% eluted in the monomeric region (f_a). In the monomeric region, a smaller peak (d_s 7.7 nm) with two shoulder peaks at shorter elution times (lower d_s values) was evident (Fig. 1D). The smaller monomeric protein peak corresponds in size to that obtained previously (7.4 nm) by flow FFF for the major Osborne alcohol soluble gliadin peak. The small shoulder peaks at 4.1 and 5.0 nm eluted at similar positions to Osborne salt soluble albumins and globulins (Stevenson et al 1999). A single large broad peak was evident in the polymeric protein region with d_s 13.8 nm at maximum absorbance. The polymeric protein peak at 13.8 nm corresponds in size to smaller glutenin polymeric proteins (Stevenson et al 1999). This broad peak showed a very wide size range, with d_s \approx 9.5 nm to d_s >80 nm. The latter number suggests the presence of some very large glutenin polymers. The wide size range of the polymeric protein peak and the presence of some very large polymers is consistent with previous flow FFF studies of these proteins (Stevenson and Preston 1996; Wahlund et al 1996; Stevenson et al 1999).

Most of the AS protein (60.2%) eluted in the small polymeric protein region (f_b 9.5–19 nm). In contrast to the AN extract, the distribution of this fraction was not skewed toward the lower end of the size range, indicating a larger average size. Considerable amounts of protein were also eluted in the large (f_c 19–37.5 nm) and very large (f_d >37.5 nm) polymeric fractions. The f_c fraction accounted for 24.2%. The f_d fraction accounted for 9.1% of the AS protein. Good reproducibility was obtained for all fractions (Table I).

The size ranges selected for small, large, and very large polymeric proteins, although somewhat arbitrary, were based on previous flow FFF results of wheat protein solubility fractions (Stevenson and Preston 1996; Wahlund et al 1996; Stevenson et al 1999) and flow FFF results obtained for SE-HPLC AS subfractions. Figure 2A shows the location of SE-HPLC subfractions obtained from a Katepwa AS extract. Figure 2B shows the flow FFF profiles of the larger molecular size subfractions after drying and reconstitution in 0.05M acetic acid. The SE-HPLC profile is somewhat different than that shown in Fig. 1C due to the use of a preparative-scale column. The subfraction (c_1) eluting at the void volume peak showed a major flow FFF peak with d_s 26 nm extending to well over d_s 50 nm as well as smaller peaks with d_s 12, 7, 5, and 4 nm. The SE-HPLC shoulder peak (c_2) on the trailing edge of the void volume peak showed a single major peak with d_s >20 nm. Small polymeric protein subfractions (c_3 and c_4) eluting at 27.5 and 30 min showed d_s values of 16 and 11 nm, respectively. Based on these results, the flow FFF large polymeric size range was chosen to reflect the major polymeric protein peaks eluting at or near the SE-HPLC void volume; the small polymeric size range reflects the proteins eluting after the SE-HPLC void volume peak. The minimum for the very large polymeric size range (>37.5 nm) was chosen at the point

where the void volume subfraction (c_1) peak (d_s 26 nm) decreased to 50% of its absorbance value.

The smaller flow FFF peaks in the SE-HPLC void volume subfraction (c_1) with d_s values of 12, 7, 5, and 4 nm correspond in size to small polymeric glutenins, gliadins, and albumins and globulins (last two peaks), respectively (Stevenson et al 1999). The appearance of these smaller proteins in the SE-HPLC void volume suggests that they may form stable complexes with the larger polymers or among themselves. Gel filtration studies have demonstrated the release of smaller proteins from void volume peaks by re-eluting the isolated peak with stronger chaotropic eluents (Rao and Nigam 1987). The emergence of these smaller sized peaks from the SE-HPLC void volume peak using flow FFF cannot be attributed to the use of a more chaotropic eluent. The most likely explanation for the dissociation of these complexes in flow FFF is the very low concentration of protein (\approx 1 μ g) loaded onto the channel that forces the equilibrium between the protein complexes and the individual components toward the latter. These results also suggest that estimates of the amount of larger polymeric proteins in samples based on the area of the SE-HPLC void volume are overestimated. In fact, based on integration of flow FFF peak areas from c_1 , only 64% eluted in the large and very large polymeric protein range. About 14% of the SE-HPLC void volume eluted in the monomeric protein range (d_s <9.5 nm) while 22% eluted in the small polymeric glutenin range (d_s 9.5–19 nm).

Extraction and Properties of 1% DDT and 50% Propanol Extractable AR proteins

When the AS residue fraction was extracted with 1% DDT and 50% propanol, an average of 1.2% (SD 0.52) of the total extractable protein was removed. SE-HPLC of this acetic acid sonicated residue protein (AR) showed several very small peaks in the monomeric protein range (\approx 34–46 min) corresponding in elution time to ω -gliadins or HMW glutenin subunits and to the major gliadin peak or LMW glutenin subunits (Fig. 1E). A very large peak in the very low size range attributable to nonprotein nitrogen was also observed. The monomeric protein peaks probably represent subunits derived from highly insoluble large polymeric glutenins. The small amount of protein extractable with 1% DDT and 50% propanol after removal of the AS fraction, as well as the very small amount of N_2 remaining in the pellet after this (AR) extraction, confirms the high efficiency of the extraction protocol.

Comparison of AN and AS with Equivalent SDS-Phosphate Extracts

Extractability and SE-HPLC size distribution profiles of AN and AS were compared with a buffer of 2% SDS and 0.05M phosphate, pH 6.9, using the same extraction protocol for a Katepwa flour sample. For duplicate extractions, the SDS buffer without sonication removed 87.8% of the protein compared with 80.8% for AN. With sonication, the SDS buffer removed an additional 9.4% compared with 17.3% for AS. These results indicate that SDS is a more efficient extractant than 0.05M acetic acid without sonication, but that with the sonication step, similar overall amounts of protein can be extracted. It should be noted that the decrease in AN and increase in AS extractability relative to results obtained during optimization of the extraction procedure (Table I) can probably be attributed to the use of a Katepwa flour sample derived from a different wheat sample.

Figure 3 compares SE-HPLC profiles of AN and AS with the corresponding SDS-phosphate buffer extracts. The increase in protein extractability with SDS-phosphate buffer without sonication relative to AN could be primarily attributed to an increase in polymeric glutenins, and in particular, those eluting at the void volume (Fig. 3A). With SDS-phosphate, the larger f_1 polymeric fraction accounted for 25.8% of the extract, while the smaller f_2 polymeric fraction accounted for 12.4% (average of two replicates). Corresponding distributions obtained for the f_1 and f_2 AN extracts were

18.0 and 8.8%, respectively. Monomeric fractions from SDS-phosphate buffer accounted for 50.0% (f_3) and 11.8% (f_4); corresponding values for AN were 59.8 and 13.4%, respectively.

Comparison of the SE-HPLC profiles of AS and SDS-phosphate sonicated extracts (Fig. 3B) showed that large polymeric proteins predominated both profiles. These proteins accounted for 84.8 and 87.8% of the SDS-phosphate sonicated and AS extracts, respectively. The smaller polymeric proteins represented 12.4% of the SDS-phosphate sonicated extract and 8.8% of the AS extract. The monomeric protein fractions (f_{3-4}) accounted for <4% of either extract.

Flow FFF analysis was not performed due to precipitation of the SDS extractable protein in the elution buffer (0.05M acetic acid, 0.002% FL-70). This precipitation can probably be attributed to SDS acting as a salt at low concentrations, resulting in neutralization of the charged amino acids that prevent aggregation of the gluten proteins through electrostatic repulsion (Preston 1981).

Relationship of AN, AS, AR and Their SE-HPLC and Flow FFF Fractions to Farinograph Mixing Properties

Eleven hard red and white spring wheat flour samples milled from cultivars grown in three advanced plant breeder co-operative tests were used to assess the relationship between amounts of AN, AS, AR and their SE-HPLC and flow FFF fractions as a % of total extractable protein (AN+AS+AR) and farinograph dough development time (FDDT). FDDT for the samples was 3.3–13.0 min. Protein content was 10.8–14.2%. No significant ($P > 0.05$) relationship was evident between FDDT and protein content.

The mean AN, AS, and AR extractability for the sample set averaged 77.8, 19.2, and 3.0%, respectively. Both AN (73.7–81.5%) and AS (15.9–22.1%) showed fairly wide ranges in extractability. AR values were 2.4–4.1%. A highly significant negative correlation was obtained between FDDT and AN extractability ($r = -0.75$, $P < 0.01$). Highly significant positive correlations were obtained between FDDT and AS extractability ($r = 0.72$, $P < 0.01$) and between FDDT and AR extractability ($r = 0.78$, $P < 0.01$). These data are consistent with previous studies that showed a strong positive relationship between mixing properties and the proportion of less extractable protein (Orth and Bushuk 1972; review by MacRitchie 1992).

The AN distribution of size fractions from SE-HPLC and flow FFF generally showed no significant relationship to mixing time. The only exception was the large polymeric f_1 SE-HPLC protein peak (9.5–13.8%) which showed a negative correlation to FDDT ($r = -0.67$, $P < 0.05$). Much stronger relationships were found between mixing time and the proportions of polymeric proteins in the AS protein extracts. Both the larger f_1 (8.5–12.7%) and the smaller f_2 (4.4–5.9%) polymeric protein fractions from SE-HPLC showed highly ($P < 0.01$) significant correlations to FDDT ($r = 0.74$ and 0.71 , respectively). Similar results were obtained using flow FFF. Both the smaller AS f_b (7.1–10.7%) and larger AS f_c (6.2–8.2%) polymeric protein fractions showed significant correlations to FDDT ($r = 0.74$, $P < 0.01$ and $r = 0.67$, $P < 0.05$, respectively). However, no significant correlation was evident between the very large polymeric fraction (f_d) and DDT. This was probably due to the small range of values obtained with this fraction (1.2–2.2%).

These results, while based on a limited sample set, are generally consistent with previous studies showing a strong positive relationship between the size distribution of the polymeric glutenin proteins and dough strength properties (reviews by MacRitchie 1992; Southan and MacRitchie 1999). Both of the less extractable AS SE-HPLC polymeric protein fractions (f_1 and f_2) as well as the less extractable flow FFF major AS polymeric fractions (f_b and f_c) were significantly positively correlated to FDDT. The more extractable AN polymeric f_1 SE-HPLC fraction showed a significant negative correlation to FDDT. Although this latter fraction eluted at the void volume, the flow FFF results suggest that these proteins are smaller in size than either of the AS SE-HPLC polymeric fractions (Fig. 1).

Significant positive correlations were also obtained between FDDT and the less soluble AS monomeric gliadin-sized f_3 SE-HPLC ($r = 0.58$, $P < 0.05$) and f_a flow FFF ($r = 0.65$, $P < 0.05$) monomeric peaks. These significant correlations with FDDT are probably a result of the significant correlations between AS f_3 and the major AS polymeric SE-HPLC fractions and between AS f_a and the major AS polymeric flow FFF fractions ($r > 0.65$, $P < 0.05$). In particular, the SE-HPLC AS f_3 fractions showed very high correlations to AS f_1 ($r = 0.91$) and AS f_2 ($r = 0.94$), suggesting a very strong relationship. This result is consistent with the presence of smaller monomeric proteins in the flow FFF profile of the void peak SE-HPLC subfraction (c_1) (Fig. 2) and supports the supposition that these smaller proteins may form complexes with the larger polymeric proteins. An increase in polymeric f_1 and f_2 protein would increase the amount of binding sites proportionately for monomeric f_3 or f_a protein. The nature of these smaller binding proteins is not known and requires further study. It should be noted that SE-HPLC and flow FFF profiles of AS (and AN) extracts from chloroform defatted flours were essentially identical to those of untreated flours (data not shown), indicating that interactions were not influenced by lipids.

CONCLUSIONS

The simple two-step extraction procedure using dilute acetic acid without (AN) then with sonication (AS) is a highly efficient and reproducible method for fractionating wheat flour proteins that can potentially replace the use of more chaotropic SDS and phosphate buffer extraction procedures. SE-HPLC and flow FFF results show that the predominant AN extract consists primarily of monomeric protein while the AS extract contains mainly polymeric protein. Flow FFF results demonstrate that the AN polymeric protein, including that eluting at the SE-HPLC void volume, has smaller Stokes diameters than the AS polymeric protein. Flow FFF analysis of the AN SE-HPLC subfraction eluting at the void volume suggests the presence of monomeric, smaller polymeric, and large polymeric proteins that may form complexes involving some or all of these components. Examination of 11 hard red and white spring wheat flours shows a fairly wide variation in AN and AS and their SE-HPLC and flow FFF fractions. The proportion of larger polymeric protein fractions in these samples shows a high positive correlation to farinograph mixing time.

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