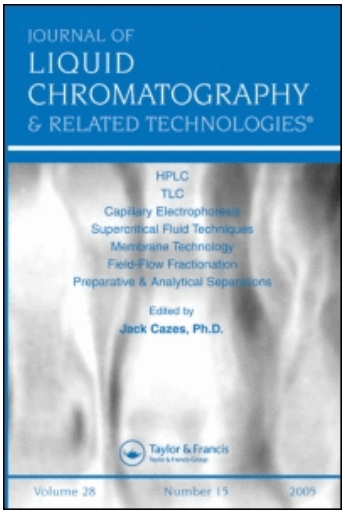


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S. G. Stevenson[a]; S. You[b]; M. S. Izydorczyk[a]; K. R. Preston[a]
[a] Grain Research Laboratory, Canadian Grain Commission, Winnipeg, MB, Canada [b] Department of Food Science, University of Manitoba, Winnipeg, MB, Canada

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# Characterization of Polymeric Wheat Proteins by Flow Field-Flow Fractionation/MALLS

**S. G. Stevenson,[1] S. You,[2] M. S. Izydorczyk,[1] and K. R. Preston[1,*]**

[1]Grain Research Laboratory, Canadian Grain Commission, Winnipeg, MB, Canada
[2]Department of Food Science, University of Manitoba, Winnipeg, MB, Canada



## ABSTRACT

Flow field-flow fractionation (FFF) combined with multiangle laser light scattering (MALLS) was used to investigate the size properties of monomeric and polymeric wheat flour protein fractions from common and durum wheat flours obtained by sequential extraction with dilute acetic acid with and without sonication. The major FFF peak obtained for the monomeric protein fraction showed $M_w$ values in the 31,000–33,000 range plus some larger monomeric and smaller polymeric protein eluting later in the fractogram. The major peak in the polymeric protein fraction showed $M_w$ values in the 300,000 range. At later elution times, $M_w$ values for the polymeric proteins approached 10,000,000. Radius of gyration

*Correspondence: K. R. Preston, Grain Research Laboratory, Canadian Grain Commission, Winnipeg, MB, Canada R3C 3G8; E-mail: kpreston@grainscanada.gc.ca.



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values showed relatively small changes for the polymeric protein fraction with increasing elution time, suggesting that the larger $M_w$ proteins tend towards a more compact structure than smaller $M_w$ polymeric proteins.

*Key Words:* Polymeric wheat proteins; Flow FFF; MALLS; Multiangle laser light scattering; Wheat varieties; Molecular weight.

## INTRODUCTION

The amount and size distribution of the polymeric proteins in wheat gluten play a major role in determining the processing characteristics of wheat flour.[1] Gel filtration and size exclusion (SE)-HPLC studies have shown high positive correlations between the proportion of the larger more insoluble polymers and dough strength and/or baking quality.[2,3] Recent studies using SE-HPLC combined with multiangle laser light scattering (MALLS), indicate that these larger glutenin polymers may range upwards of 10 million in molecular weight at or near the exclusion limit.[4,5]

Flow field-flow fractionation (FFF) has a number of advantages over SE-HPLC for measuring the size distribution properties of the larger polymeric wheat proteins. With SE-HPLC, the larger polymeric proteins elute at or near the void volume, whereas with flow FFF these proteins are resolved.[6–8] Recent flow FFF studies also indicate that the larger glutenin polymers eluting at the SE-HPLC void volume may be complexed with lower molecular weight monomeric and polymeric proteins,[9] which influence size measurements. These complexes appear to undergo dissociation at the very low ($\approx$1 μg) protein concentration required to prevent overloading with flow FFF.

In the present study, we report on the use of flow FFF combined with MALLS to characterize wheat polymeric protein fractions using four common spring wheat varieties and one durum wheat varying in dough strength. Dilute acetic acid was used with sonication to extract the polymeric proteins in order to reduce changes in protein structure and eliminate micelle formation associated with the use of the more commonly used chaotropic detergent, sodium dodecyl sulfate (SDS).

## EXPERIMENTAL

Four common Canadian spring wheat varieties (Katepwa, Laura, Glenlea, and AC 2000) and a Canadian durum wheat variety (AC Avonlea) were obtained from advanced Canadian plant breeder trials. Common wheat was tempered to optimum and then milled to straight grade flour on an Allis-Chalmers laboratory





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mill.[10] Durum wheat was tempered to optimum then milled to semolina.[11] After milling, the straight grade flours or semolina were tested for protein content ($N \times 5.7$) using combustion nitrogen analysis (LECO Model FP-428 Dumas Analyser, St. Joseph, MI).[12] Dough strength was estimated by farinograph dough development time using AACC.[13] Standard Methods with a 50-g bowl. All flour results are reported on a 14% moisture basis.

Flour (0.1 g) was extracted twice with 0.05 M acetic acid (4 then 2 mL) to remove the monomeric protein fraction, then, the residue from centrifugation was extracted with the same solution (4 mL) using sonication and centrifuged to obtain the polymeric protein extract, as described previously.[9] Protein in the residue was estimated by combustion nitrogen analysis. Fractions were subjected to SE-HPLC on a SEC-S4000 column (Phenomenex, Torrance, CA) to estimate protein yields based on absorbance at 210 nm. Automated flow FFF of the extracts was carried out using a conventional FI/FO channel (Postnova Analytics USA, Salt Lake City, UT) with a channel flow of 0.2 mL/min, recirculating cross flow of 5.0 mL/min, and recirculating frit inlet/frit outlet flow of 1.4 mL/min with 0.05 M acetic acid containing 0.002% FL-70 as eluent, as described previously.[7] Column eluent was monitored by absorbance at 210 nm, refractive index (Waters 410, Waters, Mississauga, ON, Canada) and by MALLS using a DAWN DSP Laser Photometer (Wyatt Technology, Santa Barbara, CA). Stokes diameters of the components from FFF were obtained by calibration of retention time ($t_r$) against a set of standard proteins of known molecular weight, as previously described.[7] The relationship between retention time and Stokes diameter was $d_S = 0.789 \cdot t_r - 1.209$ ($r^2 = 0.97$). The distribution of FFF size fractions within the polymeric protein extract was determined by integration of the FFF absorbance signal in four regions defined as monomeric (<8 nm), small polymeric (8–19 nm), large polymeric (19–37.5 nm), and very large polymeric (>37.5 nm), based on Stokes diameter, as previously described.[9]

Weight average molecular weight ($M_w$) was determined using ASTRA software (V4.72, Wyatt Technology) from the equation $R_\theta/K^*c = MP(\theta) - 2A_2cM^2P^2(\theta)$ from Zimm,[14] where the second virial coefficient ($A_2$) was assumed to be zero. The Debye fit method, appropriate for smaller proteins, was used for the monomeric protein fraction and the Berry fit method, appropriate for larger proteins, was used for the polymeric protein fraction for reasons described by Jeng and Balke.[15] Protein content was determined for each Katepwa solubility fraction by combustion nitrogen analysis ($N \times 5.7$). Five dilutions of each Katepwa fraction were then used to calculate $dn/dc$ values using RICAL software (V2) from Wyatt Technology for determining the optical constant ($K^*$). *Z*-average root mean square values for radius of gyration ($R_g$) were determined from the equation $P(\theta) = 1 + 16\pi^2 < R_g{}^2 > \sin^2(\theta/2)/3\lambda_0{}^2$ using ASTRA software.





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## RESULTS AND DISCUSSION

Table 1 shows straight grade flour or semolina characteristics obtained from the four common spring wheat varieties and one durum wheat variety. Common wheat flour ash content varied from 0.41% to 0.50%, while protein content ranged from 12.0% to 13.8%. Among common wheat flours, Glenlea, an extra-strong type wheat, showed the longest farinograph dough development time, while the shortest values were obtained with Katepwa, a hard red spring breadwheat, and AC 2000, a white spring wheat. The durum semolina showed an ash content of 0.61% and a protein content of 13.9%. The dough development time was similar to Katepwa and AC 2000.

Table 1 also shows the distribution of protein fractions obtained from the sequential extraction procedure. Most of the flour protein was extracted in the monomeric fraction, with AC Avonlea and Katepwa showing the highest values (82.3% and 82.5%, respectively) and Glenlea showing the lowest value (73.3%). Among the common wheat flours, Glenlea showed the largest proportion of protein in the polymeric fraction at 25.2%, while Katepwa showed the lowest proportion (16.3%). The proportion of polymeric protein in AC Avonlea was 1.0% lower than Katepwa. In general, these results are consistent with previous studies showing a close relationship between farinograph dough development time (or other strength related parameters) and the proportion of protein (primarily polymeric) that cannot be extracted without the use of sonication, chaotropic solvents or both.[1]

***Table 1.*** Physical characteristics and SE-HPLC recoveries of monomeric and polymeric protein fractions extracted from straight grade flour or semolina obtained from four common and one durum wheat variety.[a]

| Wheat variety | Flour protein[b] (%) | Flour ash[b] (%) | Farinograph DDT (min) | Protein fraction (%) | |
|---|---|---|---|---|---|
| | | | | Monomeric | Polymeric |
| Common wheat | | | | | |
| Katepwa | 13.8 | 0.35 | 5.3 | 82.5 | 16.3 |
| Laura | 14.0 | 0.41 | 7.8 | 78.8 | 19.9 |
| Glenlea | 13.1 | 0.48 | 10.8 | 73.3 | 25.2 |
| AC 2000 | 12.0 | 0.46 | 5.0 | 79.9 | 18.9 |
| Durum wheat | | | | | |
| AC Avonlea | 13.9 | 0.61 | 5.3 | 82.3 | 15.3 |

*Note:* DDT, dough development time.
[a]Protein fractionation data based on triplicate results.
[b]Flour results reported on 14% moisture basis.





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Figure 1 shows the flow FFF RI and $M_w$ profiles for the monomeric protein fraction from Katepwa at several injection volumes. The $dn/dc$ of this fraction was calculated as 0.208 mL/g, which is somewhat higher than values (0.18–0.20) reported previously.[4] Column loads of approximately 1 μg (5 μL) protein were required with the monomeric fraction to obtain sufficient light scattering intensity for $M_w$ calculations. Injections much above this amount resulted in a shift of the major peak, indicating overloading of the channel. The $M_w$ profile at the lower injection levels (5 or 10 μL) showed similar patterns but at the higher concentration (20 μL), higher $M_w$ values were apparent after the main peak. This can probably be attributed to a higher concentration of larger monomeric and small polymeric proteins eluting earlier in the fractogram due to overloading. At the peak, $M_w$ values varied from 31,000–33,000, consistent with the $M_w$ values previously obtained for the major gliadin proteins by SE-HPLC/MALLS[4] and by mass spectrometry.[16] At later elution times, $M_w$ values were evident up to about 110,000. These proteins probably consist of higher MW $\omega$-gliadin and small glutenin polymers.[7] Results for the monomeric protein extracts from the other wheat varieties were very similar (data not shown).

Figure 2 shows the flow FFF RI and $M_w$ profiles of the polymeric protein fraction from Katepwa at several concentrations. The $dn/dc$ value calculated for this fraction was 0.169 mL/g, which is slightly lower than previously reported values,[5] but considerably lower than values reported in the presence

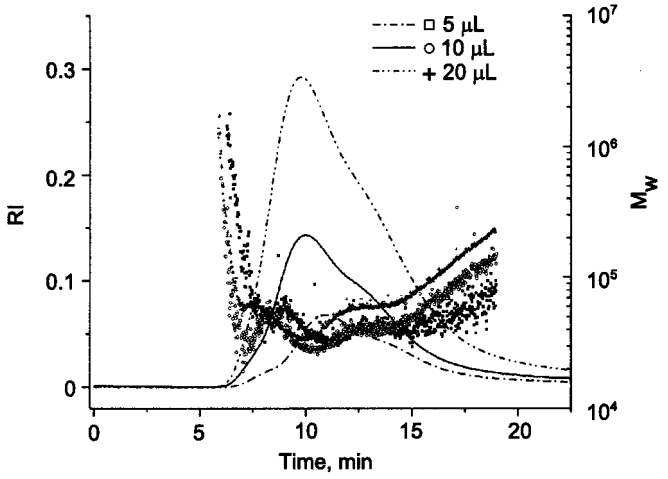


***Figure 1.*** Effect of the injection volume of the monomeric protein fraction on FFF fractograms and MALLS $M_w$ profiles.





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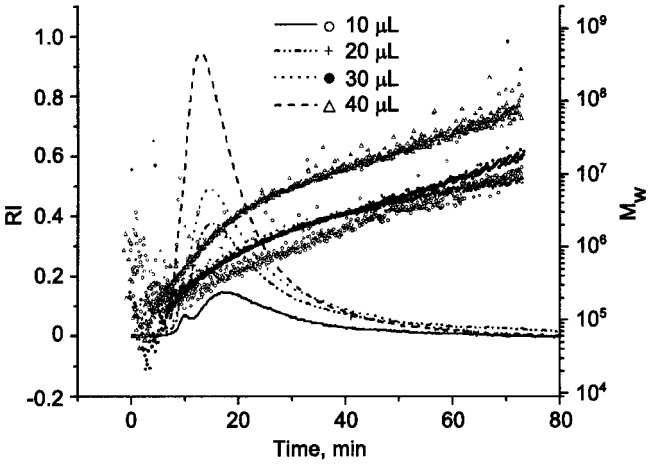


***Figure 2.*** Effect of the injection volume of the polymeric protein fraction on FFF fractograms and MALLS $M_w$ profiles.

of SDS.[4] At 10 µL (~0.6 µg), variability in the $M_w$ profile was apparent indicating less accurate values due to insufficient scattering intensity. At 40 µL, there was a definite shift of the peak towards shorter elution time indicating overloading. The higher $M_w$ values in the 40 µL $M_w$ profile are probably related to the earlier elution of higher $M_w$ polymers associated with this overloading. The 20 and 30 µL injections show little sign of overloading and had comparable $M_w$ profiles. The peak at about 17 min showed $M_w$ values close to 300,000, suggesting the predominance of polymers composed of a relatively small number of disulfide linked low and/or high molecular weight glutenin subunits. However, at later elution times $M_w$ increased to values approaching 10,000,000. These values are probably an underestimate of the upper limit, since the sonication step used to extract these polymeric proteins causes some cleavage of the largest polymers.[9] These values are generally consistent with $M_w$ values obtained for polymeric wheat protein by MALLS at or near the exclusion limit of SE-HPLC columns.[4,5]

Figure 3 shows the $M_w$ profiles of the polymeric protein fractions obtained for the four common and one durum wheat flour at optimum loading (20–30 µL), which provided the highest light scattering intensity without overloading the channel. The $M_w$ values at the peaks for all wheat varieties showed a fairly narrow range of variation, ranging from about 225,000–300,000. The $M_w$ profiles were also generally similar with $M_w$ values increasing to values of about 7,000,000 after 60 min of elution.





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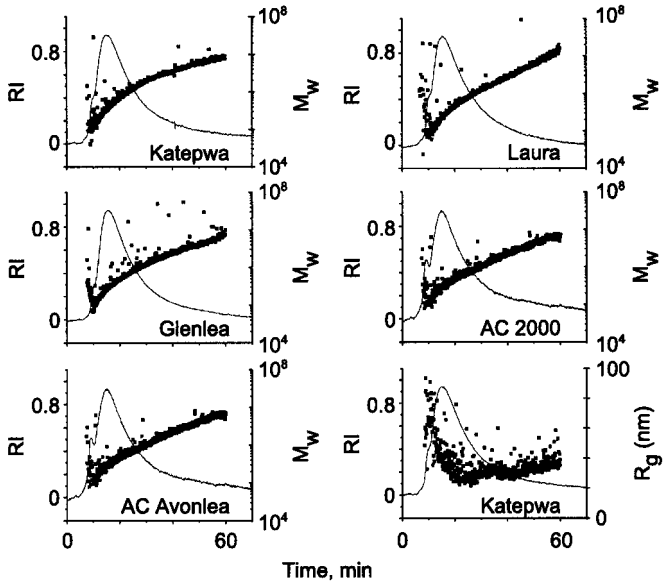


***Figure 3.*** Flow field-flow fractionation fractograms and MALLS $M_w$ profiles of polymeric protein fractions from five wheat varieties and a MALLS $R_g$ profile for the variety, Katepwa.

Table 2 shows the distribution of the FFF size fractions based on Stokes diameter where monomeric, small polymeric, large polymeric, and very large polymeric proteins have been arbitrarily defined as components eluting at <8 nm, 8–19 nm, 19–37.5 nm, and above 37.5 nm.[9] Since there was no evident difference in the $M_w$ profile among any of the varieties, the $M_w$ ranges for these size fractions can be estimated at about <125,000 for monomeric proteins, 125,000–800,000 for small polymeric proteins, 800,000–4,000,000 for large polymeric proteins, and >4,000,000 for very large polymeric proteins. It should be noted that these values are biased towards larger molecular weight species, since light scattering calculations are based upon weight average rather than number average values.

No significant differences ($p < 0.05$) were evident in the distribution of these size fractions among the common wheat flours. As shown previously,[9] the small polymeric size fraction dominated followed by the large polymeric fraction. The other two fractions represented less than 15%. Thus, differences in strength among these varieties appear to be primarily determined by the amount of polymeric protein requiring sonication for extraction rather than the size distribution within this extract. The durum wheat semolina showed a

***Table 2.*** Distribution of FFF size fractions in the polymeric protein extract of straight grade flour or semolina obtained from four common and one durum wheat variety.[a]

| Wheat variety | Monomeric (<8 nm) (%) | Small polymeric (8–19 nm) (%) | Large polymeric (19–37.5 nm) (%) | Very large polymeric (>37.5 nm) (%) |
|---|---|---|---|---|
| Common wheat | | | | |
| Katepwa | 6.4 ± 0.7 | 55.9 ± 5.1 | 29.7 ± 3.6 | 8.1 ± 2.3 |
| Laura | 5.7 ± 0.7 | 57.5 ± 5.4 | 27.7 ± 4.1 | 9.0 ± 2.0 |
| Glenlea | 5.4 ± 0.6 | 58.7 ± 3.1 | 27.4 ± 2.6 | 8.5 ± 1.2 |
| AC 2000 | 6.2 ± 0.8 | 57.0 ± 5.6 | 29.0 ± 4.4 | 7.7 ± 2.0 |
| Durum wheat | | | | |
| AC Avonlea | 9.3 ± 1.5 | 42.8 ± 7.1 | 33.7 ± 3.7 | 14.3 ± 3.0 |

[a]Data based on mean of three values ± standard deviation.

higher proportion of monomeric, large, and very large polymeric proteins and a corresponding smaller proportion of smaller polymeric proteins. Although, the total amount of these larger size fractions based on total flour protein are somewhat reduced relative to common wheat flours when corrected for extractability, values are still within the same range obtained with the latter. These results suggest that the poor baking quality of this variety, and durum wheat in general,[17] is probably not related to protein size distribution. Recent studies by Ammar et al.[18] suggest that the lack of dough extensibility due to loss of high molecular weight glutenin subunits associated with the absence of the *D* genome rather than dough strength factors, plays a major role in the poor baking performance of durum wheat.

Figure 3 also shows a $R_g$ profile of the polymeric protein extract for Katepwa. The $R_g$ profiles were very similar for all the common wheat flours and durum semolina (data not shown). There was a decrease in $R_g$ values during the first 20 min elution of the protein samples. $R_g$ then increased slightly, from about 28 nm to about 36 nm, over the next 40 min of elution. The slope of $R_g$ vs. $M_w$ plot provides an estimate of the conformational properties of polymers.[19] The slope of the lower molecular weight fraction at the peak ($t_r = 13.3$–$17.3$ min) of the Katepwa polymeric protein extract was 0.60, indicating a random coil conformation. The intermediate molecular weight fraction ($t_r = 17.3$–$23.5$ min) of the protein extract showed a slope of 0.39, indicative of spherical conformation, while the higher molecular weight fraction ($t_r = 23.5$–$60$ min) exhibited a more compact spherical conformation (slope = 0.17). The results suggest that the lower molecular weight protein polymers are in loose conformation, while the higher molecular weight protein exists in a more entangled and compact conformation, probably due to more intra-molecular interactions. This is consistent with SE-HPLC/MALLS results obtained by Carceller and Aussenac,[5] who also found an increase in the compactness of the polymeric proteins with increasing $M_w$.

## CONCLUSIONS

Flow FFF combined with MALLS is a promising technique for assessing the size and shape of polymeric wheat proteins. Care must be taken to inject the appropriate amount of protein to ensure sufficient light scattering intensity to obtain accurate $M_w$ values without overloading the channel. The wheat monomeric fraction obtained by extraction with dilute acetic acid, consists primarily of proteins with $M_w$ values in the 31,000–33,000 range plus some larger monomeric and smaller polymeric protein. The wheat polymeric protein fraction obtained after removal of monomeric proteins by sonication with dilute acetic acid, shows a major peak with $M_w$ of about 225,000–300,000.





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A large increase in $M_w$ is evident with increasing elution time, with values approaching 10,000,000 after 60 min. The relatively small increase in $R_g$ with increasing elution time, suggests that the larger polymeric proteins tend towards a more compact shape than the smaller polymeric proteins.

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