Equivalence of High Molecular Weight Glutenin Subunits Prepared by Reversed-Phase High-Performance Liquid Chromatography and Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis[1]

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ABSTRACT

High molecular weight (HMW) glutenin subunits of three cultivars were isolated from reduced glutenin by two techniques: reversed-phase high-performance liquid chromatography (RP-HPLC) and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. By using one method for the preparation of the subunits and the other for analysis, and vice versa, the identity of the subunits by the two techniques was established. RP-HPLC results showed that in general, surface hydrophobicity of the subunits increased with increasing molecular weight and with complexation with SDS. In most cases, the increment of change in surface hydrophobicity after complexation with SDS decreased with increasing molecular weight.

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Interest in improved methods for wheat protein analysis has increased recently with the discovery of the association of glutenin subunit composition and breadmaking potential of flour (Branlard and Dardevet 1985, Campbell et al 1987, Payne et al 1987, Pogna et al 1987, Ng and Bushuk 1988). Polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS) has quickly become the most common technique for fractionating and identifying wheat proteins on the basis of apparent molecular weight (Mapp) (for a review, see Wrigley and Bietz 1988). Reversed-phase high-performance liquid chromatography (RP-HPLC), which separates proteins on the basis of surface hydrophobicity, was recently introduced to research on wheat proteins (for a review, see Bietz 1986). The two methods complement each other.

Burnouf and Bietz (1985), Marchylo et al (1989), and Wieser et al (1989) used a combination of the two methods to relate RP-HPLC peaks to the high molecular weight (HMW) subunits of glutenin as determined by SDS-PAGE. The peak fractions were collected by RP-HPLC and analyzed by SDS-PAGE. The

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pioneering work by these scientists has been extended in our laboratory. Additionally, we separated single bands of HMW glutenin subunits by preparative SDS-PAGE and analyzed them by RP-HPLC.

MATERIALS AND METHODS

Materials

Three Canadian hard red spring wheat cultivars—Neepawa, Roblin, and Selkirk—were selected for this study because collectively they contained all but one of the HMW subunits present in the 21 cultivars registered for the Canada western red spring class (Ng et al 1988). The only exception is the subunit we have named "1/2*", of the cv. Chinook, which migrates in SDS-PAGE between subunits 2* and 1, named according to the nomenclature of Payne and Lawrence (1983). The HMW subunit compositions of the three cultivars Neepawa, Roblin, and Selkirk are, respectively, 2*, 5, 7, 9, and 10; 2*, 5, 7, 8, and 10; and 1, 5, 6, 8, and 10.

Flour was milled from the grain on a Buhler pneumatic laboratory mill after tempering overnight to 15.5% moisture.

Acetonitrile and 2-propanol, both of HPLC grade, were obtained from Fisher Scientific (Fair Lawn, NJ). Sequanal grade trifluoroacetic acid was purchased from Pierce Chemical Co. (Rockford, IL). All other chemicals used were of analytical reagent grade. Water was distilled and then purified with a Millipore Milli-Q system (Mississauga, ON).

Protein Extraction for RP-HPLC

Most of the gliadins were first removed and discarded by extracting 1.0 g of flour with 6 ml of 50% (v/v) 2-propanol (Sugimoto et al 1986). The mixture was vortexed in 15-ml glass centrifuge tubes at 5-min intervals for 15 min and then centrifuged for 10 min at 30,500 × *g* at 24°C. The wet residue was washed with 6 ml of water for 15 min as described above. The washed residue was extracted with 2 ml of buffer solution (pH 7.5) containing 0.082*M* Tris-HCl, 2*M* urea, and 1% (w/v) dithiothreitol for 60 min at 60°C. This mixture was vortexed at 15-min intervals and centrifuged for 15 min at 30,500 × *g* at 24°C. Supernatant was collected and alkylated according to Marchylo et al (1989). The clear alkylated supernatant was filtered through a 0.45-μm nylon filter (Millipore) into a disposable microvial in preparation for RP-HPLC.

Protein Extraction for Electrophoresis

Flour protein extracts for SDS-electroendosmotic preparative electrophoresis (SDS-EPE, analogous to SDS-PAGE) were prepared by suspending 150 mg of flour in 3 ml of SDS-PAGE buffer solution (Ng and Bushuk 1987). For SDS-PAGE, flour proteins were extracted according to Ng and Bushuk (1987).

RP-HPLC

Analyses were performed on a Hewlett-Packard 1090M chromatograph according to Scanlon et al (1989) with some minor modifications. A 25-cm Supelcosil LC-308 reversed-phase column (C_8, 300-Å pore size, 5-μm particle size, 4.6-mm i.d.) (Supelco Inc., Bellefont, PA) was used in conjunction with a 5-cm guard column with the same packing and specifications. Chromatography was carried out using distilled-purified water as solvent A and acetonitrile as solvent B, both containing 0.1% (v/v) trifluoroacetic acid. Column temperature was 50.0°C, solvent flow rate was maintained at 1.0 ml/min, and 25 μl of protein extracts were chromatographed. Proteins were eluted over 85 min using the conditions shown in Table I and monitored at 210 nm. Peak fractions were collected manually, dried in a vacuum concentrator (Speedvac Concentrator, Savant Instruments, Inc., Farmingdale, NY), and stored at −20°C until used for SDS-PAGE analysis.

TABLE I
Solvent Composition Changes for Chromatography

Time (min)	Solution B (Acetonitrile) (%)	Explanation
0	23	Initial conditions
0–3.0	23	Isocratic
3.0–49.8	23–35	Linear
49.8–50.0	35–48	Linear
50.0–60.0	48	Isocratic
60.0–75.0	48–60	Linear
75.0–85.0	60–23	Linear
85.0–95.0	23	Equilibration at initial conditions

Electrophoresis

SDS-EPE was carried out with an ELFE electrophoresis unit (Genofit, Geneva, Switzerland). The procedure was that of Curioni et al (1989) with some minor modifications. The separating gel (2-cm diameter cylinder) was prepared with 5 ml of solution containing 9.87% (w/v) acrylamide and 0.13% (w/v) bisacrylamide and was polymerized with 25 μl of 10% (w/v) ammonium persulfate and 1.5 μl of TEMED. The stacking gel was prepared with 10 ml of solution containing 4.0% (w/v) acrylamide and 0.06% (w/v) bisacrylamide and was polymerized with 66 μl of 10% (w/v) ammonium persulfate and 6 μl of TEMED. Electrophoresis was at room temperature at a constant current of 20 mA for 5–6 hr. Proteins eluted from the gel were detected by measuring absorbance at 280 nm with a Pharmacia LKB Uvicord SII (Montreal, PQ). Fractions (500 μl) were collected manually, dried in a vacuum concentrator, and stored at −20°C. The dry fractions were analyzed by SDS-PAGE (Ng and Bushuk 1987) and alkylated before being subjected to RP-HPLC analysis as described above.

RESULTS AND DISCUSSION

RP-HPLC

Figure 1 shows a complete RP-HPLC chromatogram of reduced-alkylated glutenin of cv. Roblin. In a preliminary study, all peaks were collected and analyzed by SDS-PAGE. Peaks (Fig. 1) that eluted at approximately 52 min were mainly gliadins and low molecular weight glutenins, and those at 55 min were nonprotein components of the buffer or phenolic compounds of the flour since no protein bands were detected upon SDS-PAGE of these peaks (data not shown). The nature of these components was not determined. All of the HMW subunits of glutenin were eluted between 20 and 40 min. This is in general agreement with the results of Burnouf and Bietz (1984, 1985).

Figure 2 shows all HMW subunit peaks from RP-HPLC chromatograms of the cultivars examined. The SDS-PAGE electrophoregrams of the RP-HPLC fractions of cv. Neepawa, which contained the HMW subunits (Fig. 3) are in general agreement with the results reported by Marchylo et al (1989) for the same wheat variety. The HMW subunits clearly differ sufficiently in hydrophobicity and therefore are well resolved by RP-HPLC.

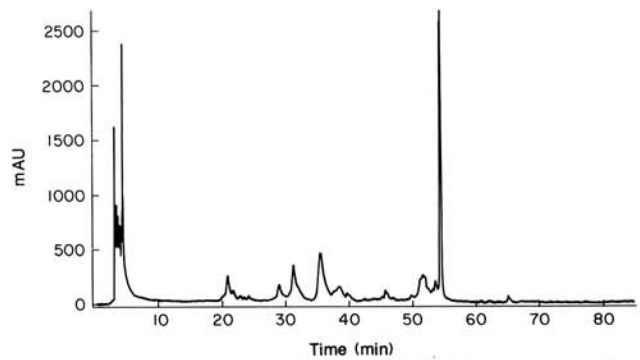

Fig. 1. Reversed-phase high-performance liquid chromatogram of total reduced-alkylated glutenin of cv. Roblin. mAU = milliabsorbance units.

SDS-EPE

The elution profiles of the HMW subunits of cv. Roblin from SDS-EPE (Fig. 4) were generally similar to those reported by Curioni et al (1989) for other wheat varieties. The arrow in Figure 4 indicates the tracking dye (Pyronin Y) being eluted off the gel and followed by most of the low molecular weight components. Although there were only three distinct peaks, all five of the HMW subunits were resolved in the fractions collected, as indicated by the horizontal bars in Figure 4. The HMW-subunit fractions from SDS-EPE were alkylated and analyzed by RP-HPLC. Peak fractions from the SDS-EPE after RP-HPLC analysis, subjected to SDS-PAGE (Fig. 5), had the same SDS-PAGE patterns as those in the same fractions subjected to SDS-PAGE directly from SDS-EPE (i.e., without prior alkylation and RP-HPLC analysis).

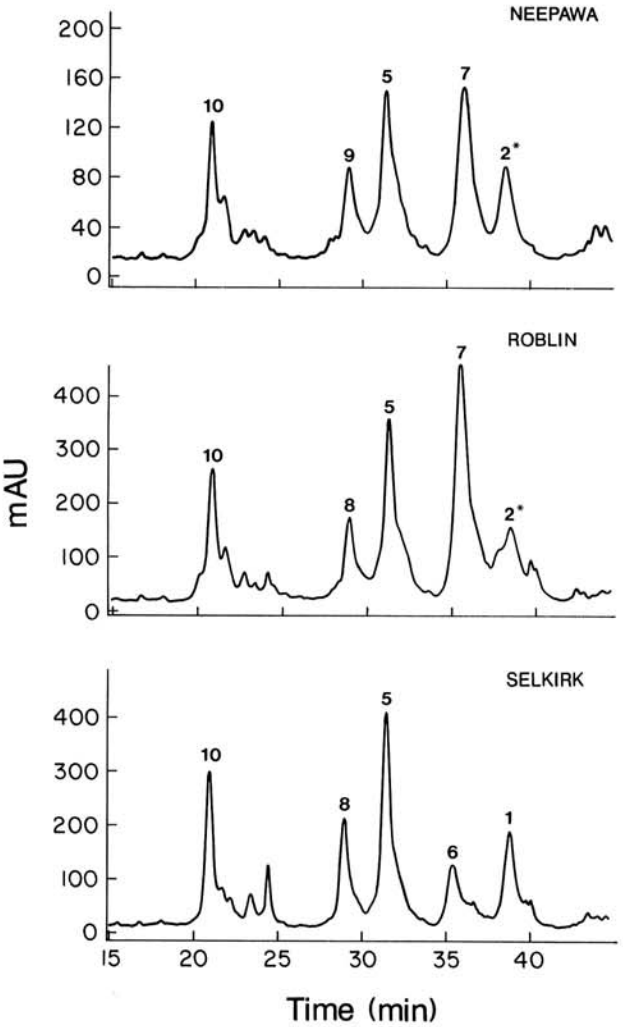

Fig. 2. Reversed-phase high-performance liquid chromatograms of total reduced-alkylated glutenin of cvs. Neepawa, Roblin, and Selkirk. Five high molecular weight glutenin subunits of each cultivar are indicated, using nomenclature of Payne and Lawrence (1983). mAU = milliabsorbance units.

By way of example, Figure 6 compares the RP-HPLC chromatograms of alkylated subunit 7 fraction of Roblin (from SDS-EPE) with that of the total alkylated glutenin fraction of Roblin. Subunit 7, obtained from SDS-EPE fractionation (already complexed with SDS), eluted later (had a higher surface hydrophobicity) than the same subunit in total glutenin (indicated by arrows in Fig. 6). The extracts of these two separations were all alkylated first, thus the difference in the elution time of the same subunit (7) in the two extracts must be due to the complexation with SDS. This result is in agreement with the observation by Bietz (1983) that glutenin subunits extracted with a solvent containing SDS had a higher hydrophobicity by RP-HPLC (due to complexation of SDS) than those extracted without SDS.

RP-HPLC retention times for the HMW subunits from the analysis of total alkylated glutenin (TG) and of the SDS-EPE alkylated subunit fractions (in which protein is complexed with SDS) are given in Table II. In most cases, the retention time of the peaks (i.e., surface hydrophobicity) of TG increased significantly with Mapp ($r = 0.85$, $P < 0.01$). This would be expected because of the increase in surface area with increasing Mapp.

Complexation with SDS increased retention time and variation in retention time for all the HMW subunits (Table II). For example, the retention times of subunit 5 prepared by SDS-EPE for the three cultivars yielded a coefficient of variation of 1.11% but only 0.13% when subunit 5 was prepared by TG. A significant correlation was also obtained between Mapp and retention time of the subunit fractions after complexation with SDS ($r = 0.74$, $P < 0.01$). Although this correlation coefficient is lower than that obtained in the correlation of Mapp versus retention time of peaks of TG (0.74 vs. 0.85), it is still significant at the 1% level. The correlation coefficient suggests that after SDS complexation, differences in the overall hydrophobicity of the subunits are reduced. It is unlikely that SDS in the sample extract would modify the hydrophobicity of the column, since the amount of the extract used was only 25 μl, compared with the large volume of solvent used in the chromatographic separation (1 ml/min for 85 min).

However, it is not possible to relate this effect to an increase in either surface area or specific hydrophobicity (i.e., surfacing of more hydrophobic side chains due to the unfolding of the protein). The effect of SDS, as indicated by the EPE-TG ratio (Table II), generally decreased with increasing Mapp. This is consistent with the fact that without SDS the larger subunits have a higher hydrophobicity; that is, more of the hydrophobic residues are already exposed on the surface. Further research of this point is obviously required to confirm these speculative

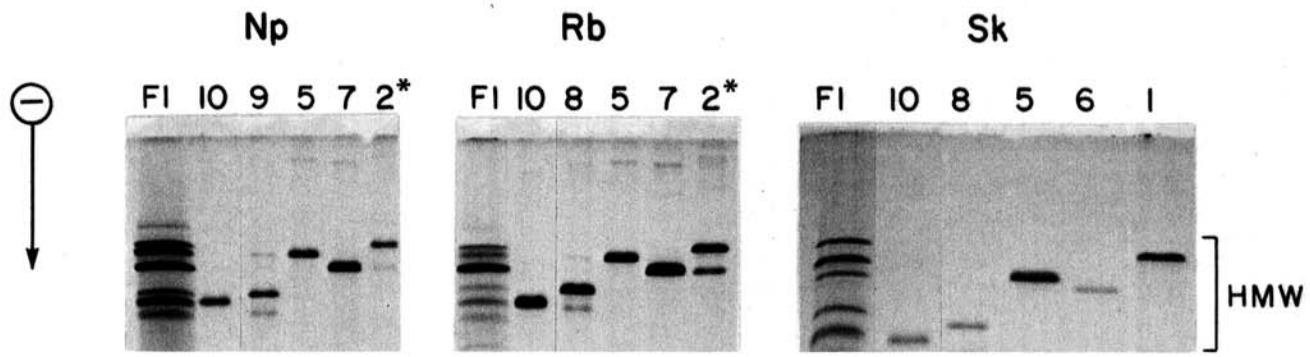

Fig. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoretic patterns of the high molecular weight (HMW) glutenin subunits in peaks resolved by reversed-phase high-performance liquid chromatography in Figure 2. Lane numbers are the subunit numbers according to Payne and Lawrence (1983) and correspond to peak numbers in Figure 2. Np = Neepawa, Rb = Roblin, Sk = Selkirk, Fl = flour.

TABLE II
Reversed-Phase High-Performance Liquid Chromatography Retention Time (min) of Alkylated High Molecular Weight Glutenin Subunits from Total Glutenin (TG) and Protein Fractionated from Sodium Dodecyl Sulfate-Electroendosmotic Preparative Electrophoresis (EPE)

		Neepawa			Roblin			Selkirk		
Subunit	Mapp[a]	TG	EPE	EPE-TG Ratio	TG	EPE	EPE-TG Ratio	TG	EPE	EPE-TG Ratio
10	92.1	20.9	34.7	1.7	20.9	33.1	1.6	20.9	35.4	1.7
9	95.7	29.1	39.8	1.4	...	...	...	...	...	...
8	101	...	...	...	28.9	38.1	1.3	28.9	40.2	1.4
7	115	36.0	40.8	1.1	35.5	40.0	1.1	...	...	...
6	121	...	...	...	...	...	...	35.3	42.0	1.2
5	128	31.3	38.9	1.2	31.3	38.3	1.2	31.3	38.1	1.2
2*	136	38.5	43.7	1.1	38.5	43.1	1.1	...	...	...
1	149	...	...	...	...	...	...	38.7	42.9	1.1

[a] Apparent molecular weight (kDa) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Ng and Bushuk 1989).

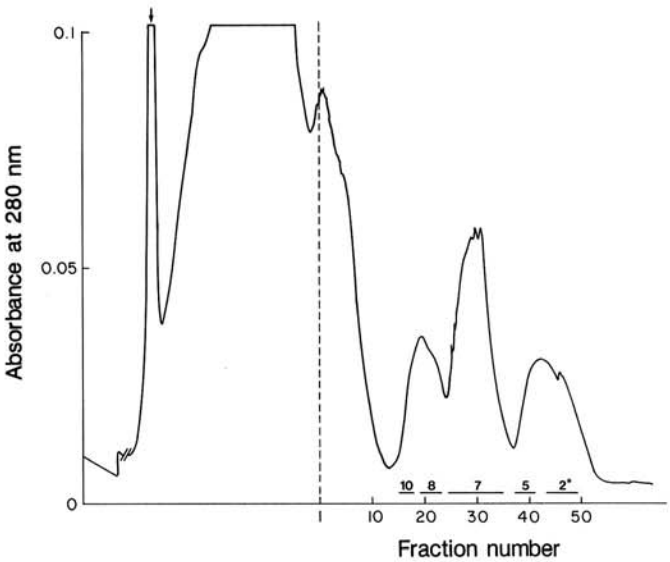

Fig. 4. Elution profile of reduced proteins from cv. Roblin separated by electroendosmotic preparative electrophoresis in the presence of sodium dodecyl sulfate. Arrow indicates where Pyronin Y dye eluted off the gel. The volume of each fraction was 500 μl. Solid lines at the base indicate the pooled fractions for each subunit.

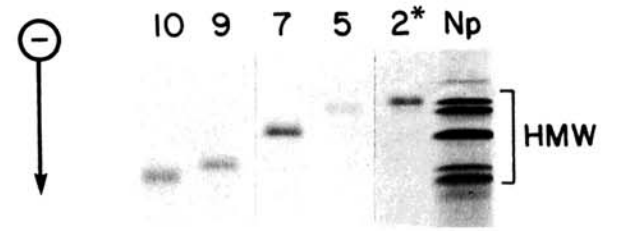

Fig. 5. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoretic patterns of the high molecular weight (HMW) glutenin subunits in peaks resolved by reversed-phase high-performance liquid chromatography after alkylation from pooled fractions of SDS-electroendosmotic preparative electrophoresis of cv. Neepawa.

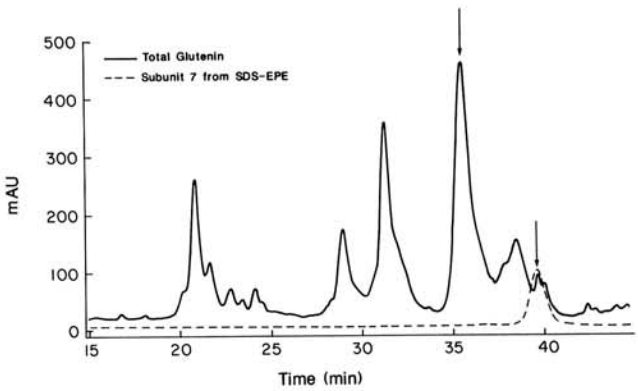

Fig. 6. Reversed-phase high-performance liquid chromatograms of total reduced-alkylated glutenin and subunit 7 obtained from sodium dodecyl sulfate-electroendosmotic preparative electrophoresis of cv. Roblin. Arrows indicate subunit 7, mAU = milliabsorbance units.

comments. It would appear that this line of research could provide useful information relevant to the effects of surfactants on the functionality of glutenin in dough.

CONCLUSION

HMW glutenin subunits of three cultivars were prepared by two techniques: RP-HPLC and SDS-PAGE. The HMW subunits differ sufficiently in hydrophobicity to be well resolved by RP-HPLC. In general, peak retention time increased with Mapp of the HMW subunits. Subunits (complexed with SDS) obtained from SDS-EPE had higher retention times (i.e., higher surface hydrophobicity) than did the equivalent subunits before complexation with SDS. The effect of SDS decreased with increasing Mapp. Whether this is due to unfolding of the protein in the presence of SDS and exposing more hydrophobic side chains in the smaller subunits than larger ones remains to be investigated.

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