

Characterization of Glutenin Protein Fractions from Sequential Extraction of Hard Red Spring Wheats of Different Breadmaking Quality¹

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ABSTRACT

Cereal Chem. 81(6):681–685

Gluten proteins from two cultivars of hard red spring (HRS) wheat with good and poor breadmaking quality were fractionated into 13 fractions by sequential extraction with dilute hydrochloric acid. Each sub-fraction was characterized by multistacking (MS) SDS-PAGE under nonreducing conditions, followed by imaging densitometry. The glutenin polymers from the origins of MS-SDS-PAGE were analyzed by SDS-PAGE under reducing conditions to determine the composition of high and low molecular weight subunits. The results showed that fractions differed significantly in glutenin-to-gliadin ratios and in the size distribution of glutenin polymers. The earlier precipitated fractions were composed of more gliadins but fewer glutenin polymers. However, the glutenin polymers gradually increased in their relative quantities with the

residue having the largest glutenin-to-gliadin ratio. The size distribution of glutenin polymers differed significantly from early precipitated to later fractions. The relative quantities of glutenin aggregates at the 4% origins increased significantly. The ratio of high molecular weight (HMW) to low molecular weight (LMW) glutenin subunits increased significantly from early to intermediate fractions. Between the two cultivars, significant differences were found in the ratio of HMW to LMW glutenin subunits and quantity of SDS insoluble glutenin polymers in the residue fraction with the better breadmaking quality cultivar ND706 having a greater ratio than the cultivar Sharp. It was concluded that the size distribution of glutenin polymers played an important role in determining the differences in breadmaking quality between the good and poor HRS wheat cultivars.

Hard red spring (HRS) wheats grown in North Dakota are known for their strong dough properties and are primarily used for breadmaking. For many years, there have been continuing studies investigating the factors that determine the breadmaking quality differences of HRS wheats (Busch et al 1969; McGuire and McNeal 1974; Khan et al 1989; Huang and Khan 1997b, 1997c; Zhu and Khan 1999, 2001, 2002; Johansson et al 2001; Uthayakumaran et al 2000). These investigations have unfolded the complexity of glutenin proteins that are most responsible for dough strength and hence breadmaking quality. In our continuing characterization of gluten proteins of HRS wheats, research results have shown that genotype, environment, and genotype-by-environment interaction all contributed to the complexity of glutenin proteins in determining breadmaking quality (Zhu and Khan 1999, 2001, 2002). Characteristics of gluten proteins, including ratio of glutenin polymers to gliadins, the size distribution of glutenin polymers (SDS soluble and insoluble glutenin polymers), and the ratios of HMW and LMW glutenin subunits, to name a few, were investigated for their relationship to breadmaking quality with respect to genotype and environment (Zhu and Khan 2002). Most HRS wheats grown in North Dakota contained the same HMW glutenin subunits such as 5+10 that are characteristic of good breadmaking quality (Khan et al 1989). But large differences still exist in breadmaking quality among these wheat cultivars, even when their protein contents are similar (Khan et al 1989; Zhu and Khan 2001). Previous studies indicated that the quantities of both total HMW glutenin subunits (Huang and Khan 1997b) and individual subunits (Huang and Khan 1997c) were highly associated with dough mixing strength and breadmaking performance of HRS wheat cultivars. In another study, we indicated that the difference in breadmaking quality might result from the ratio of SDS soluble to insoluble glutenins between HRS wheat cultivars with similar protein contents (Zhu and Khan 2001). But more detailed information of the size differences of the glutenin proteins is necessary to fully understand the difference in breadmaking quality of HRS wheats.

The effects of different protein fractions from sequential extraction (MacRitchie 1987) or salt precipitation (Jood et al 2001) on breadmaking quality were studied by fractionation and reconstitution methods (MacRitchie 1985). These protein fractions were also characterized by gliadin-to-glutenin ratios, size distribution of glutenin polymers by SE-HPLC (Cornec et al 1994), and ratios of HMW to LMW glutenin subunits (Jood et al 2001) to explain their contributions to breadmaking quality. Multistacking (MS) SDS-PAGE, which separates glutenin polymers into various aggregates of different molecular weights (Khan and Huckle 1992), is a good approach for determining the size distribution of glutenin polymers in relation to dough properties and breadmaking quality (Huang and Khan 1997a; Zhu and Khan 1999; Zhu et al 1999). The size distribution of glutenin polymers could be obtained under nonreduced conditions by using MS-SDS-PAGE to analyze the different gluten fractions and the composition of their HMW to LMW glutenin subunits by SDS-PAGE under reducing conditions. The results of MacRitchie (1987) indicated that different protein fractions, obtained by successive extraction with dilute HCl, contributed differently to dough mixing and breadmaking properties. These protein fractions also showed a significant difference in their amino acid composition from fraction to fraction (MacRitchie 1987). However, no information was available regarding the size distribution of these protein fractions in relation to dough properties and breadmaking quality. In the study by Cornec et al (1994), the size distribution of those protein fractions obtained using the same procedure of MacRitchie (1987) was investigated using SE-HPLC in relation to dough properties. But only two adjacent peaks could be identified, one as larger-size glutenin polymers and the other as medium-size glutenin polymers from each glutenin fraction (Cornec et al 1994). The quantitative composition of HMW and LMW glutenin subunits was not determined for these two glutenin fractions in the study (Cornec et al 1994). These glutenin polymers, however, might still be a mixture of different sized polymers. Therefore, it would be of interest to further characterize these glutenin fractions into more (different-sized) subfractions and their composition of HMW and LMW glutenin subunits in relation to breadmaking quality. Hence, the objective of this study was to fractionate glutenin into different molecular sizes and to characterize the different protein fractions for ratios of glutenin polymers to gliadins. Also the size distribution of glutenin polymers was examined by MS-SDS-PAGE under nonreducing conditions and the composition of HMW and LMW glutenin subunits was determined by SDS-PAGE under

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reducing conditions. The information obtained would help us understand the influence of glutenin size differences in determining the differences in breadmaking quality between HRS wheat cultivars with similar protein contents and similar HMW glutenin subunit composition. It also would provide useful information for fractionation and reconstitution of protein fractions from HRS wheat flours.

MATERIALS AND METHODS

Hard Red Spring (HRS) Wheats

For this study two HRS wheat cultivars, ND706 and Sharp, were chosen from Carrington field trial location in North Dakota in 1998. They contained the same HMW glutenin subunit composition 2*, 7+9, and 5+10 according to the nomenclature of Payne et al (1987). The average protein contents of the two cultivars were both 14.6% of three replicates (Zhu and Khan 2001).

Preparation of Gluten Fractions

Flour was defatted by successive extraction with chloroform according to MacRitchie (1987). In brief, 50 g of flour was extracted with 100 mL of chloroform at room temperature and then filtered through filter paper. This extraction was repeated two more times for a total of three extractions. The defatted flour was left to stand at room temperature until dry. Gluten was prepared from the defatted flour by hand-washing with distilled water at room temperature. The gluten was then freeze-dried for subsequent further fractionation.

Gluten was fractionated into subfractions according to MacRitchie (1987) by sequential extraction using dilute hydrochloric acid (HCl) of four different concentrations: 0.3 mM, 0.625 mM, 1.5 mM, and 5 mM (Cornec et al 1994). Gluten sample (1.8 g) was mixed with 40 mL of HCl and then homogenized with a Janke and Kunkel Ultraturrax homogeniser at 8,000 rpm for 2 min followed by centrifugation at 6,000 × *g* for 15 min. The supernatant was collected and adjusted to pH 5.8 using dilute sodium hydroxide solutions and then freeze-dried. Three fractions were ob-

tained with 0.3 mM HCl and combined as fraction I (Fr I) in this study, and three fractions with 0.625 mM HCl as fraction II (Fr II), three fractions with 1.5 mM HCl as fraction III (Fr III), and the last two fractions with 5 mM HCl as fraction IV (Fr IV). The residue protein was designated as fraction V (Fr V).

MS-SDS-PAGE of Gluten Fractions and Quantification of Their Composition by Densitometry

MS-SDS-PAGE of the above gluten fractions was performed and followed by quantification of their composition according to our previously reported procedure (Zhu and Khan 2002). Samples (15 mg) of each sequentially extracted fraction (Fr I through Fr V) were extracted with SDS phosphate buffer for electrophoresis. All the other steps were the same as reported previously (Zhu and Khan 2002).

Statistical Analyses

Statistics were analyzed using SAS software (Windows 8.2, SAS Institute, Cary, NC). Comparison was made using least significant difference (LSD) at a probability level of 0.01.

RESULTS

Characterization of Different Gluten Fractions by Multistacking SDS-PAGE

The five protein fractions obtained from sequential dilute HCl extraction were solubilized in SDS phosphate buffer for MS-SDS-PAGE. The gliadin-to-glutenin ratio was determined as a percentage of gliadin in total proteins from densitometry of MS-SDS-PAGE patterns (Zhu and Khan 1999). The results showed the gliadin-to-glutenin ratio from both wheat cultivars decreased significantly from Fr I through Fr V (residue); the residue had the smallest ratio (Table I). This indicated that more glutenin polymers were obtained by the successive extractions with increasing concentration of the dilute HCl. The first fraction, Fr I, from the most dilute acid contained predominately gliadins. The size distribution of glutenin polymers also changed significantly from fraction to fraction (Table II). Generally, it was observed that the

TABLE I
Gliadin-to-Glutenin Ratios^a in Sequentially Extracted Fractions of Two Hard Red Spring Wheat Cultivars Determined from Densitometry of MS-SDS-PAGE Patterns

Protein Fraction	ND706	Sharp
Fr I	95.5a ^b	92.2a
Fr II	76.1b	74.7b
Fr III	67.5c	66.7c
Fr IV	66.5c	63.5d
Fr V	53.5d	45.1e
LSD ^c	2.83	1.45

^a Determined as a percentage.

^b Values followed by the same letter in the same column are not significantly different ($P < 0.01$).

^c Least significant difference at 0.01 level.

TABLE III
Proportion of HMW Glutenin Subunits from Glutenin Aggregates at 4% Origins of Multistacking SDS-PAGE in Sequentially Extracted Fractions of Two Hard Red Spring Wheat Cultivars

Protein Fraction	ND706	Sharp
Fr I	2.2e ^a	3.3e
Fr II	5.7d	6.1d
Fr III	12.9c	12.1c
Fr IV	15.8b	15.4b
Fr V	21.9a	20.3a
LSD ^b	1.20	0.94

^a Values followed by the same letter in the same column are not significantly different ($P < 0.01$).

^b Least significant difference at 0.01 level.

TABLE II
Size Distribution by MS-SDS-PAGE of Glutenin Polymers Under Nonreducing Conditions in Sequentially Extracted Fractions of Two Hard Red Spring Wheat Cultivars

Protein Fraction	ND706						Sharp					
	4%	6%	8%	10%	12%	14%	4%	6%	8%	10%	12%	14%
Fr I	3.8d ^a	7.2c	2.4c	8.6d	25.4c	52.7a	4.5d	3.8d	2.8c	4.8d	30.3b	53.8a
Fr II	13.1c	7.3c	2.4c	10.7c	37.7a	28.9b	11.4c	9.9c	5.9c	13.2a	31.0b	28.5b
Fr III	19.5a	12.9a	7.7a	14.6ab	28.4b	17.7d	18.8a	13.8b	8.0a	13.0a	26.2c	20.2c
Fr IV	18.3a	11.8b	7.3ab	13.7b	29.7b	19.3d	16.6b	11.0c	6.6b	11.0b	31.7b	23.0c
Fr V	16.6b	8.2c	6.4b	14.8a	30.8b	23.2c	11.1c	17.3a	6.8b	8.2c	36.2a	20.5c
LSD ^b	1.54	1.08	0.99	1.12	2.52	3.59	1.11	1.20	0.86	0.87	1.72	3.57

^a Values followed by the same letter in the same column are not significantly different ($P < 0.01$).

^b Least significant difference at 0.01 level.

relative proportion of the largest extracted glutenin polymers at the 4% origins of MS-SDS-PAGE increased from Fr I to III in both cultivars, while the proportion of the smallest glutenin polymers at the 14% origins decreased significantly, suggesting that more of the larger sized glutenin polymers were obtained by the successive extractions with increasing HCl concentrations (Table II). The relative proportions of the glutenin polymers at the 4% origins from Fr IV and V fractions decreased significantly compared with that of Fr III fraction but still were greater than those from the first two fractions, Fr I and Fr II. The reason for the decrease in solubility of glutenin polymers from Fr III to Fr V is most likely due to much larger sized glutenin polymers.

Proportions of HMW glutenin subunits obtained from 4% origins, the largest sized glutenin polymer species, also increased significantly from Fr I to V (Table III). Many previous studies have shown that the greater ratio of HMW to LMW glutenin subunits from total glutenin fraction (Payne and Corfield 1979; Graveland et al 1985; Gupta et al 1993; Popineau et al 1994) and from MS-SDS-PAGE (Huang and Khan 1997a) was associated with larger

sizes of glutenin polymers. The increase, therefore, suggested that glutenin polymers not only increased their relative proportions but also their molecular weights/sizes.

The extractability of different fractions sequentially extracted (Fr I through Fr V) with the SDS phosphate buffer without reducing agent differed from fraction to fraction. The first fraction, Fr I, was completely soluble, while most of the residue fraction, Fr V, was insoluble (data not shown). The SDS insoluble proteins from Fr II through Fr V were analyzed for HMW glutenin subunit composition using SDS-PAGE containing reducing agent according to our previous report (Zhu and Khan 1999). The results indicated that the proportion of HMW glutenin subunits increased significantly in the SDS insoluble proteins from Fr II to Fr V (Table IV). The greatest proportion was found in fraction Fr V, indicating that Fr V contained the largest glutenin polymers (least soluble in the SDS-phosphate buffer without reducing agent). Both cultivars showed a similar trend in which proportions of HMW glutenin subunits increased in SDS-insoluble glutenin polymers from Fr I through Fr V in this study. It was also observed that the quantity of HMW glutenin subunits in the SDS insoluble glutenin polymers was greater than that in the SDS soluble glutenin polymers, as determined from the relevant 4% origins of MS-SDS-PAGE (Table V). Therefore, the quantity of HMW glutenin subunits was highly associated with the size and the extractability of glutenin polymers.

TABLE IV
Proportion of HMW Glutenin Subunits in SDS Insoluble Proteins of Sequentially Extracted Fractions of Two Hard Red Spring Wheat Cultivars

Protein Fraction	ND706	Sharp
Fr I	0.0e ^a	0.0e
Fr II	9.9d	10.9d
Fr III	14.9c	15.1c
Fr IV	21.6b	20.1b
Fr V	28.4a	23.3a
LSD ^b	1.50	1.40

^a Values followed by the same letter in the same column are not significantly different ($P < 0.01$).

^b Least significant difference at 0.01 level.

Characteristics of Gluten Fractions in Relation to Breadmaking Quality

The two cultivars used in this study showed a significant difference in their loaf volume, even though they had similar protein contents with the same composition of HMW glutenin subunits (Zhu and Khan 2001). Results from this study showed that the proportions of glutenin aggregates at the 4% origins of MS-SDS-PAGE from fraction Fr V were significantly greater in ND706 than in Sharp (Table VI [A]). This is in agreement with the results of

TABLE V
Proportions of HMW Glutenin Subunits in Sequentially Extracted Fractions Between Glutenin Polymers at (A) 4% Origins of MS-SDS-PAGE and (B) from SDS Insoluble Fractions

Protein	ND706				Sharp			
	Fr II	Fr III	Fr IV	Fr V	Fr II	Fr III	Fr IV	Fr V
A	5.7b ^a	12.9b	15.8b	21.9b	6.1b	12.1b	15.4b	20.3b
B	9.9a	14.9a	21.6a	28.4a	10.9a	15.1a	20.1a	23.3a
LSD ^b	0.55	1.09	1.23	2.70	0.98	1.80	0.49	1.87

^a Values followed by the same letter in the same column are not significantly different ($P < 0.01$).

^b Least significant difference at 0.01 level.

TABLE VI
Comparisons of Glutenin Characteristics of Sequentially Extracted Protein Fractions of Two HRS Wheat Cultivars^a

Proportion	Fr I	Fr II	Fr III	Fr IV	Fr V
A					
ND706	3.8a ^b	13.1a	19.5a	18.3a	16.6a
Sharp	4.5a	11.4b	18.8a	16.6a	11.1b
LSD ^c	1.43	0.91	1.58	1.91	1.09
B					
ND706	2.2b	5.7a	12.9a	15.8a	21.9a
Sharp	3.3a	6.1a	12.1a	15.4a	20.3a
LSD	0.34	0.93	0.81	0.72	2.09
C					
ND706	0a	9.9b	14.9a	21.6a	28.4a
Sharp	0a	10.9a	15.1a	20.1a	23.3b
LSD	0.00	0.63	1.94	1.11	2.53

^a **A**, proportions of glutenin polymers (nonreduced) at the 4% origins of MS-SDS-PAGE of the sequentially extracted fractions. **B**, proportions of HMW glutenin subunits from the 4% origins of MD-SDS-PAGE of the sequentially extracted fractions. **C**, proportions of HMW glutenin subunits from the SDS insoluble proteins (insoluble proteins left after solubilizing the sequentially extracted fractions Fr I through Fr V with SDS-phosphate buffer without reducing agent for electrophoresis).

^b Values followed by the same letter in the same column are not significantly different ($P < 0.01$).

^c Least significant difference at 0.01 level.

Huang and Khan (1997a). However, the proportions of HMW glutenin subunits at the 4% origins of MS-SDS-PAGE from fraction Fr V showed no significant difference between the two cultivars (Table VI [B]). In contrast, Huang and Khan (1997a) found significant differences between good and poor breadmaking quality HRS wheat cultivars for the proportion of HMW glutenin subunits at 4% origins, but not for total SDS-soluble glutenin proteins at 91% extraction rate. The different results might be due to the difference in glutenin species in total soluble glutenin (Huang and Khan 1997a) versus the soluble portion from insoluble glutenin (Fr V) in this study. The similar proportion between the two cultivars in this study might indicate that the average sizes of both glutenin polymers of Fr V of both cultivars were similar. Therefore, the higher proportion of glutenin aggregates at the 4% origins of MS-SDS-PAGE for cultivar ND706 might indicate that more smaller glutenin polymers were extracted in this fraction for this cultivar and resulted in glutenin polymer sizes similar to those for cultivar Sharp. It still could be concluded that ND706 had a larger size distribution of SDS soluble glutenin polymers than Sharp because the 4% origins contained larger SDS soluble glutenin polymers than the rest of the origins of MS-SDS-PAGE. This observation is in accordance with the previous finding that flours of good breadmaking quality contained a higher proportion of glutenin aggregates at the 4% origins of MS-SDS-PAGE compared with flours of poor quality (Huang and Khan 1997a). However, the more significant difference between the two cultivars might come from the SDS-insoluble glutenin polymers, as these glutenin polymers contained the largest sized polymers. The SDS-insoluble glutenin polymers of fraction Fr V showed a significant difference in the proportions of HMW glutenin subunits between the two cultivars (Table VI [C]). The better breadmaking cultivar, ND706, had a greater ratio than Sharp. The higher proportion of HMW glutenin subunits might indicate that ND706 also had larger sized SDS insoluble glutenin polymers than Sharp. Therefore, it was concluded that the difference in the size distribution of glutenin polymers most likely determined the difference in breadmaking quality between the two cultivars.

DISCUSSION

Characterization of gluten fractions from successive extraction using MS-SDS-PAGE in this study seemed to support the previous findings of similar studies that earlier fractions contained more gliadins, while later ones had more glutenin proteins (MacRitchie 1987; Cornec et al 1994). In this study, each of the five fractions obtained by sequential extraction with dilute HCl was further separated into six different sized molecular weight species by MS-SDS-PAGE to give additional information on glutenin polymer size and its relationship to breadmaking quality. In contrast, only two different sized glutenin polymer fractions were obtained from SE-HPLC in the study by Cornec et al (1994). Furthermore, in our study, proportions of HMW glutenin subunits were determined for both glutenin polymers at the 4% origins of MS-SDS-PAGE and for SDS insoluble glutenin polymers. It was established that proportions of glutenin polymers at 4% origins of MS-SDS-PAGE as well as the size of the SDS insoluble glutenin polymers from Fr V played a significant role in determining the breadmaking quality differences between the two cultivars. Therefore, more detailed information was obtained for the size distribution of glutenin polymers with respect to breadmaking quality compared with the results of previous studies (Cornec et al 1994).

The extractability of glutenin polymers was associated with molecular weights and sizes. However, physical vortexing and extraction reagents could also modify the extraction rate, as shown by homogenization and different concentrations of hydrochloric acids used in this study. As a result, different extraction procedures might result in different information. Therefore, care must be taken when comparing results of different studies. The present study was

conducted to compare the differences in the characteristics of successively extracted and different sized gluten fractions. This would allow us to see which fraction was more important in determining differences in breadmaking quality among wheat cultivars. In our previous studies, only two distinct protein components, SDS soluble and insoluble glutenin polymers, were compared in characterizing HRS wheats in relation to breadmaking quality (Zhu and Khan 1999, 2001, 2002). The results of the present study with five different sized protein fractions confirmed the hypothesis of Southan and MacRitchie (1999) that the size distribution of glutenin polymers may be a key factor in determining differences in breadmaking quality of HRS wheats. The present study also supported the results of many previous studies that the greater proportion of HMW glutenin subunits was associated with the larger sized glutenin polymers (Payne and Corfield 1979; Gupta et al 1993; Popineau et al 1994; Huang and Khan 1997a; Zhu and Khan 2002). Hence, the quantity of HMW glutenin subunits also is important in relation to breadmaking quality of HRS wheats.

CONCLUSIONS

Various protein fractions from dilute hydrochloric acid sequential extraction differed significantly in glutenin-to-gliadin ratio and the size distribution of glutenin polymers. The quantity of HMW glutenin subunits was highly associated with the size of glutenin polymers they formed. The difference in breadmaking quality of HRS wheat cultivars of similar protein contents was mainly determined by the size distribution of glutenin polymers, especially the size of SDS insoluble glutenin polymers. These results will help in our understanding of the complexity of the gluten proteins and their functionalities in relation to differences in breadmaking quality of HRS wheats. The fractions from dilute HCl sequential extractions are being used directly in reconstitution studies to evaluate the contributions of glutenin size and molecular weight differences in influencing breadmaking quality differences of HRS wheat cultivars.

ACKNOWLEDGMENTS

The NRICGP through grant #2000-01601 is thanked for financial support of this research.

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[Received July 9, 2003. Accepted April 6, 2004.]