

# Characterization of Monomeric and Glutenin Polymeric Proteins of Hard Red Spring Wheats During Grain Development by Multistacking SDS-PAGE and Capillary Zone Electrophoresis<sup>1</sup>

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## ABSTRACT

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Three cultivars of hard red spring (HRS) wheats with identical high molecular weight (HMW) glutenin subunit composition (5+10 type, *Glu-D1d*) but different dough properties and breadmaking quality were used in this study. The synthesis and accumulation characteristics of different protein fractions during grain development were examined. Samples were collected at three-day intervals from anthesis to maturity between day 10 to day 37. The nonreduced SDS-extractable glutenin aggregates of developing grains were characterized by a multistacking SDS-PAGE procedure to obtain information on the size distribution and polymerization of glutenin aggregates. The HMW to low molecular weight (LMW) glutenin subunit ratio was determined for its relationship to polymerization of the various glutenin aggregates of different molecular sizes. Glutenin proteins were quantified using an imaging densitometer. In addition, albumins and globulins,  $\alpha$ - and  $\beta$ -gliadins,  $\gamma$ -gliadins, and  $\omega$ -gliadins were separated by capillary zone electrophoresis. The results indicated that albumins-globulins, gliadins, and glutenins in developing grains were present at 10 days after anthesis or earlier. Albumin-globulins decreased in proportion, while gliadins increased in proportion during grain development. Polymerization of glutenin aggregates occurred 10 days after anthesis or earlier and increased significantly throughout the grain-filling period until maturity. Larger aggregates of glutenin increased in proportion, while smaller ones decreased in proportion during grain development. Ratio of polymers to monomers increased signi-

ficantly from day 10 to day 22 of grain development and then remained constant until grain maturity. Glutenin polymers arrived at their maximum in proportion to total SDS-extractable proteins or monomers at day 22 after anthesis while the molecular size of these polymers continued to increase, as indicated by a rapid increase in proportion of HMW to LMW glutenin subunits. Significant differences were found in accumulation rates of glutenin polymers among the three cultivars. Cultivars Kulm and Grandin, with better breadmaking quality, appeared to have greater rates of accumulation and HMW subunit synthesis or formation of larger polymers than did Sharp, a cultivar with poorer quality. Significant differences were found among the three cultivars in the proportion of albumins-globulins and gliadins during grain development. However, no significant differences were found among the cultivars in the proportion of albumins-globulins,  $\alpha$ -,  $\beta$ -,  $\gamma$ -, and  $\omega$ -gliadins at grain maturity. Varietal differences in breadmaking quality were due mainly to the differences in glutenin polymers such as ratio of polymeric to monomeric proteins, molecular size distribution, and ratio of HMW to LMW glutenin subunits among wheat cultivars of 2\*, 7+9, and 5+10 subunit types. The better breadmaking cultivars might be characterized with higher proportions of glutenins and greater proportion of HMW subunits in total SDS-extractable proteins than the poorer quality cultivar. However, more genotypes need to be examined.

Wheat grain quality is mainly determined by gluten proteins, which consist of monomeric gliadins and polymeric glutenins. Differences in the composition of these proteins are responsible for the characteristics of dough properties and breadmaking quality among various wheat cultivars. Polymeric glutenins, formed through intermolecular disulfide linkages, are composed of two types of subunits: high molecular weight (HMW) and low molecular weight (LMW) subunits. The contribution of HMW glutenin subunits to breadmaking quality has been extensively studied and documented throughout the world (Payne et al 1979, 1987; Khan et al 1989; Benedettelli et al 1992; Khelifi and Branlard 1992; Manley et al 1992; Shewry et al 1992; Randall et al 1992, Hou et al 1996). Breadmaking quality has been found to be associated with the different types of HMW glutenin subunits such as the 5+10 subunits (*Glu-D1d*), the existence of which was proven to be significant for breadmaking quality, in contrast to 2+12 subunits (*Glu-D1a*) (Moonen et al 1983, Payne et al 1987, Manley et al 1992, Randall et al 1992). However, it was also found that some cultivars possessing 5+10 subunits showed poorer breadmaking quality than those possessing 2+12 subunits (Wrigley et al 1982, Johansson et al 1994, Blumenthal et al 1995). Other factors such as size distribution of glutenin polymers and proportions of polymeric to monomeric proteins also played significant roles in determining breadmaking quality differences among wheat cultivars (Singh et al 1990; Gupta et al 1993; Gupta and MacRitchie 1994; Weegels et al 1995b, 1996, 1997).

Studies using size-exclusion HPLC (SE-HPLC) indicated that the amount and size distribution of polymeric proteins were important in breadmaking quality (Singh et al 1990, Gupta et al 1993). SDS-insoluble polymeric protein was more strongly related with dough strength than the total polymeric protein (Gupta et al 1993). Using reversed-phase HPLC (RP-HPLC), Wieser et al (1989) found that the HMW patterns of the ethanol-insoluble fractions showed a relationship with baking quality. Comparison between subunits in their contributions to breadmaking quality indicated that the *Glu-D1* subunits 5+10 could result in a higher molecular size distribution in a cultivar than 2+12 subunits (Gupta and MacRitchie 1994). Differences were also found in cultivars during grain filling. For example, the wheat biotype with HMW glutenin subunits 5+10 (*Glu-D1d*) accumulated larger polymers of glutenins more quickly than the biotype with allelic subunits 2+12 (*Glu-D1a*) in developing grains (Gupta et al 1996). Other studies found that the 2+12 subunit type was better than the 5+10 type, and a greater rate of polymer accumulation was found in a 2+12 subunit genotype than in a 5+10 type during grain filling (Johansson et al 1994, Stone and Nicolas 1996). However, polymeric proteins also could be different among cultivars of the 5+10 types differing in breadmaking quality. The differences might exist in polymeric glutenin proteins in their accumulation and size distribution during grain development, which would contribute to the differences in mature grains which, in turn, could be related to breadmaking quality.

Nonreduced glutenin proteins could be characterized by a multistacking SDS-PAGE procedure to separate the glutenin aggregates into fractions of different sizes (Khan and Huckle 1992). The different glutenin aggregates then could be extracted separately and their subunit compositions determined under reducing conditions for further characterization. This procedure has proven to be very helpful for studying size distribution of nonreduced glutenins and their respective subunit compositions from different aggregates of different sizes (Huang and Khan 1997a,b). Good quality flour had a higher total

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glutenin protein and higher proportion of glutenins with the largest molecular sizes at the 4% origins of multistacking SDS-PAGE gels than did the poor quality flour sample (Huang and Khan 1997c).

Capillary electrophoresis (CE) has been a very powerful tool for studying protein structure-function relationships (Ganzler et al 1992). It incorporates speed, high resolution, minimal sample requirement, and direct quantitative digital analysis (Lookhart and Bean 1995a). Over the last few years, high-performance capillary electrophoresis (HPCE) has been introduced for the separation of cereal proteins. Wheat proteins recently have been examined by capillary zone electrophoresis (CZE) (Werner et al 1994; Bietz and Schmalzried 1995; Lookhart and Bean 1995a,b; Weegels et al 1995a; Lookhart and Bean 1996; Lookhart et al 1996; Bean and Lookhart 1997; Bietz and Lookhart 1997; Sutton and Bietz 1997). However, no report has been published on investigations of protein accumulation characteristics at various stages of grain development by CE in relation to grain quality. Because of its high resolution with automatic and quantitative analysis capabilities, CE could be quite useful for studying the accumulative behavior of proteins during grain development.

The objective of this study was to investigate the accumulative characteristics of polymeric proteins and monomeric proteins during grain development of hard red spring (HRS) wheats and their relationships with breadmaking quality by multistacking SDS-PAGE and HPCE.

## MATERIALS AND METHODS

### Wheat Samples

Three HRS wheat cultivars of 5+10 subunit type, differing in dough property and breadmaking quality, were chosen for this study: Sharp, Kulm, and Grandin. According to the nomenclature of Payne and Lawrence (1983), the three cultivars have identical HMW glutenin subunit composition: 2\* (*Glu-A1b*), 7+9 (*Glu-B1c*) and 5+10 (*Glu-D1d*). Wheats were grown in 1996 at Casselton, ND. All grain samples were collected at three-day intervals from day 10 until day 37 after anthesis. The spikes collected were immediately frozen and stored at  $-20^{\circ}\text{C}$  to prevent or minimize further changes in the developing grains. When needed, grain samples were freeze-dried and ground into a fine meal using a mortar and pestle before analysis.

### Protein Extraction and Gel Electrophoresis

A multistacking SDS-PAGE procedure was used to characterize the polymeric proteins under nonreducing conditions according to Khan and Huckle (1992). Gels 1.5 mm thick were used to fractionate nonreduced total SDS-extractable glutenin proteins and to quantify the glutenins at the different origins (origins refer to the beginning of each stacking gel) after electrophoresis. Gels 3 mm thick were used for preparative electrophoresis to isolate the glutenins at the different origins.

Total proteins for electrophoresis (nonreducing conditions) were extracted from 60 mg of whole meal for each sample with 0.05M sodium phosphate buffer (pH 6.8) containing 0.5% SDS (w/v) as described by Khan and Huckle (1992) for 24 hr at room temperature in 1.5-mL Eppendorf test tubes. The sample was then centrifuged for 10 min at 10,000 rpm on an Eppendorf 5415C mini-centrifuge with an 18-place rotor (F-45-18-11). The protein content of the supernatant was determined using a Bio-Rad DC (detergent compatible) protein assay kit (Bio-Rad, Hercules, CA). The supernatant containing 100  $\mu\text{g}$  of protein was loaded onto each well of 1.5 mm thick gel in duplicate and electrophoresed. The residue was washed twice by the same 0.05M sodium phosphate buffer (pH 6.8) and was then sonicated in the buffer (1 mL) for 30 sec at a power output of 10W using a Braun Sonic (model 2000) according to Gupta et al (1993). The protein content of the extracts was determined by the Bio-Rad DC protein assay kit.

Subunit composition of glutenin aggregates was determined by SDS-PAGE according to Huang and Khan (1997c) for the different origins from the preparative gels under reduced conditions. The

various origins were cut out and extracted with the same volume of sample buffer containing 65 mM Tris-HCl (pH 6.8), 20% glycerol (v/v), 2% SDS (w/v), and 1% DDT (w/v) at  $60^{\circ}\text{C}$  for 2 hr. The samples were centrifuged for 10 min at 14,000 rpm on an Eppendorf 5415C mini-centrifuge with an 18-place rotor (F-45-18-11). Then, an equal volume of the extracts was loaded onto each well for the different samples.

### Densitometry and Quantification of Glutenin Proteins

After electrophoresis, the multistacking SDS-PAGE gels (containing nonreduced glutenin aggregates) were stained with 0.25% (w/v) Coomassie Brilliant Blue R-250 in a solution of 50% (v/v) methanol and 10% (v/v) acetic acid for 1 hr with constant shaking. Destaining was achieved in a solution of 7% (v/v) acetic acid and 20% (v/v) methanol.

The SDS-PAGE gels for determining subunit composition of various glutenin aggregates from multistacking SDS-PAGE were stained with Coomassie Brilliant Blue G-250 according to the procedure of Neuhoff et al (1988, 1990).

All gels were scanned using a Bio-Rad imaging densitometer (model GS-670), and volume (area) analysis was performed to quantify proteins according to the procedure in the manufacturer's manual (Bio-Rad). The wavelengths used were 520–570 nm (green filter recommended by Bio-Rad) for gels stained with Coomassie R-250, and 595–750 nm (red filter recommended by Bio-Rad) for gels stained with Coomassie G-250. From the multistacking gels, the proportions of total SDS-extractable glutenin aggregates (i.e., all the multistacking gel origins plus the 14% resolving gel origin) relative to total proteins (i.e., the entire lane including the multistacking layers) in each lane were quantified. Also, the proportions of glutenins at the individual origins relative to the total glutenin aggregates of all origins of each lane were quantified. From the SDS-PAGE gels containing reduced glutenins from the various origins of multistacking SDS-PAGE gels, the proportions of individual or combined HMW glutenin subunits were determined relative to total glutenin subunits, and the ratio of HMW glutenin subunits was measured relative to LMW glutenin subunits.

### Gliadin Extraction

Gliadins were extracted from 40 mg of whole meal from developing grains in 1 mL of 70% aqueous ethanol in Eppendorf tubes by vortexing for 1.5 hr at room temperature. Samples were then centrifuged for 10 min at 14,000 rpm on an Eppendorf 5415C mini-centrifuge with an 18-place rotor (F-45-18-11). The supernatant was filtered through 0.45- $\mu\text{m}$  filters (Gelman Sciences) and used for subsequent CZE. Because albumins-globulins cannot be completely extracted in 70% aqueous ethanol, the albumins-globulins in this study are those extracted in 70% aqueous ethanol.

### CZE

Beckman P/ACE 5000 equipment (Beckman Instruments, Inc., Fullerton, CA) was employed for the CE separation according to the procedure of Bean and Lookhart (1997). Separations were achieved using 25- $\mu\text{m}$   $\times$  27-cm uncoated fused capillaries (Polymicro Technologies, Phoenix, AZ) in cartridges with a detection aperture of 100  $\times$  800  $\mu\text{m}$ . Samples were run for 20 min at 12.5 kV and  $45^{\circ}\text{C}$  and were detected by UV absorbance at 214 nm. Separation buffer and rinse procedure were from Scott Bean (*personal communication*). The separation buffer was 100 mM phosphoric acid/ $\beta$ -alanine (pH 2.5), 20% (v/v) acetonitrile, and 0.05% (w/v) HPMC (hydroxypropylmethyl-cellulose [Sigma] viscosity of 2% aqueous solution at  $25^{\circ}\text{C}$   $\approx$ 4,000 cp). Capillaries were rinsed with 1M  $\text{H}_3\text{PO}_4$  for 2 min and then with separation buffer for 4 min before each separation. Before using a capillary for the first time each day, the capillary was equilibrated with 1M  $\text{H}_3\text{PO}_4$  for 30–60 min until retention time of a standard (cultivar Len) was stabilized. Temperature was maintained at  $45^{\circ}\text{C}$  throughout. Gliadin extract was injected by pressure at 0.5 psi for 10 sec.

## RESULTS

### Glutenin Content per Kernel

A number of freeze-dried grain kernels (60–80) were ground into whole meal and the average kernel weight was determined. Native (nonreduced) SDS-extractable proteins were extracted from a 60-mg whole meal sample, and the protein content of the supernatant was determined after extraction. An equal amount (100 µg) of supernatant proteins was loaded onto each well of the multistacking SDS-PAGE gel. After electrophoresis under nonreducing conditions, the total glutenin aggregates in proportion to total proteins was quantified by scanning each sample lane, which represented 100 µg of total native proteins. Total SDS-extractable glutenin aggregates were determined and divided by the average number of kernels used in the extraction of total native proteins to determine the average amount of total extractable glutenin proteins in one kernel. Glutenin per kernel increased rapidly and accumulated progressively during grain development as shown in Fig. 1. Differences were found in the pattern of accumulation among cultivars. Kulm had a significant and higher quantity of extractable glutenins than Sharp at day 10 and day 19. This difference increased significantly from day 28 to maturity stage (Fig. 1). Although there were no significant differences between Grandin and Sharp at day 10, synthesis and accumulation rate of glutenins showed significant and higher amounts for Grandin per kernel from day 13 until maturity (Fig. 1). These results indicated that significant differences in accumulation rates of glutenins existed among the different cultivars. The cultivars of better breadmaking quality seemed to have a greater amount of glutenins than did the poorer quality cultivars. However, more genotypes need to be examined in more environments of different growing conditions.

### Ratio of Polymeric Proteins to Monomers

The extractability of total nonreduced proteins from mature grain (37 days after anthesis) by SDS buffer for each cultivar was determined after measurements of protein content in the supernatants and residues. Sharp, a poorer quality cultivar, had the highest extractability (94.3%) and was significantly different from the other two

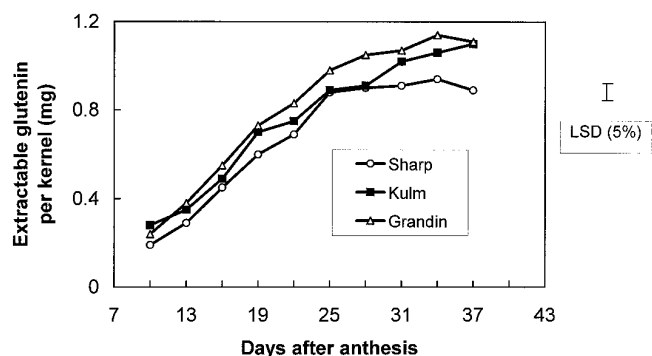


Fig. 1. Glutenin content per kernel of hard red spring wheats during grain development. Least significant difference (LSD at  $P = 0.05$ ): 0.07.

TABLE I  
Quality Data for Three Hard Red Spring Wheat Cultivars at Maturity<sup>a</sup>

Cultivar	Flour Protein (14% mb)	Farinograph (min)		Loaf Volume (cm <sup>3</sup> )
		Peak Time	Mix Tolerance	
Sharp	14.3a <sup>b</sup>	6.0b	14.0c	1025c
Kulm	14.7a	10.0a	18.5a	1135a
Grandin	14.3a	10.0a	16.0b	1080b

<sup>a</sup> Data from 1996 field plot variety trials, Department of Cereal Science, Agricultural Experimental Station, North Dakota State University.

<sup>b</sup> Values followed by the same letter in the same column are not significantly different ( $P < 0.05$ ).

cultivars (Grandin 92.4%, Kulm 91.7%, LSD = 1.41,  $\alpha=0.05$ ). But no significant difference was found between Grandin and Kulm in their extractability. This was in agreement with previous studies that poorer quality cultivars had higher extractability than stronger quality cultivars (Orth and Bushuk 1972, Kruger et al 1988, Gupta and MacRitchie 1994, Huang and Khan 1997a, Sapirstein and Fu 1998).

The ratio of polymeric proteins to monomers in total SDS-extractable proteins was determined for each sample of each wheat cultivar from the densitometry scanning values. During grain development, the ratio initially increased rapidly between day 10 and day 22 and then remained relatively constant for these cultivars (Fig. 2). The proportion of glutenin in total proteins during grain development demonstrated a similar accumulation pattern as observed for the ratio of polymeric to monomeric proteins. However, differences existed among the cultivars in the magnitude of ratio values of polymeric proteins to monomers. Even though Sharp had the highest protein extractability of these three cultivars, it appeared to have a lower ratio, while Kulm and Grandin remained at a higher ratio, indicating that Grandin and Kulm had a higher rate of glutenin accumulation and polymerization than did Sharp. However, these differences in amount of polymeric proteins cannot be explained by differences in protein content among the three cultivars because protein content was very similar among these cultivars (Table I).

### Size Distribution of Glutenins

The proportions of glutenin aggregates from 4 to 14% origins relative to total SDS-extractable glutenins increased gradually, with the 14% origin having the greatest proportion among them (Fig. 3). This is just opposite to the size distributions among these origins as explained below. The unreduced proteins from the different origins of multistacking SDS-PAGE reflected differences in the size of glutenin polymers because different gel concentrations had different pores of sieving formed by polyacrylamide and bis-acrylamide. The larger the gel concentration, the smaller the molecule size of the protein separated at the origin of this concentration. Therefore, the molecular size decreased as the gel concentration increased for the five stacking gels of different concentration (4, 6, 8, 10, 12, and 14%).

Glutenins were examined by multistacking SDS-PAGE to investigate the size distribution of native (nonreduced) SDS-extractable glutenin proteins during grain development. The accumulation patterns of glutenin proteins in proportion from the six origins are

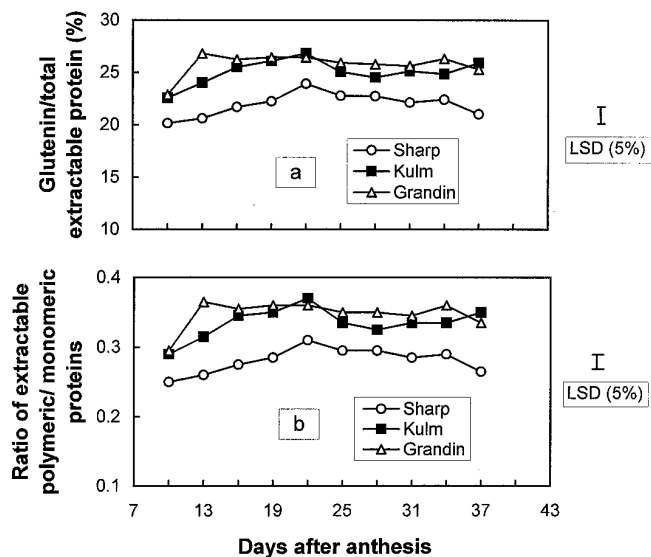
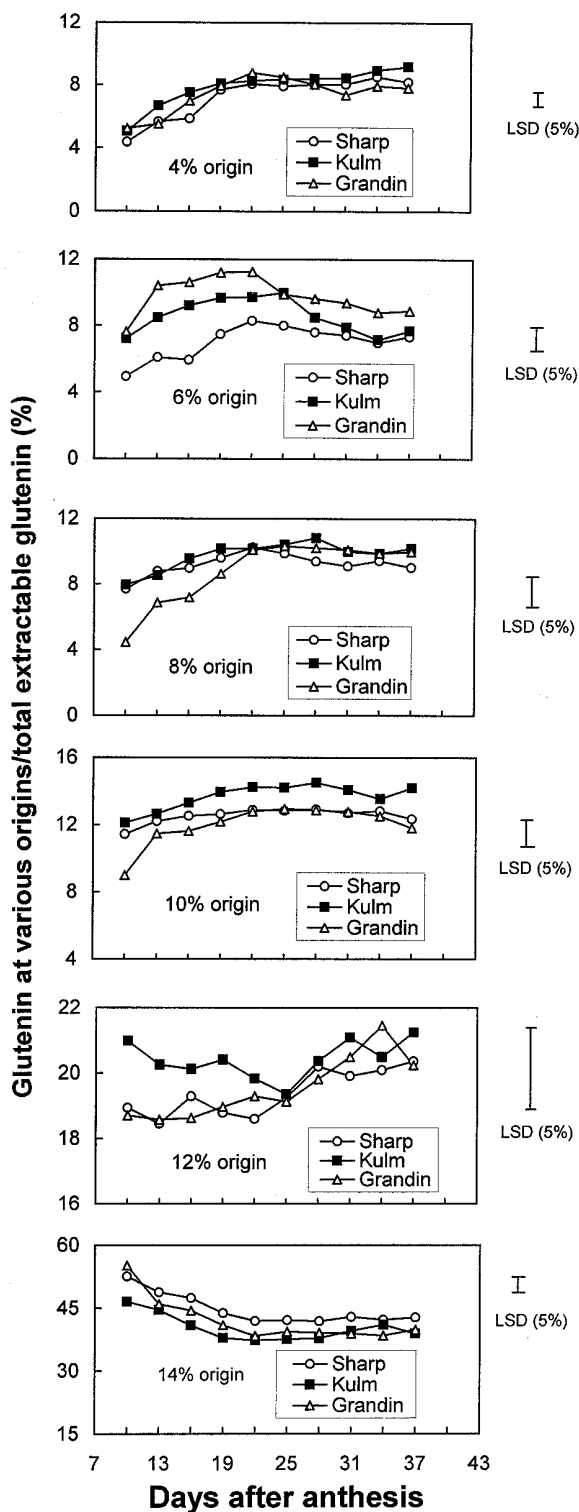


Fig. 2. Glutenin proportion in total proteins (a). Ratio of polymeric glutenin to monomeric proteins of hard red spring wheats during grain development quantified from multistacking SDS-PAGE patterns of unreduced total protein extracts (b). Least significant difference (LSD at  $P = 0.05$ ): 1.37 (a); 0.02 (b).

shown in Fig. 3. The proportion of large size glutenin protein polymers at the 4, 6, and 8% origins increased significantly up to 22 days after anthesis for all three cultivars and then leveled off. At 10% origin, from day 10 to day 22, Grandin showed significant increase but Kulm and Sharp did not (Fig. 3). Concomitantly, there was a significant decrease in the amount of glutenin polymers at 14% origins up to 22 days and then a leveling off, while no sig-



**Fig. 3.** Proportions of glutenin at different origins in total glutenins of hard red spring wheats during grain development quantified from multistacking SDS-PAGE patterns of unreduced total protein extracts. Least significant difference (LSD at  $P = 0.05$ ): 0.79 (4%); 1.38 (6%); 1.80 (8%); 1.54 (10%); 2.46 (12%); 3.33 (14%).

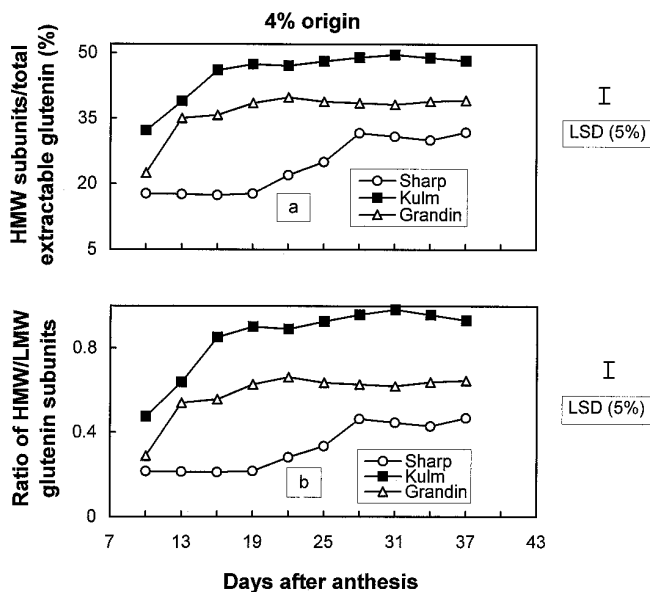
nificant decrease was found in the proportion of the glutenin polymers from day 10 to day 22 for all the three cultivars (Fig. 3). All these results indicated that glutenin proteins increased their molecular sizes as the grain developed and, as a result, more large glutenin polymers were formed.

There also existed significant differences among the three cultivars during grain development (Fig. 3). Sharp, the poorest quality cultivar, had more of the lower molecular weight type glutenins at the 14% origin with Grandin midway between Sharp and Kulm, while Kulm had the highest amount of largest molecular weight species at the 14% origin. This would seem to indicate that better bread-making quality cultivars have more of the highest molecular weight glutenin species. The results agree with the findings of other studies (Dachkevitch and Austran 1989, Gupta et al 1993, Gupta and MacRitchie 1994, Huang and Khan 1997a).

### HMW and LMW Glutenin Subunits

The HMW and LMW glutenin subunits were those obtained from reduction of the different origins of multistacking SDS-PAGE. The proportion of HMW glutenin subunits in total glutenin subunits or the ratio of HMW/LMW subunits at 4% origin increased significantly during grain development for the three cultivars (Fig. 4). Sharp, a cultivar with the poorest breadmaking quality, showed a very slow increase at the early stages of grain development, while Kulm and Grandin, with better breadmaking quality, showed a more rapid and higher rates of increase for HMW subunits in proportion (Fig. 4).

Glutenin accumulation in proportion arrived at maximum on day 22 (Fig. 2), while HMW glutenin subunits continued to increase (Fig. 4a), indicating that the polymerization of HMW subunits continued after glutenin accumulation in proportion slowed down. The ratio of HMW to LMW subunits at the 4% origin (Fig. 4b) showed an accumulation pattern similar to the proportion values. In contrast, at 14% origin, the HMW glutenin subunits in proportion to total glutenin subunits and the ratio of HMW to LMW subunits showed different proportion patterns among these cultivars (Fig. 5) during grain development. Sharp decreased in its proportion of the HMW subunits, while Kulm increased and Grandin showed more constant values during the period. In addition, the glutenin aggregates at 14%

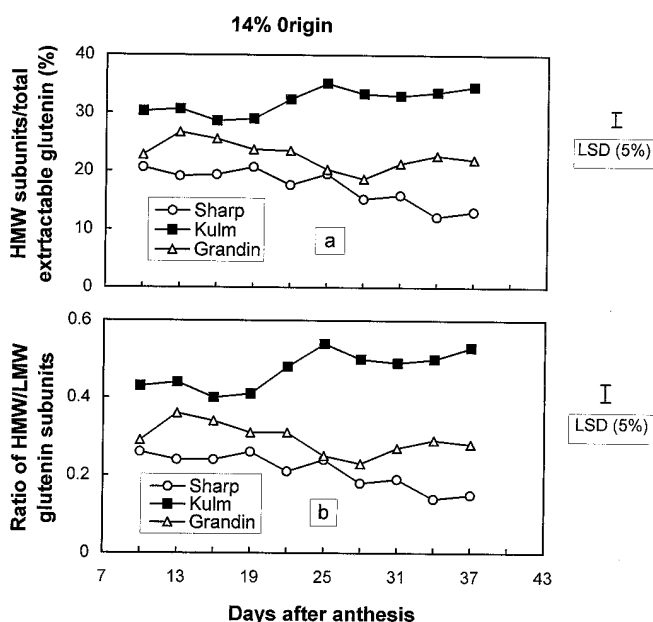


**Fig. 4.** High molecular weight (HMW) glutenin subunits in proportion to total extractable glutenin (a). Ratio of HMW to low molecular weight (LMW) glutenin subunits of hard red spring wheats during grain development quantified from SDS-PAGE patterns of reduced extracts from 4% origins of multistacking SDS-PAGE (b). Least significant difference (LSD at  $P = 0.05$ ): 3.12 (a); 0.07 (b).

origin differed in their composition of HMW and LMW subunits and ratio of HMW to LMW subunits (Fig. 5). The HMW subunits at 14% origin in proportion to total glutenin subunits at this origin had much lower values (Fig. 5) than at the 4% origin (Fig. 4), indicating that the smallest glutenin aggregates at 14% origin had fewer HMW subunits and more LMW subunits, while the largest glutenin aggregates at 4% origin had more HMW subunits and fewer LMW subunits.

Significant varietal differences existed in which Sharp, the cultivar with the poorest breadmaking quality, displayed a slower increase and a lower proportion of HMW glutenin subunits at 4% origin and a decrease at 14% origin during grain development (Figs. 4 and 5). In comparison with Sharp, the cultivar with the best breadmaking quality, Kulm, showed a more rapid increase and a higher proportion of HMW subunits at both 4 and 14% origins (Figs. 4 and 5), indicating that Kulm synthesized a greater proportion of larger polymeric glutenin molecules than Sharp. Grandin showed values midway between those of Kulm and Sharp. These differences of HMW glutenin subunits would contribute to the differences in breadmaking quality for these three cultivars (Table I).

The HMW glutenin subunits at 4% origin were further examined to investigate the accumulation patterns in the proportion of individual subunits compared to total glutenin subunits (Fig. 6). All the HMW glutenin subunits showed an increase in proportion to total glutenin at 4% origin, indicating that polymeric glutenins increased their molecular sizes until the very late stages of grain development, although their total glutenin arrived to a maximum at an earlier stage (day 22, Fig. 2). Differences were found among cultivars in the accumulation rates and proportions of different HMW glutenin subunits for the largest size of the HMW subunits (2\*+5) (Fig. 6a), medium size HMW subunit (7) (Fig. 6b), and the smallest HMW subunits (9+10) (Fig. 6c). Kulm had the highest rates and proportions, Grandin had intermediate rates, and Sharp, of the poorest quality, had the slowest rates and smallest proportions. These results were in high agreement with the ratings of the breadmaking quality of the three cultivars (Table I), suggesting that higher proportions of HMW glutenin subunits in glutenins contribute to better breadmaking quality.

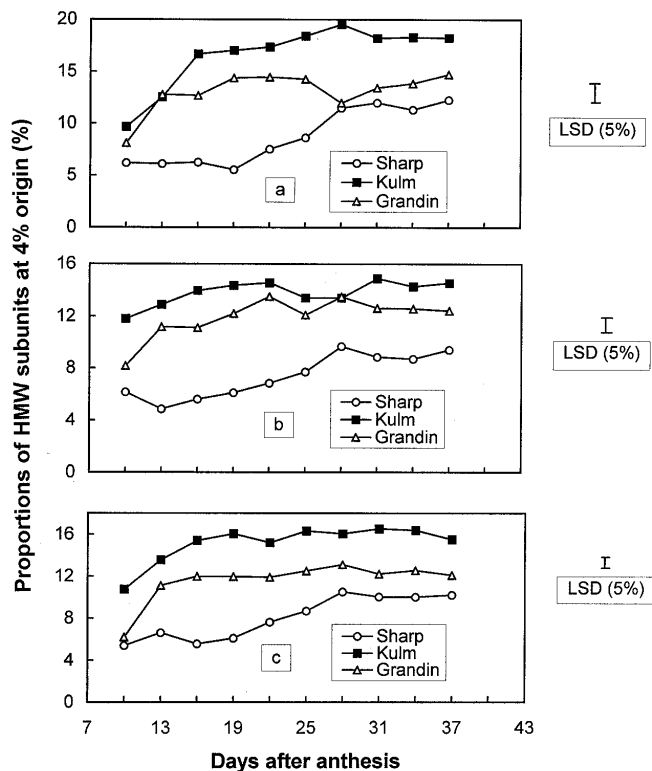


**Fig. 5.** High molecular weight (HMW) glutenin subunits in proportion to total extractable glutenins (a). Ratio of HMW to low molecular weight (LMW) glutenin subunits of hard red spring wheats during grain development quantified from SDS-PAGE patterns of reduced extracts from 14% origins of multistacking SDS-PAGE (b). Least significant difference (LSD at  $P = 0.05$ ): 2.75 (a); 0.05 (b).

### Albumins-Globulins and Gliadins

According to Lookhart and Albers (1988), the migration order of gliadins extracted with 70% ethanol was the same in HPCE as in acid PAGE. Werner et al (1994) also found the same HPCE assignments with those of single purified gliadins. The  $\alpha$ -gliadins migrated the earliest, followed by the  $\beta$ -,  $\gamma$ -, and  $\omega$ -gliadins (Lookhart and Bean 1995b). Monomeric proteins in this study included albumins-globulins,  $\alpha$ -,  $\beta$ -,  $\gamma$ -, and  $\omega$ -gliadins. These proteins were examined during grain development by CZE and classified according to Lookhart and Bean (1995a,b). These proteins were present at 10 days after anthesis or earlier during grain development. A general accumulation pattern from CZE of monomeric proteins during grain development for Grandin is shown in Fig. 7. Proteins increased their absolute amounts and more components appeared during grain development. The albumins-globulins, which migrated between 2.4 and 5.1 min, showed some changes in components (peaks) as the grain matured. For example, a peak at  $\approx 3.3$  mins became larger, while a peak at  $\approx 5.3$  min became much smaller as the grains matured. The gliadins, however, which migrated beginning at  $\approx 6.3$  min, showed a general increase in all peaks that was retained throughout the stages of grain development.

The various monomeric proteins separated by CZE were quantified using corrected areas (Altria 1993) and values plotted as shown in Fig. 8. The 70% ethanol solubilized only a portion of the albumins-globulins present in the sample. Albumins-globulins decreased rapidly in proportion, especially at the early stages of grain development (up to 19 days), and then remained relatively constant (Fig. 8). Kulm at day 10 showed a much larger proportion of albumin-globulin proteins than did the other two cultivars, but this larger amount decreased rapidly from day 16 to day 19. However, the final proportion of albumin-globulin from day 19 onwards appeared to be quite similar among the cultivars.



**Fig. 6.** Proportions of different high molecular weight (HMW) glutenin subunits in total glutenins quantified from SDS-PAGE patterns of reduced extracts from 4% origins of multistacking SDS-PAGE of hard red spring wheats during grain development. (a) 2\*+5, highest HMW subunits; (b) 7, medium HMW subunit; (c) 9+10, lowest HMW subunits. Least significant difference (LSD at  $P = 0.05$ ): 1.76 (a); 0.98 (b); 1.04 (c).

The  $\alpha$ - and  $\beta$ -gliadins had the largest proportions (63–68% at maturity) of total monomeric proteins (Fig. 8). The accumulation of these proteins was relatively constant throughout the grain-filling period, except for Kulm. Differences were observed in the early stage of grain development among cultivars in which Kulm had a much lower value than the other two cultivars, while the final proportion from day 19 onwards appeared to be similar among all three cultivars.

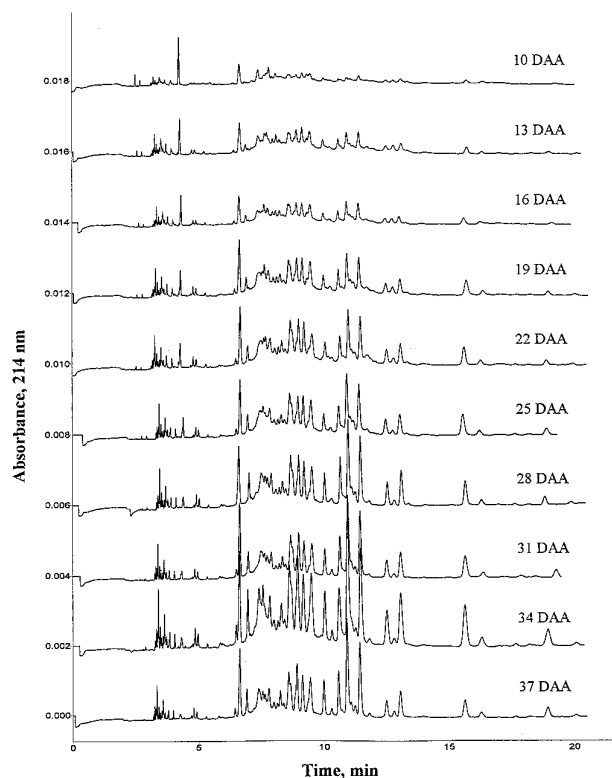
The  $\gamma$ - and  $\omega$ -gliadins showed different accumulation patterns compared to albumins-globulins and  $\alpha$ - and  $\beta$ -gliadins. The proportion of  $\gamma$ -gliadins increased rapidly in the early stage between day 10 and day 22, except there was a decrease for Sharp from day 16 to day 19. The three cultivars showed different patterns of increase or decrease from day 22 onwards (Fig. 8). However, the final proportions of these proteins were quite similar among the cultivars.

The  $\omega$ -gliadins increased in proportion during grain development (Fig. 8). Kulm had a very small proportion of  $\omega$ -gliadins in the initial stages (day 10–16) of grain development, but the proteins increased rapidly to their maximum at day 19 after anthesis and then decreased until day 28. The final proportion of these proteins was similar among the cultivars.

It is interesting to note that most variations in accumulation patterns of the monomeric proteins occurred before 19 days after anthesis. Kulm, the better quality variety, seemed to show the greatest variation up to 19 days after anthesis. However, the final proportion showed no significant differences among the three cultivars.

### Cultivar Differences in Glutenin and Breadmaking Quality

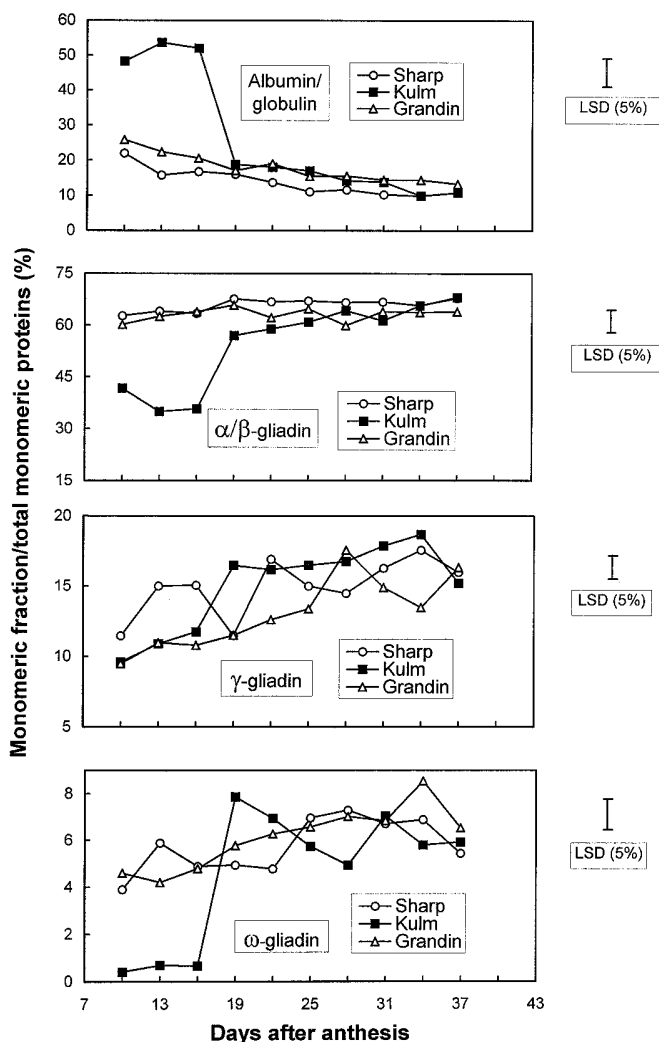
In this study, comparisons were based on a limited number of wheat cultivars. However, certain trends may be established for use in future studies. The three cultivars used in this study differed



**Fig. 7.** High-performance capillary electrophoresis patterns of albumins-globulins and gliadins extracted with 70% ethanol from wholemeal samples of hard red spring wheat Grandin at different stages of grain development. Designation of albumins-globulins,  $\alpha$ -,  $\beta$ -,  $\gamma$ -, and  $\omega$ -gliadins according to Lookhart and Bean (1995b). DAA = days after anthesis. Buffer: 100 mM phosphoric acid and  $\beta$ -alanine (pH 2.5), 20% (v/v) acetonitrile, 0.05% (w/v) hydroxypropylmethyl-cellulose. Pressure-injection 0.5 psi for 10 sec. Separation with uncoated fused capillaries of 25 mm i.d.  $\times$  27 cm (20 cm to detector) at 45°C and 12.5 kV for 20 min. UV absorbance at 214 nm.

in their breadmaking quality as shown in Table I. Comparisons were made among the three cultivars of different protein fractions at maturity (Table II, III). Comparing glutenin in total proteins, ratio of polymer to monomer, and glutenin content per kernel, Kulm and Grandin had significantly greater values than did Sharp (Table II). This indicated that the former two cultivars had greater proportions of polymeric proteins that could contribute to stronger dough properties than did Sharp. The only difference inbetween Kulm and Grandin was found between glutenin content per kernel (Fig. 1) in the above three parameters, which might explain why the dough properties and breadmaking quality of Kulm is superior to that of Grandin (Table I).

Differences also were found among these cultivars for HMW glutenin subunits in total SDS-extractable glutenins and HMW-to-LMW glutenin subunits ratio at 4% origins from multistacking SDS-PAGE (Table II). These parameters were all significantly different among the three cultivars; Kulm had the largest values, followed by Grandin, and then Sharp with the smallest values, indicating that Kulm had the largest proportion of HMW glutenin subunits. HMW glutenin subunits, either individual or combined, relative to total proteins, total glutenins, or LMW glutenin subunits, also showed highly significant differences among the three cultivars (Tables II and III). The ratings of these three cultivars in terms of their breadmaking qualities suggest that higher proportions of HMW glutenin



**Fig. 8.** Albumins-globulins and gliadins of hard red spring wheats during grain development separated by capillary zone electrophoresis. Each fraction was quantified as a proportion of all fractions. Least significant difference (LSD at  $P = 0.05$ ): 8.01 (albumin-globulin); 6.39 ( $\alpha$ -,  $\beta$ -gliadin); 1.68 ( $\gamma$ -gliadin); 1.38 ( $\omega$ -gliadin).

subunits were significant for better dough properties and breadmaking quality. However, within the HMW glutenin subunits themselves, no significant differences were found among individual or combined HMW glutenin subunits in their proportion relative to total HMW glutenin subunits. Only Grandin showed significant differences from the other two cultivars for individual subunit 7 or 9 (Table III). However, this difference could not explain their differences in breadmaking quality. Because these cultivars all had the same HMW glutenin subunit composition, the contribution of HMW glutenin subunits to breadmaking quality may not be due to their quality but to their quantity. Therefore, among cultivars of 2\*, 7+9 and 5+10 HMW subunit types, differences in breadmaking quality might be mainly due to the different quantity of HMW glutenin subunits that are required to form larger glutenin polymers.

## DISCUSSION

This study has shown that the different protein fractions in three HRS wheat cultivars during grain development differed in the timing of their deposition, maximization, and their relative proportions in total proteins. CZE showed that albumins-globulins and  $\alpha$ - and  $\beta$  gliadins accumulated in the greatest proportions at the early stages of grain development. This is in agreement with other studies which showed that albumin-globulin proteins were formed before other proteins (Gupta et al 1996, Stone and Nicolas 1996). These two protein fractions (albumins-globulins and  $\alpha$ - and  $\beta$ -gliadins) appeared to arrive at their stable proportion between day 19 and day 25 as shown in Fig. 7, while  $\gamma$ - and  $\omega$ -gliadins continued to increase in proportion until later stages.

The accumulation of glutenin proteins also showed different trends for the various protein fractions. As a proportion of total SDS-extractable proteins, glutenin proteins arrived at a maximum at day 22 after anthesis (Fig. 2). However, when glutenin proteins were fractionated into aggregates of different sizes by multistacking SDS-PAGE, the accumulation patterns differed. The largest glutenin aggregates (unreduced) from 4% origin of multistacking SDS-PAGE increased progressively to a maximum until grain maturity (37 days after anthesis), while the smallest glutenin aggregates from 14% origin concomitantly progressively decreased in proportion. The 4% origin had more HMW subunits joined to form larger

polymers, while the 14% origins had fewer HMW subunits and shorter polymers (Figs. 4 and 5). This was in agreement with the previous findings of Huang and Khan (1997a). Also, the 4% origin has fewer LMW subunits as part of the polymers, while the 12 and 14% origins have more LMW subunits in their shorter polymers (Figs. 4 and 5). Therefore, the ratio of HMW to LMW subunits is altered. Based on these data, it would seem that, at later stages of grain development, larger glutenin aggregates were produced with the larger subunits (HMW) and fewer LMW subunits joined by disulfide bonds, while smaller aggregates were produced with more LMW subunits and fewer HMW subunits (Figs. 4–6).

Previously, it has been suggested that a proportion of proteins that decrease during grain development may be broken down for reconstitution into other protein fractions at a later stage (Flint et al 1975, Cressey et al 1987). The results of our study do not seem to support this hypothesis. The results in Fig. 3 show that all polymer species from smallest molecular size (14% origin) to largest molecular size (4% origin) are synthesized from the very earliest stages of grain development through to maturity. It seems that both HMW and LMW subunits are synthesized at the same time, but with more LMW subunits being synthesized. However, the mechanisms of polymerization seem to favor polymerization with more LMW subunits, resulting in more LMW polymer species at the 12 and 14% origins ( $\approx 61\%$  of total extractable glutenin) (Fig. 3) compared to  $\approx 10\text{--}12\%$  of total extractable glutenin at each of the other four origins at maturity (total of  $\approx 39\%$  at all four origins). Lew et al (1992) suggested that gliadin subunits might become incorporated into glutenin polymers. However, our results did not suggest that gliadin subunits might participate in the formation of larger glutenin polymers because the ratio of polymer to monomer was highly constant in the later stages of grain development, while larger glutenin proteins continued increasing their proportion significantly at this stage (Fig. 4).

Contradictory results have been reported about the ratio of glutenins to monomers during grain development. Some reports showed an increased ratio toward grain maturity (Cressey et al 1987, Panozzo et al 1994, Stone and Nicolas 1996). Others found the ratio was either stable (Kaczkowski et al 1986), decreased toward maturity (Kapoor and Heiner 1982, Dell'Aquila et al 1983, Stenram et al 1990), or varied during grain filling (Bushuk and Wrigley 1971, Huebner

**TABLE II**  
Comparison of Various Relationships of Protein Components at Maturity in Three Hard Red Spring Wheats

Protein Component <sup>a</sup> (%)	Kulm	Grandin	Sharp	LSD <sup>b</sup>
Glutenin/kernel (mg)	1.06a <sup>c</sup>	1.11a	0.89b	0.10
Glutenin/total protein	25.29a	25.73a	21.87b	1.61
Polymer/monomer ratio	0.34a	0.35a	0.28b	0.04
HMW-GS/glutenin	48.92a	38.79b	30.91c	3.12
HMW/LMW-GS ratio	0.96a	0.63b	0.45c	0.09
2*+5/Glutenin <sup>d</sup>	18.24a	14.00b	11.85c	1.59
7/Glutenin <sup>e</sup>	14.56a	12.53b	8.99c	1.10
9+10/Glutenin <sup>f</sup>	16.12a	12.26b	10.07c	1.31
2*/Total protein	2.09a	1.59b	1.30c	0.27
2*/Glutenin	8.25a	6.19b	5.70b	1.34
2*/HMW-GS	16.86a	18.43a	15.96a	2.55
2*/LMW-GS	16.16a	10.12b	8.25b	2.89
5/Total protein	2.53a	2.00b	1.40c	0.21
5/Glutenin	9.99a	7.81b	6.15c	1.57
5/HMW-GS	20.42a	20.12a	19.90a	2.05
5/LMW-GS	19.55a	12.76b	8.71c	0.99

<sup>a</sup> Glutenin subunits (GS) were reduced from the glutenin aggregates at 4% origin of multistacking SDS-PAGE. HMW = high molecular weight.

<sup>b</sup> Least significant difference at  $P = 0.01$ , significance is indicated between wheat cultivars with different letters for each protein component.

<sup>c</sup> Values followed by the same letter in the same column are not significantly different ( $P < 0.05$ ).

<sup>d</sup> Highest mol. wt. of HMW subunits.

<sup>e</sup> Medium mol. wt. of HMW subunits.

<sup>f</sup> Lowest mol. wt. of HMW subunits.

**TABLE III**  
Comparison of Various Relationships of Protein Components at Maturity in Three Hard Red Spring Wheats

Protein Component <sup>a</sup> (%)	Kulm	Grandin	Sharp	LSD <sup>b</sup>
7/Total protein	3.68a <sup>c</sup>	3.22b	2.05c	0.26
7/HMW-GS	29.76a	32.30b	29.07a	2.30
7/LMW-GS	28.51a	20.46b	13.01c	2.66
9/Total protein	1.74a	1.26b	0.98c	0.11
9/Glutenin	6.89a	4.92b	4.31c	0.45
9/HMW-GS	14.09a	12.69b	13.96a	0.86
9/LMW-GS	13.50a	8.04b	6.24c	1.19
10/Total protein	2.33a	1.89b	1.32c	0.17
10/Glutenin	9.23a	7.34b	5.76c	1.01
10/HMW-GS	18.86a	18.94a	18.64a	0.89
10/LMW-GS	18.08a	12.00b	8.34c	2.64
7+9/Total protein	5.42a	4.48b	3.03c	0.37
7+9/Glutenin	21.45a	17.45b	13.30c	1.54
7+9/HMW-GS	43.85a	44.98a	43.02a	3.09
7+9/LMW-GS	42.01a	28.50b	19.26c	3.83
5+10/Total protein	4.86a	3.89b	2.72c	0.19
5+10/Glutenin	19.22a	15.15b	11.91c	1.28
5+10/HMW-GS	39.28a	39.06a	38.54a	1.47
5+10/LMW-GS	37.63a	24.76b	17.05c	3.42

<sup>a</sup> Glutenin subunits (GS) were reduced from the glutenin aggregates at 4% origin of multistacking SDS-PAGE. HMW = high molecular weight.

<sup>b</sup> Least significant difference at  $P = 0.01$ , significance is indicated between wheat cultivars with different letters for each protein component.

<sup>c</sup> Values followed by the same letter in the same column are not significantly different ( $P < 0.05$ ).

et al 1990). These differences in results among various laboratories might be due to variations in genotype, environment, and method of analysis (Stone and Nicolas 1996) such as using the aggregate amount of the various dissociated glutenin subunits (Skerritt et al 1988, Johansson et al 1994) or the amount of actual polymeric proteins (Bushuk and Wrigley 1971, Panozzo et al 1994, Stone and Nicolas 1996).

Regarding changes in molecular size of proteins, previous studies found that glutenin polymers increased their sizes during grain filling (Gupta et al 1996, Stone and Nicolas 1996). Significant changes occurred during the late stages with a rapid increase in the amounts of glutenin subunits in which HMW glutenin subunits accumulated to a greater degree than LMW glutenin subunits (Gupta et al 1996). Our results are in close agreement with these previously published data. Larger aggregates of glutenins and their HMW glutenin subunits increased in proportion, while the smaller aggregates of glutenins decreased in proportions during grain development. Our results clearly indicated that the size increase was mainly due to the increase in ratio of HMW to LMW glutenin subunits.

Because there were no significant differences among the cultivars in proportions of albumins-globulins and  $\alpha$ -,  $\beta$ -,  $\gamma$ -, and  $\omega$ -gliadin fractions at maturity, breadmaking quality differences may be due mainly to the differences in glutenin fractions. Quality differences were highly correlated with parameters of glutenins such as their content, relative proportions, and size distributions as shown in Tables II and III. However, contradictory results were reported concerning whether the genotype differences in polymer (glutenin) accumulation during grain development was related to the allelic composition at the *Glu-D1* locus. Some studies showed that 5+10 subunits were superior to 2+12 subunits in increasing glutenin polymers (Gupta and MacRitchie 1994, Gupta et al 1996). Other studies showed the opposite results, in which 2+12 subunits tended to have a higher proportion of glutenin polymer than their counterpart 5+10 subunits (Johansson et al 1994, Stone and Nicolas 1996). Our current results showed that significant differences existed even among the cultivars with the HMW glutenin subunits 5+10. Obviously, it is not so simple to explain the behavior of polymer accumulation and dough properties by using data from correlations with one pair of subunits. More factors such as genotype, physiology of plants, and environment in which wheats grow might be involved in the relationships between accumulation of polymer proteins and dough properties.

The accumulation patterns of different protein fractions and the rates of polymer formation during grain development might help explain the effects of environmental factors on breadmaking quality of wheats. Environmental factors may modify or change proportions of different protein fractions and the molecular sizes of polymer proteins (Ciaffi et al 1995, Blumenthal et al 1998). This type of information would help our understanding of structure and functionality of wheat grain proteins in relation to breadmaking quality.

## CONCLUSIONS

The differences in size distribution of glutenin protein at maturity resulted from the differences in accumulation and polymerization rates among different cultivars. Glutenin proteins increased their sizes mainly by increase in ratio of HMW to LMW subunits. The results seem to indicate that higher proportion and larger size of glutenin proteins contribute to stronger dough properties and better breadmaking quality.

There are two possible mechanisms affecting the accumulation and polymerization of glutenin proteins during grain development. One mechanism proposes that certain proteins break down and reconstitute to form other protein fractions at a later stage of grain development (Flint et al 1975, Cressey et al 1987). From the results of our study, we propose that a switch in mechanism in the rate of synthesis and incorporation of HMW subunits to form polymers determines the size distribution of glutenin polymers.

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