

Protein Changes During Various Stages of Breadmaking of Four Spring Wheats: Quantification by Size-Exclusion HPLC¹

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ABSTRACT

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Protein changes for four hard red spring wheat genotypes (Len, Marshall, 215, and Butte 86) were assessed at various stages of breadmaking using a size-exclusion HPLC technique. Breadmaking stages considered were flour, after mixing, before punching, after punching, after fermentation, and after proofing. Quality and functional characteristics of the four wheat genotypes were determined. The three main protein groups isolated by SE-HPLC were further characterized by SDS-PAGE. A direct relationship between polymeric glutenin (peak I of SE-HPLC fractions) in flours and loaf volume was found for the three wheat genotypes with identical high molecular weight glutenin subunit (HMW-GS) composition (2*,

7+9, 5+10) and one line with similar HMW-GS composition (2*, 7+9, 2+12), differing in the *Glu-D1* locus. Quantitative changes in the distribution of SDS-soluble proteins fractionated by SE-HPLC were also examined. Peak I proteins (polymeric proteins) from SDS-extractable proteins tend to decrease during breadmaking, while peak III proteins (low molecular weight) tend to increase. Peak II (monomeric proteins, medium molecular weight) did not show a change in quantity during breadmaking. These results seem to indicate that some type of rearrangement took place during the breadmaking process to release proteins of smaller molecular weight.

Wheat endosperm proteins are important because of their influence on the baking characteristics of flour. The major group of proteins are gliadin, a mixture of single polypeptides, and glutenin, a complex group of polypeptides joined together by interpolypeptide disulfide bonds (MacRitchie et al 1990). When glutenin is treated with reducing agents and analyzed by SDS-PAGE, two groups are obtained based on molecular weight: high molecular weight glutenin subunits (HMW-GS) and low molecular weight glutenin subunits (LMW-GS) (Payne et al 1979). Many studies (Payne et al 1979, 1981, 1987; Moonen et al 1982; Campbell et al 1987; Ng and Bushuk 1988; Khan et al 1989) have established correlations between particular HMW-GS and breadbaking quality.

Bietz (1984) analyzed unreduced SDS extracts from wheats differing in breadmaking quality by size-exclusion HPLC (SE-HPLC) and found an inverse relationship between HMW native (nonreduced) glutenin and flour quality. However, in another set of wheats, Huebner and Bietz (1985, 1986) found a direct relationship. They reported that the ratio of peak I (size > 800,000) from unreduced extracts was directly related to mixing time, indicating a possible use of this technique for breeding purposes. Dachkevitch and Autran (1989) analyzed 30 genotypes and found that the amount of fraction 2 (size 115,000–650,000) and the peak I to peak II ratio were related to the French baking score and gluten elastic recovery, respectively. Dachkevitch and Autran (1989) found that excluded proteins in peak I (F1) and intermediate aggregates (F2) were highly correlated with baking quality data. They also showed that the F1/F2 ratio was the best indicator of the potential baking strength (measured by alveograph *W* index, mixograph index, or gluten viscoelasticity). Recently, Singh et al (1990a,b) extracted gluten proteins using sonication to aid in protein solubilization. They separated proteins from flour samples using SE-HPLC to evaluate the relative quantity of glutenin (peak I) as a measure of breadmaking quality. They found that the relative quantity of glutenin was highly positively correlated with loaf volume ($r = 0.72$), extensigraph dough resistance ($r = 0.84$), extensibility ($r = 0.84$), and mixograph peak development time ($r = 0.84$). However, caution must be exercised when sonication is used due to the possibility that disulfide bonds may be cleaved (Khan et al 1989).

Most studies report using SE-HPLC to analyze only flour or gluten proteins. The objective of this study was to evaluate quantitative differences in gluten proteins among wheat cultivars with good and poor breadmaking quality at each stage of the breadbaking process. Since compositional differences in gluten proteins are correlated with differences in breadmaking quality, we hypothesized that those compositional changes may be more apparent during the actual breadmaking process which is the final quality evaluation test for flours. The SE-HPLC technique was used to quantify different gluten protein groups.

MATERIALS AND METHODS

Wheat Samples

Four hard red spring (HRS) wheat genotypes were used in this study. Three (Len, Marshall, and Butte 86) have the same HMW-GS composition (2*, 7+9, 5+10) and one (215) has a different but similar HMW-GS composition (2*, 7+9, 2+12). Wheat genotypes were supplied by the North Dakota Agriculture Experimental Station (R. Froberg). Wheats were grown at the same location during 1992 and 1995. During 1995, the wheats were grown in two different plots to replicate the experiment. Although three of the wheat genotypes have the same HMW-GS composition, they differ in rheological and breadbaking properties.

Milling and Quality Assessment

Wheat samples were milled on a Bühler laboratory experimental mill (Bühler Co., Minneapolis, MN) according to a standard procedure currently used at the Department of Cereal Science at North Dakota State University based on Approved Method 26-20 (AACC 1995).

Moisture, protein, ash, and falling number (Approved Methods 44-15A, 46-11, 08-81, and 56-81B, respectively [AACC 1995]) were determined for the flours. Rheological properties were assessed using farinograph and mixograph techniques (Approved Methods 54-21, and 54-40, respectively). The baking test was a straight 3-hr fermentation (Approved Method 10-09) using 25 g of flour at 14% mb. The formulation included yeast (Instant Dry Yeast, 1.0%), sugar (5.0%), salt (1.0%), and yeast food (ammonium phosphate, 0.1%). No other additive was used to avoid interfering effects on breadmaking.

Dough Sampling, Protein Extraction, and SE-HPLC

Doughs were collected at different stages of the baking procedure. The stages considered for dough sampling were: after mixing, before second punch, after second punch, after fermentation, and

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after proofing. Dough at each of the these stages of the baking procedure were collected in plastic containers and immediately frozen at -40°C . The frozen doughs were freeze-dried and ground using a coffee grinder.

Unreduced proteins were extracted in sodium phosphate buffer (0.05M) containing 0.5% SDS. Flour (2 g) or freeze-dried dough was extracted in 40 mL of phosphate buffer for 12 hr (20°C) with constant stirring. After extraction, soluble proteins were separated by centrifugation at $27,000 \times g$ for 20 min (20°C). Extracts were immediately analyzed or kept at 4°C until needed (not more than 24 hr).

Unreduced proteins were analyzed by SE-HPLC using the method of Singh et al (1990a,b) as modified by Batey et al (1991). Solutions of 1 mg/mL of extracted proteins were prepared. Protein solutions were filtered using 0.45-mm filters (Gelman LC30) before 20- μL aliquots were applied for SE-HPLC analysis. The samples were analyzed using liquid chromatography (Hewlett Packard 1090) consisting of a PV5 solvent delivery system, a filter photometric system, and an integrator (Hewlett Packard 3393). The samples were loaded onto a column 7.5mm (i.d.) \times 300 mm (Waters Protein Pack

300SW). The elution solvent used was 50% acetonitrile in water (v/v) with 0.1% of trifluoroacetic acid (v/v). Flow rate was 0.5 mL/min for a total run time of 30 min. The solvent was previously filtered (0.45 mm), degassed, and continually sparged with helium. Detection was at 210 nm.

Characterization of Protein Fractions Separated by SE-HPLC

Major protein groups separated by SE-HPLC were further characterized by SDS-PAGE using the procedure of Khan et al (1989) under both reduced and unreduced conditions. To apply the sample to the gel on an equal protein basis, protein solutions of 1 mg/mL were made. Ten sample injections (100 μL) of each protein solution were applied to the same column under the conditions explained above. The proteins separated from these runs were collected (0.5 mL each). The fractions that exhibited the highest absorbance (210 nm) were pooled. Acetonitrile was removed by evaporation under nitrogen and frozen immediately at -80°C . Frozen samples were freeze-dried and stored at -40°C until needed.

SDS-PAGE of SE-HPLC Fractions

Freeze-dried residues were dissolved (10 mg/mL final concentration) in sample buffer (nonreducing conditions [62.5 mM Tris-HCl, 20% glycerol, 2% SDS]; reducing conditions [62.5mM Tris-HCl, 20% glycerol, 2% SDS, 5% mercaptoethanol]) and applied (50 μL) to 0.75-mm polyacrylamide gels (upper stack 4%, resolving gel 14%). Gels were electrophoresed overnight at 5 mA per gel at 20°C . Gels were stained with Coomassie Blue R-250 (0.25% in 50% methanol and 10% acetic acid) for 30 min and destained (7% acetic acid, 20% methanol) until a clear gel background was obtained.

Experimental Design and Statistical Analysis

A randomized complete block design (Cochran and Cox 1950, Steel and Torrie 1980) was used in this study. Three true replicates (blocks) were analyzed. Each block represented a different environment (a combination of location and year of planting) where the four wheat cultivars were grown (all cultivars were grown with the same environmental conditions within each block).

Each analysis was performed in triplicate, except for HPLC analysis, which was performed in quadruplicates (two extracts per sample, two injections per extract), and baking analysis, which was performed in duplicate. Mean, standard deviations and errors, and correlation coefficients were calculated using the Analytical Tool Pack of the Microsoft Excel software package. Reproducibility of the areas from SE-HPLC data was 2% between injections of the same extract and 4% between extracts of the same flour. Analysis of variance (ANOVA) and least square differences (LSD) were made using the SAS statistics software version 6.11 (SAS Institute Inc., Cary, NC). A level of significance of 95% was used on all statistical analysis.

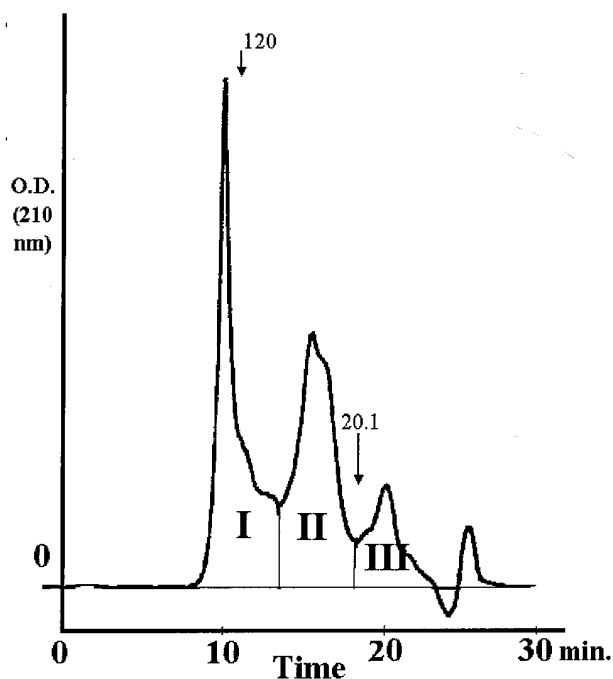


Fig. 1. Size-exclusion HPLC results of unreduced proteins from wheat flour (Len) extracted with phosphate buffer (pH 6.9) containing SDS (0.5%). Chromatograms were divided into peaks I, II, III. Arrows indicate elution times for standard proteins β -galactosidase (MW 120,000), trypsin inhibitor (MW 20,100).

TABLE I
Quality Attributes of Flours from Four Hard Red Spring Wheat Cultivars^a

Cultivar		Moisture (%)	Protein ^b (%)	Ash ^b (%)	Falling Number (sec)	Loaf Volume (cm ³)	Farinograph			Mixograph		
							WA (%)	DT (min)	S (BU)	WA (%)	PT (min)	MH (mm)
Len	Mean	12.8a	13.6a	0.46a	420a	183.3a	64.5a	9.1a	14.1a	67.0a	7.5a	79.7a
	SD	0.4	1.1	0.02	11.3	3.1	0.3	1.2	4.2	2.6	0.9	4.7
Marshall	Mean	12.8a	12.4b	0.46a	442b	164.0b	58.9b	5.5b	9.2b	62.5a	4.5b	70.3b
	SD	0.7	1.0	0.02	8.9	6.6	0.7	0.5	2.0	0.9	0.5	2.1
215	Mean	13.0a	13.7a	0.46a	436b	167.7b	66.5c	6.3c	8.8b	68.7a	4.2b	77.7a
	SD	0.4	1.3	0.06	9.7	5.5	1.0	1.6	1.4	7.5	0.3	8.5
Butte 86	Mean	13.1a	13.7a	0.43a	470c	166.3b	66.7c	7.4c	9.6b	68.7a	5.7b	79.0a
	SD	0.3	1.5	0.03	4.3	3.5	0.3	0.5	4.1	6.7	1.5	9.5

^a WA = water absorption, DT = dough development time, S = stability, PT = peak time, MH = maximum height. Values are means of three replicates. SD = standard deviation. Means followed by different letters in the same column are significantly different ($P < 0.05$).

^b 14% moisture basis.

RESULTS AND DISCUSSION

Variation in Flour Quality

Table I shows various flour quality parameters for the wheat genotypes used in this study. Many statistically significant differences were found among flours. Farinograph and mixograph data characterize Len as a strong wheat cultivar with high values of dough development time (DT), stability (S), peak time (PT), and maximum mixograph height (MH). Len also exhibited the highest loaf volume (LV) of all the wheats used in this study. Marshall, 215, and Butte 86 had lower values for these parameters and could be considered medium strength wheats. Although the wheat flours had similar protein contents (12–13%), they exhibited different rheological and functional characteristics. Butte 86 had the highest protein content (13.7%) but lower LV, DT, S, PT, and MH than Len, which had the highest values for these characteristics. These results indicate that other factors different than, or in addition to, protein content are responsible for wheat flour functionality differences.

Quantification of Unreduced Proteins in Flours by SE-HPLC

Gluten proteins are the most important factor in determining bread-making quality differences (MacRitchie et al 1990). In an attempt to further investigate the protein factors that may be related to bread-making quality, we studied the different quantities of major protein groups in flours with different rheological and breadmaking qualities. We selected the SE-HPLC technique for this study because of its speed, small size requirements, good size-separation, and quantitative capabilities.

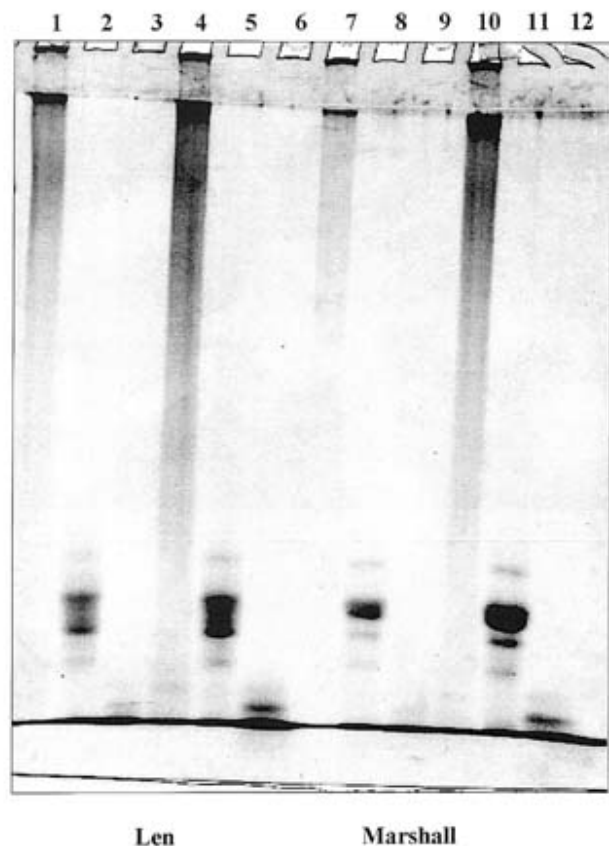


Fig. 2. SDS-PAGE patterns of unreduced proteins separated by size-exclusion HPLC from two wheat cultivars. Lanes 1–6 Len, lanes 7–12 Marshall. Lanes 1, 2, 3 and 7, 8, 9 are from doughs taken after mixing. Lanes 4, 5, 6 and 10, 11, 12 are from doughs taken after proofing. Lanes 1, 4, 7, and 10 are peak I proteins. Lanes 2, 5, 8, and 11 are peak II proteins. Lanes 3, 6, 9, and 12 are peak III proteins.

Proteins from the wheat flours were fractionated by SE-HPLC into three main peaks (I–III in Fig. 1). SE-HPLC separates proteins according to molecular size. The chromatography column used in this study has a claimed separation range of 10–400 kDa for globular proteins and 2–150 kDa for random coils (Waters Chromatography Division, Millipore Corp., Milford, MA).

An estimation of the molecular weight range of proteins was made using data of runs of standard proteins previously made by L. Huckle (*personal communication*, Department of Cereal Science, NDSU). Standards included trypsin inhibitor (20.1 kDa) and β -galactosidase (120 kDa). On the basis of the elution times of these standard proteins made under the same conditions as the gluten proteins, it was estimated that peak I proteins include proteins >120 kDa, peak II proteins range between 20–100 kDa, and peak III proteins range between 2 kDa (lowest possible according to manufacturer) and 20 kDa. A comparison with previously published data on molecular weight size distribution of proteins (Huebner and Wall 1976, Kasarda et al 1976) would indicate that peaks I, II, and III are glutenin, gliadin, and globulins-albumins, respectively. SDS-PAGE data presented by Singh et al (1990a,b) and Batey et al (1991) also confirm the composition of the three main peaks obtained by SE-HPLC.

Our gel results (Fig. 2, nonreduced conditions) further confirmed the composition of the proteins present in each major SE-HPLC peak. As seen in Fig. 2, the proteins present in peak I consist of polymeric polypeptides of very large sizes that are retained at both the 4% stacking gel and the 14% resolving gel origins. There is some streaking in the upper part of the resolving gel in lane 1. This streaking is low molecular weight native glutenins as shown by Khan et al (1994). No other bands appear throughout lane 1. This indicates that peak I proteins are composed mainly of disulfide-bonded polypeptides that are not able to penetrate the resolving or stacking gels due to high molecular weight. However, on reduction with mercaptoethanol, peak I proteins are cleaved into many subunits that are able to migrate into the resolving gel (Fig. 3). Peak II proteins are of a lower molecular weight, as evidenced by

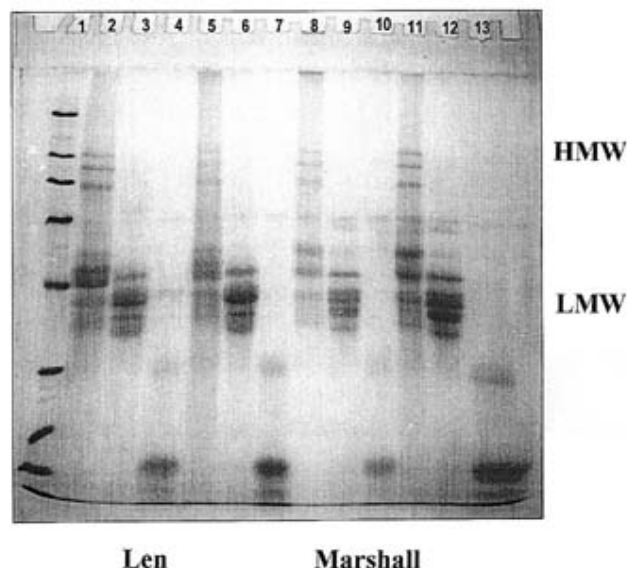


Fig. 3. SDS-PAGE patterns of reduced proteins separated by size-exclusion HPLC from two wheat cultivars (Len and Marshall). Lane 1 = molecular markers (top to bottom: myosin, 200,000; β -galactosidase, 116,250; phosphorylase b, 97,400; serum albumin, 66,200; ovalbumin, 45,500; carbonic anhydrase, 31,000; trypsin inhibitor, 21,500; lysozyme, 14,400; aprotinin, 6,500); Lanes 2–7 Len; lanes 8–13 Marshall. Lanes 2, 3, 4 and 8, 9, 10 are from doughs taken after mixing. Lanes 5, 6, 7 and 11, 12, 13 are from doughs taken after proofing. Lanes 2, 5, 8, and 11 are peak I proteins; Lanes 3, 6, 9, and 12 are peak II proteins. Lanes 4, 7, 10, and 13 are peak III proteins.

the ability to penetrate the resolving gel (Fig. 2). Proteins present in peak III are polypeptides that migrate in the lowest part of the gel and with the dye front (Fig. 2).

The relative quantities (expressed as percent of the total area) of the proteins for the four flours are reported in Table II. The values shown in Table II represent average relative proportions (calculated as the average of the area under the peak for the same cultivar grown in each block) of protein extracted from the respective flour samples. We found that only peak I showed statistically significant differences among the four flours (Table II). Len had the highest value for peak I. Earlier it was shown that Len also had the highest value for loaf volume and other rheological parameters (Table I) and that those values were statistically different from the other wheat cultivars. We also examined the statistical correlations (Table III) between the percentages of the major groups of gluten proteins with the different quality attributes. There was a highly significant and positive correlation between the percentage of peak I proteins and loaf volume ($r = 0.73$) (Fig. 4) and a highly significant and negative correlation between loaf volume and the peak III proteins ($r = -0.64$) (Fig. 4). The percentage of proteins in peak I also correlated positively with the farinograph dough development time ($r = 0.46$) and the stability to mechanical mixing ($r = 0.61$). The total quantity of proteins (determined by Kjeldahl procedure) was negatively correlated to the tolerance index ($r = -0.73$, data not shown) and positively correlated to water absorption ($r = 0.85$). The results obtained in this study for the four wheat genotypes seem to confirm previously reported results from flour samples (Singh et al 1990a,b). Our results confirm the existence of a direct relationship between the percentage of peak I proteins and loaf volume, and between peak I proteins and rheological properties of flour samples.

Quantitative Changes of Proteins During Breadmaking

Protein extractability. Table IV shows the effect of the bread-making process on protein extractability and distribution by SE-

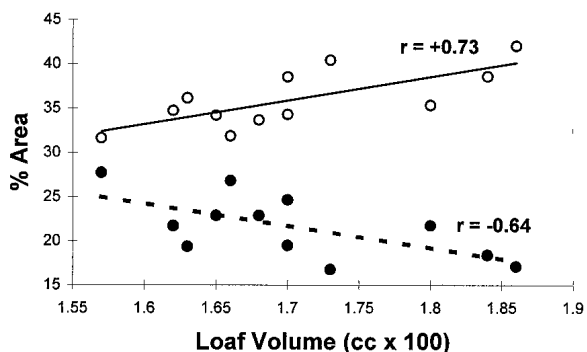


Fig. 4. Relationship between relative amounts of protein in peaks I and III with loaf volume. Area % for peaks I (○, —) and III (●, - - -).

TABLE II
Percentage of Major Protein Groups Determined by Size-Exclusion HPLC of Four Wheat Flours^a

Cultivar		Area Percent			Ratio		
		Peak I	Peak II	Peak III	Peaks I/II	Peaks I/III	Peaks I/II
Len	Mean	38.7a	42.3a	19.1a	0.92a	2.0a	2.2a
	SD	3.4	1.2	2.4	0.1	0.4	0.2
Marshall	Mean	34.8b	41.9a	23.4a	0.83b	1.5a	1.8a
	SD	3.5	1.1	4.1	0.1	0.4	0.4
215	Mean	36.3ab	43.3a	20.5a	0.84b	1.8a	2.1a
	SD	3.6	0.4	3.2	0.1	0.5	0.4
Butte 86	Mean	34.1ab	42.3a	23.6a	0.81b	1.4a	1.8a
	SD	2.1	1.9	3.8	0.0	0.4	0.4

^a Peak I = polymeric/glutenins, peak II = monomeric/gliadin, peak III = albumin/globulins. Values are means of three replicates. SD = standard deviation. Means followed by different letters in the same column are significantly different ($P < 0.05$).

HPLC. Extractions >94% were obtained for flours under the conditions of this study. Proteins from freeze-dried doughs at the different breadmaking stages are more difficult to extract, as evidenced by lower recoveries. This variable extractability of unreduced gluten proteins is not a new problem in wheat protein research. Variable extractability has prevented broad use of techniques such as SE-HPLC due to difficulty in interpreting data (Bietz 1986). Singh et al (1990a) claimed that they have solved this problem by using sonication. Extractions of ≈99% were obtained when sonication was used for times as low as 30 sec. Although sonication facilitates protein extraction from flours and doughs, it is also true that glutenins are broken down to smaller aggregates (Singh et al 1990b). We decided not to use sonication because we wanted to study gluten proteins under extraction conditions that minimized changes from its native (in vivo) state.

In this study, mixing decreased protein extractability. Other authors (Weegels et al 1996) have obtained increased protein extractabilities after mixing (flour-water dough) and quantitation of residue protein by the Kjeldahl method. They hypothesized that mixing causes depolymerization of glutenin aggregates by physical separation and breaking noncovalent or covalent bonds. However, changes in the extractability of dough seem to be related to the strength of the flour. Higher protein extractabilities are obtained with weaker flours (Tsen 1967, Tanaka and Bushuk 1973, Huang 1995). Rheological data presented in Table I showed that all the cultivars used in this study are fairly strong with high values of farinograph stability. Len is considered a strong wheat cultivar with a farinograph

TABLE III
Simple Correlation Coefficients Between Peak Areas (%) from SE-HPLC and Various Functional Properties in Flour and During Two Stages of the Breadmaking Process^a

Variable ^b	Flour			After Mixing			Before Second Punch		
	Peak I	Peak II	Peak III	Peak I	Peak II	Peak III	Peak I	Peak II	Peak III
Protein		0.69							
LV	0.73		-0.64				0.56		
FWA									
Arrival time									-0.54
FDT				0.66		-0.65			
Tol. index		-0.54							
Stability	0.61		-0.61						
MWA									
MPT									-0.69
MMH	0.54		-0.61						

^a Correlation coefficients >0.532 or <-0.532 are statistically significant at $P < 0.05$, $n = 12$. Correlation coefficients >0.661 or <-0.661 are statistically significant at $P < 0.01$, $n = 12$.

^b LV = loaf volume, FWA = farinograph water absorption, FDT = farinograph development time, MWA = mixograph water absorption, MPT = mixograph peak time, MMH = mixograph maximum height.

stability >14 min (Table I). Stability values of 20 min are not rare for Len. Possibly the fact that we used strong wheat cultivars in this study explains the observed decrease in protein extractability after mixing. It is also necessary to mention that we took the dough samples after optimal dough development. Optimal dough development involves the creation of the gluten network that later on, during fermentation and proofing, will retain gas and oven spring, so that a bread of optimal loaf volume will be obtained (Hoseney 1980). If a good gluten network is formed (by polymerization of gluten proteins), then we would expect to observe a decrease in the protein extractability after an optimum mixing. Godon and Herard (1984) suggested that interactions (hydrophobic) increase during mixing. Also, the dough formula contained 1% salt (sodium chloride), which most likely promoted protein aggregation and insolubility. Another factor that may have contributed to protein insolubility could be the freeze-drying of the dough before extraction for SE-HPLC. These differences may help to explain the observed decrease in protein extractability at the dough mixing stage.

Protein extractability continues to decrease during fermentation (before and after punching) (Table IV). Weegels et al (1996) also observed decreased solubility of glutenin macropolymer after dough resting. The practice of punching is widely used in breadmaking. Punching subdivides gas cells, redistributes flour components, and optimizes fermentation by allowing the yeast to get the nutrients it needs for the fermentation process (Pylar 1988). The effect of punching was to further decrease protein extractability for wheat cultivars Len and 215. For Butte 86, punching increased protein extractability, while Marshall did not show a significant change in protein extractability at the punching stage (Table IV). When protein recovery after fermentation is compared with the protein recovery at the initial stage of mixing, the protein extractability decreased for Len and Marshall but did not change significantly for 215 and Butte 86 (Table IV). Hoseney et al (1979) described fermentation as an oxidizing process that may involve cross-linking. A possible difference in the amount of cross-linking that occurs during fermentation for different wheat cultivars may explain the

TABLE IV
Changes of Major Protein Groups (as Determined by SE-HPLC) During Breadmaking^a

Cultivar	Baking Stage	Protein Recovery	Area Percent		
			Peak I	Peak II	Peak III
Len	Flour	94.7a	38.7a	42.3a	19.1a
	After mixing	83.9b	34.4ab	41.1a	24.5ab
	Before second punch	88.8c	34.4ab	39.8a	25.8ab
	After second punch	81.2b	32.3bc	40.5a	27.2b
	After fermentation	76.6d	30.6bc	39.1a	30.3b
	After proofing	88.4c	28.5c	40.9a	30.6b
Marshall	Flour	97.8a	34.8a	41.9a	23.4a
	After mixing	98.5a	29.9a	41.5a	28.7a
	Before second punch	92.7b	30.4a	42.4a	27.2a
	After second punch	91.2b	26.3a	43.2a	30.5a
	After fermentation	83.3c	27.6a	42.3a	30.1a
	After proofing	87.6d	27.4a	42.8a	29.7a
215	Flour	94.9a	36.3a	43.3a	20.5a
	After mixing	84.9b	30.3ab	40.9a	28.8ab
	Before second punch	96.1a	30.1ab	42.4a	27.5ab
	After second punch	90.2c	29.5bc	41.4a	29.1bc
	After fermentation	84.8b	27.4bc	40.4a	32.2bc
	After proofing	82.3b	23.1c	39.6a	37.3c
Butte 86	Flour	94.7a	34.1a	42.3a	23.6a
	After mixing	92.6a	28.9b	42.6a	28.7ab
	Before second punch	72.5b	26.5bc	40.1a	33.4bc
	After second punch	77.6c	24.8c	40.3a	34.9bc
	After fermentation	95.0a	23.7c	38.4a	37.9c
	After proofing	91.5a	23.6c	39.4a	37.0bc

^a Values are means of three replicates. SD = standard deviation. Means followed by different letters in the same column are significantly different ($P < 0.05$).

observed differences in protein extractabilities. Cultivars that are able to cross-link more protein will form bigger aggregates, and this may be reflected as less extractable protein upon solubilization with SDS.

Quantification of SE-HPLC fractions. SE-HPLC was used to quantify major proteins at each stage of the baking process. Table IV also shows the three major protein groups and their distribution during the breadmaking process. Many statistical differences were found between baking stages within each wheat cultivar. Marshall was the only cultivar that did not show statistical differences at the 95% level of confidence. However, if the level of confidence is slightly decreased to 92%, statistical differences can be found for the cultivar Marshall (data not shown). The general trend of the changes of the protein groups can be seen better in Fig. 5. Although only cultivar, Len, is shown, the trend is similar for Marshall, 215, and Butte 86. There is a definite and statistically significant decrease of the relative quantity of peak I proteins and an increase in the relative amount of peak III proteins as the breadmaking process progresses. Peak II remained statistically unchanged during the different baking stages (Table IV). Overall, it seems that during breadmaking there is a process by which the wheat proteins are rearranged to release proteins of smaller molecular weight that elute as an increase in peak III.

SDS-PAGE of Reduced Proteins from SE-HPLC peaks. Figure 3 shows electrophoretic patterns of reduced proteins isolated by SE-HPLC from cultivars Len and Marshall at two different baking stages (after mixing and after proofing). Lanes 2, 5, 8, and 11 are the electrophoretic patterns of reduced proteins from peak I proteins. These proteins (characterized as polypeptide aggregates that could not enter the stacking-resolving gel) (Fig. 2) are composed of many polypeptide subunits (at least 15). The protein composition is independent of the baking stage because the electrophoretic pattern of the proteins isolated at each of the stages studied are essentially the same. Peak I proteins are composed of a distinctive group of HMW polypeptides and another group of LMW polypeptides that can be seen on SDS-PAGE gels only after treatment with reducing agents. Peak II proteins (lanes 3, 6, 9, and 12) (Fig. 3) are composed of polypeptides that migrate to the LMW (mid-mobility) region. No HMW polypeptide subunits are observed in peak II fractions. The peak III fraction indicates polypeptides that migrate to the very low end of the gel (lowest

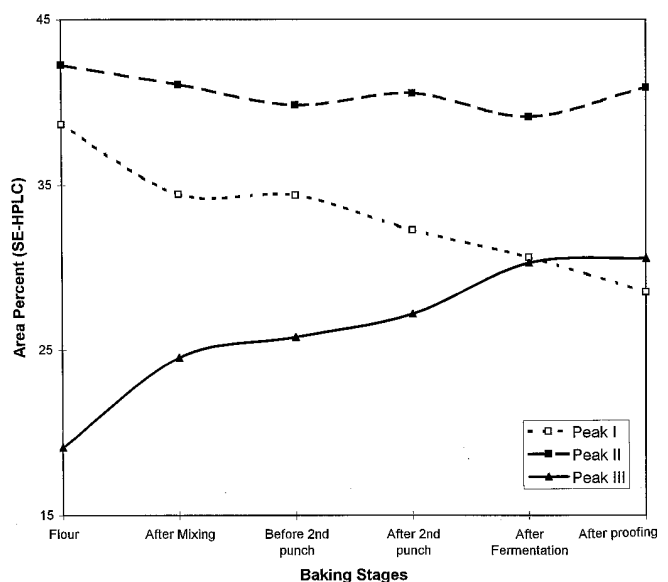


Fig. 5. Changes in major protein peaks during breadmaking as determined by size-exclusion HPLC for wheat cultivar Len. Maximum standard deviation (as a percentage of the mean) observed among replicates was 3.9%.

molecular weight species) (Fig. 3). Band patterns for peak I, II, or III proteins are similar, regardless of the baking stage from which the respective peak was isolated (compare patterns of lanes 3 and 6 in Fig. 3). However, differences in staining intensities exist (samples were applied to the gel on an equal protein basis). Peak III proteins after proofing (lanes 7 and 13) are more intensely stained than peak III proteins from the after mixing stage (lanes 4 and 10). At the same time, the intensity of peak I proteins is less after proofing (lanes 5 and 11) than after the after mixing stage (lanes 2 and 8). Peak I proteins tend to decrease as fermentation progressed, while peak III proteins increase (Table IV). Also, peak II proteins increased in intensity (Fig. 3, lane 6 compared to lane 3, and lane 12 compared to lane 9) as fermentation proceeded. However, this increase is not significant from SE-HPLC quantitative results (Table III).

One possible explanation for the changes observed during the breadmaking process in this study could be that protein-lipid aggregates are being broken down to smaller aggregates during rearrangement of the polymers as the fermentation process proceeds. As early as 1947, Olcott and Mecham showed that protein-lipid complexes form during dough mixing. This early observation has been confirmed by many researchers (Grosskretz 1960, Simmons and Wrigley 1972, Frazier et al 1981, Laszity et al 1979, Zawistowska and Bushuk 1986). Gluten is formed during mixing. Olcott and Mecham (1947) observed that wetting flour causes binding of lipid. A study of the gluten composition showed that lipid associates with glutenin. Thus, according to many authors, glutenin as it is present in gluten is a lipid-protein complex (Laszity et al 1979, Frazier et al 1981). Frazier et al (1981) reported the isolation of a low molecular weight ($\approx 9,000$) protein with very strong aggregative characteristics. This protein also complexes with triglycerides on a 1:1 molar basis. This protein, ligolin, represents 10% or more of total gluten and is responsible for most of the binding of lipids in doughs. The molecular weight of this protein corresponds closely to peak III proteins shown in Figs. 2 and 3.

Correlations of quality data and gluten proteins. In an effort to establish possible relationships between flour quality data and gluten proteins, simple correlations were made between the amounts of peak I, II, and III proteins found at different baking stages and the baking and rheological data. Tables III and V show the different correlation coefficients found at each of the baking stages. Many correlations were statistically significant. However, correlations among HPLC fractions and a particular functional characteristic are not necessarily significant through the whole baking process. For example, the correlation between loaf volume and peak I protein

is significant in flours ($r = 0.77$) (Table III) but it becomes nonsignificant at the after mixing stage ($r = 0.44$). Then, it becomes significant once more at the before second punch stage ($r = 0.56$) and changes again through the rest of the breadbaking stages (Table V). The fact that correlation between quality parameters and content of protein (area peaks) changes during breadmaking is an indication of the dynamic nature of the dough system even during resting (nonmechanical) periods where interactions still take place. These results represent additional proof of the complex nature of the relationship that exists in the breadmaking process.

CONCLUSIONS

A direct relationship between polymeric glutenin and loaf volume was found for three wheat cultivars with identical HMW-GS composition and one line with similar HMW-GS composition, differing in the *Glu-D1* locus. We studied the changes that gluten proteins undergo during the breadmaking process. Except for the last step of the breadmaking process (proofing), all of the other steps (mixing, punching, and fermentation) produced a reduction in protein extractability. This may be related to differences in the amount of cross-linking that occurs during fermentation for each particular wheat cultivar. We also studied the changes in the distribution of SDS-soluble proteins by SE-HPLC. Peak I proteins (polymeric proteins) from SDS-extractable proteins tend to decrease during breadmaking, while peak III proteins (low molecular weight) tend to increase. Peak II (monomeric proteins) do not change during breadmaking. The multistacking SDS-PAGE procedure developed by Khan and Huckle (1992) is currently being used to further separate glutenin proteins by size and to study the changes in protein composition during breadmaking.

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TABLE V
Simple Correlation Coefficients Between Peak Areas (%)
from SE-HPLC and Various Functional Properties in Flour
and During Two Stages of the Breadmaking Process^a

Variable ^b	Flour			After Mixing			Before Second Punch		
	Peak I	Peak II	Peak III	Peak I	Peak II	Peak III	Peak I	Peak II	Peak III
	Protein		-0.74						
LV	0.76		-0.61						
FWA		-0.57							
Arrival time		-0.79							
FDT									
Tol. index		0.56							
Stability									
MWA		-0.85	0.63		-0.58			-0.68	0.61
MPT		-0.70							
MMH				0.58		-0.59		0.54	

^a Correlation coefficients >0.532 or <-0.532 are statistically significant at $P < 0.05$, $n = 12$. Correlation coefficients >0.661 or <-0.661 are statistically significant at $P < 0.01$, $n = 12$.

^b LV = loaf volume, FWA = farinograph water absorption, FDT = farinograph development time, MWA = mixograph water absorption, MPT = mixograph peak time, MMH = mixograph maximum height.

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