

Analysis by Reversed-Phase High-Performance Liquid Chromatography of Changes in High Molecular Weight Subunit Composition of Wheat Storage Proteins During Germination¹

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ABSTRACT

Cereal Chem. 67(2):141-147

The breakdown of high molecular weight (HMW) glutenin subunits in Neepawa wheat was examined over a five-day germination period by reversed-phase high-performance liquid chromatography (RP-HPLC) and sodium dodecyl sulfate gradient polyacrylamide gel electrophoresis. Storage proteins were extracted sequentially (after preextraction with salt) with 50% 1-propanol, 50% 1-propanol containing 1% dithiothreitol (DTT), and 50% 1-propanol containing 4% DTT and 1% acetic acid. HMW glutenin was selectively precipitated from the 50% 1-propanol and 50% 1-propanol containing 1% DTT extracts, alkylated with 4-vinylpyridine, and individual subunits were analyzed quantitatively by RP-HPLC. The amount of HMW glutenin subunits extracted into 50% 1-propanol containing 1% DTT decreased throughout germination, whereas amounts of HMW subunits extracted into 50% 1-propanol remained nearly con-

stant. The relative composition of HMW glutenin subunits, particularly in extracts of 50% 1-propanol containing 1% DTT, changed upon germination. Subunits 9 and 10 gradually disappeared, whereas subunits 5, 7, and 2* remained after five days of germination. On the other hand, in the 50% 1-propanol extractant, subunits 5 and 10 showed the greatest increases during germination. Only small amounts of HMW glutenin subunits were extracted with 50% 1-propanol containing 4% DTT and 1% acetic acid. The subunit composition of this extractant was different from that in the extractants preceding it and showed only small changes during germination. These results provide further insight into the relationship between storage proteins and the deterioration in wheat quality concomitant with preharvest sprouting.

Biochemical changes occur in wheat with increased sprout damage, ultimately rendering the flour produced from it unsuitable for breadmaking (Buchanan and Nicholas 1980). In general, it is assumed that increased degradation of endosperm tissue by enzymes, particularly amylases during the baking stage, is the main factor responsible for quality deterioration.

Several studies show, however, that *in situ* damage to the storage proteins of wheat also may play an adverse role in affecting quality. For example, deterioration of quality has been evaluated by rheological tests (Finney et al 1981; Ciacco and D'Appolonia 1982; D'Appolonia 1983; Kulp et al 1983; Lorenz et al 1983; Lukow and Bushuk 1984a,b) showing that farinograph absorption, dough development time, and mixing tolerance index decrease with increased germination. Sedimentation test value also decreases as does the optimum mixing time on the mixograph. Furthermore, gluten isolated from sprouted wheat appears very sticky (Ciacco and D'Appolonia 1982, Kulp et al 1983).

Trying to relate such quality deterioration to specific changes in storage proteins is difficult. Lukow and Bushuk (1984b) observed that although there is a change in distribution of protein in modified Osborne solubility fractions during germination, no qualitative or quantitative changes in reduced gluten subunits could be observed by electrophoresis. Kruger and Marchylo (1985) found, using high-performance gel permeation chromatography, that no major observable shifts in molecular weights of wheat proteins with germination occur except for the formation of low molecular weight species. Using reversed-phase high-performance liquid chromatography (RP-HPLC), the overall chromatographic profile of reduced storage proteins from ungerminated and germinated wheat was identical except for the amounts of such components. Results with barley using RP-HPLC indicate that specific hordein proteins are degraded at different rates during malting (Marchylo et al 1986). The analysis was carried out, however, using a sequential extraction scheme in order to more closely quantify such changes.

The present study was undertaken to examine the preferential degradation of wheat storage proteins during germination by quantitative RP-HPLC analysis. A sequential extraction scheme (Kruger et al 1988, Marchylo et al 1989) was used to fractionate the wheat proteins, and a recently described precipitation technique (Marchylo et al 1989) was incorporated in order to quantify individual high molecular weight (HMW) glutenin subunits.

MATERIALS AND METHODS

Wheat Sample

The Canada Western Red Spring wheat cultivar Neepawa was used throughout this study.

Germination of Wheat Samples

Wheat grains were sterilized with 1% sodium hypochlorite and rinsed thoroughly with distilled water. After steeping for 16 hr, they were spread evenly on moist blotting paper, germinated at 18°C for one to five days in a germination cabinet at about 80% humidity, and then freeze-dried. The wheat samples were ground finely in a Udy cyclone sample mill (Udy Corp., Boulder, CO) equipped with a 1-mm sieve.

α -Amylase Activity

Wheats were ground and analyzed by the nephelometric method described by Kruger and Tipples (1981).

Extraction

Proteins were extracted sequentially from 1.0 g of ground wheat by a procedure similar to that described previously (Marchylo et al 1989). For each extraction step described, the grist was stirred continuously for 30 min, followed by centrifugation at 15,000 $\times g$ for 10 min.

Sequential extraction steps. 1) 0.5M Sodium chloride: 4°C, 1 \times 6.0 ml, 1 \times 3.0 ml. Prior to the next extraction step, the precipitate was washed with 6.0 ml of water at 4°C to remove occluded salt. 2) 50% (v/v) Propanol: 60°C, 1 \times 6.0 ml, 1 \times 3.0 ml. 3) 50% 1-Propanol containing 1% (w/v) dithiothreitol (DTT): 60°C, 1 \times 6.0 ml, 1 \times 3.0 ml. 4) 50% 1-Propanol containing 4% (w/v) DTT and 1% (v/v) acetic acid (HAc): 60°C, 1 \times 6.0 ml.

Aliquots of the first and second extracts were pooled proportionately (2:1) prior to RP-HPLC analysis or precipitation of

¹Paper no. 643 of the Canadian Grain Commission, Grain Research Laboratory, 1404-303 Main Street, Winnipeg, Manitoba, R3C 3G8.

HMW glutenin subunits. Proteins extracted with 50% 1-propanol were reduced with 1% DTT before RP-HPLC analysis.

For reference purposes, "total" storage proteins were extracted in a single extraction using 50% 1-propanol containing 4% DTT and 1% acetic acid.

All extractions were carried out in duplicate.

Separation and Alkylation of HMW Glutenin Subunits

HMW glutenin subunits in 50% 1-propanol and 50% 1-propanol + 1% DTT extracts as well as "total" 50% 1-propanol + 4% DTT + 1% HAc extracts were separated from other storage proteins by selective precipitation and alkylated with 4-vinylpyridine prior to RP-HPLC by a procedure similar to that of Marchylo et al (1989).

To 1.0 ml of the pooled extracts was added 0.428 ml of 1-propanol containing 1% DTT to bring the final propanol concentration to 65% (v/v). The mixture was vortexed and allowed to stand at 4°C for 60 min, during which time HMW glutenins precipitated. The precipitate was collected by centrifugation (20,000 × *g*, 10 min). The resulting pellet was washed with 0.5 ml of cold 65% 1-propanol containing 1% DTT. It was resolubilized with stirring at 60°C for 1 hr into 1.0 ml of 50% 1-propanol buffered to pH 7.5 with 0.082*M* Tris-HCl and containing 2*M* urea and 1% (w/v) DTT. The resulting clear solution was filtered through a Millex HV4 0.45- μ m filter (Millipore Ltd., Mississauga, ON), and 300 μ l was alkylated with 20 μ l of 71% (v/v) 4-vinylpyridine in 50% 1-propanol at 60°C for 15 min just prior to RP-HPLC analysis.

RP-HPLC

A Waters HPLC and a Waters 840 data and chromatography control station (Waters Associates, Inc., Milford, MA), with a Supelcosil LC-308 RP-HPLC column (*C*₈, 300 Å pore size, 5- μ m particle size, 5 × 4.6 mm i.d. [Supelco Inc., Bellefonte, PA]) maintained at a column temperature of 50°C was used for all analyses (Kruger et al 1988). A modified gradient that minimizes analysis time was employed for analysis of alkylated HMW glutenin subunits, and peaks were quantified as reported previously (Marchylo et al 1989). Protein extracts were injected using a

multiple 5- μ l injection technique to ensure complete binding of protein to the column (Marchylo and Kruger 1988), and protein was detected at 210 nm and 0.5 absorbance units full scale (AUFS; 1,000 mV = 0.5 AU).

Sodium Dodecyl Sulfate Gradient Polyacrylamide Gel Electrophoresis (SDSGPAGE)

Sequential extracts from the various stages of germination were also analyzed electrophoretically by SDSGPAGE as described previously (Marchylo 1987, Marchylo et al 1989).

RESULTS

α -Amylase levels in samples that were steeped or germinated for one, two, three, four, or five days were, respectively, 13,724, 16,105, 31,820, 45,752, and 105,775 mg/maltose per minute per gram × 10⁻³. Thus, α -amylase levels increased dramatically over the five-day germination period after an initial lag period of one day. This observed increase in α -amylase was typical of normal germination as described previously (Kruger and Marchylo 1985). The major enzymes responsible for storage protein breakdown are known to be endopeptidases that parallel α -amylase in their time of synthesis during germination (Jacobsen and Varner 1967).

Salt-Soluble Proteins

Ungerminated, steeped, and five-day germinated Neepawa wheat samples were preextracted with 0.5*M* sodium chloride prior to storage protein extraction and analysis. An extraction temperature of 4°C was used to minimize proteolytic degradation. Analyses of salt-soluble proteins after reduction with 1% DTT are shown in Figure 1. No large increases in salt-soluble hydrophobic proteins occurred during germination. The proteins elute in two major groupings, 20 to 40 min and 50 to 70 min. A continuously changing chromatographic pattern suggests the disappearance of certain protein components and formation of new ones. These results confirm previous findings, using RP-HPLC (Kruger and Marchylo 1985), that storage protein degradation must liberate predominantly hydrophilic peptide components that do not bind to reversed-phase columns under the conditions used in this study.

Sequential Extraction of Storage Proteins

Initially, "total" storage proteins from ungerminated and germinated wheat (i.e., proteins extracted solely with 50% propanol containing 4% DTT and 1% acetic acid) were analyzed by RP-HPLC. Chromatograms for ungerminated and five-day germinated wheat qualitatively were very similar (results not shown), thus confirming previous results (Kruger and Marchylo 1985).

Storage proteins of ungerminated and five-day germinated wheats after preextraction with salt were extracted sequentially with 50% 1-propanol (propanol), followed by 50% 1-propanol containing 1% DTT (propanol-DTT), and 50% 1-propanol containing 4% DTT and 1% acetic acid (propanol-DTT-HAc), and analyzed by RP-HPLC. Very little qualitative change was observed in the patterns of RP-HPLC chromatograms of the propanol extracts (reduced with 1% DTT prior to RP-HPLC) for ungerminated and five-day germinated wheat (Fig. 2A and D). In sequentially extracted samples, however, loss of an early eluting protein peak present in the propanol-DTT solubles of ungerminated wheat (Fig. 2B, arrow) and absent in the five-day germinated wheat (Fig. 2E, arrow) was suggestive of preferential breakdown. Very small amounts of protein were extracted by propanol-DTT-HAc treatment, and no obvious preferential breakdown of specific protein components was observed (Fig. 2C and F). Table I indicates the total integrated chromatographic areas of storage proteins present in the various sequential extracts at different stages of germination. A slight increase in chromatographic area is found upon steeping. There is generally a much greater decrease in integrated area in the propanol-DTT (mainly glutenins) relative to that of propanol (mainly gliadins) throughout germination. Although only a small amount of protein

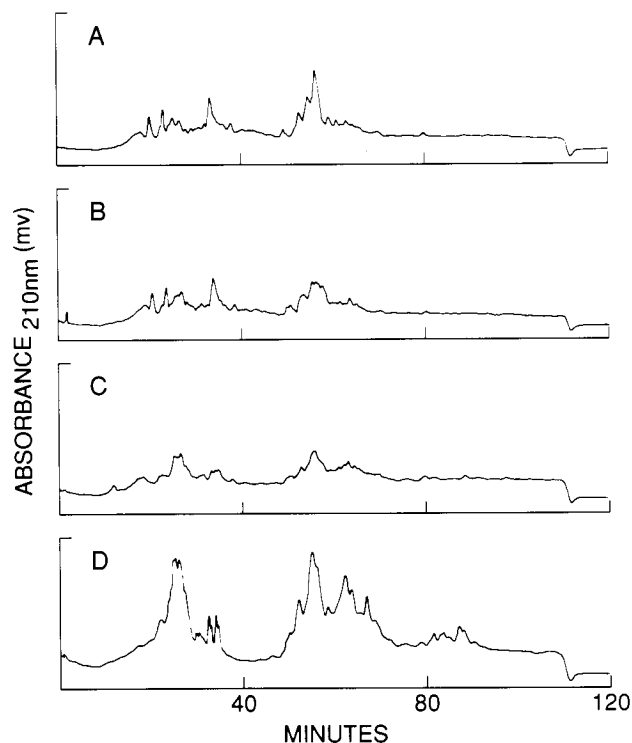


Fig. 1. Reversed-phase high-performance liquid chromatographic analysis of salt-soluble wheat proteins separated from A, ungerminated, B, one-day, C, three-day, and D, five-day germinated wheat. Injection volumes were 45 μ l for A, B, and C, and 180 μ l for D. Full scale = 350 mV.

was extracted by the propanol-DTT-HAc, the amount did not change significantly during germination.

Changes in HMW Subunits During Germination

HMW glutenins were separated from low molecular weight (LMW) glutenin and gliadin components in sequential propanol and propanol-DTT extracts using a precipitation procedure described previously (Marchylo et al 1989). This procedure precipitated all of the HMW glutenins concomitant with coprecipitation of only limited amounts of other storage proteins (see SDS-PAGE analysis). The amount of HMW glutenin extracted by propanol-DTT-HAc was small and, therefore, was not considered in further quantitative analyses.

Precipitated HMW glutenin was resolubilized and analyzed by RP-HPLC, and the total integrated area of HMW glutenin was plotted versus germination time (Fig. 3). HMW glutenin in propanol-DTT extracts decreased throughout germination. Propanol extracts, however, showed little change during germination in comparison with the propanol-DTT extracts.

It was necessary to alkylate the resolubilized HMW glutenin with 4-vinylpyridine (Burnouf and Bietz 1984, Marchylo et al 1989) prior to RP-HPLC analysis in order to follow changes in individual HMW glutenin subunits during germination. Individual HMW glutenin subunits then were resolved and separated from any contaminating LMW glutenins or gliadins using gradient conditions described previously (Marchylo et al 1989). Five HMW subunits were present in both propanol and propanol-DTT extracts (Fig. 4). Quantitatively, however, the relative proportions of individual alkylated subunits differed between the propanol and propanol-DTT extracts. For example, in propanol extracts of ungerminated grain subunit 7 was present in relatively larger amounts than subunit 5, whereas the converse was observed in the propanol-DTT extract (Fig. 4A). The HMW glutenin subunits also changed in relation to each other in a particular extractant

as germination proceeded. This was particularly evident in the propanol-DTT extracts where, for example, subunits 10 and 9 were completely absent after five days of germination (Fig. 4G).

To further illustrate the observed changes during germination, peak areas of individual subunits were quantified, and relative changes in peak area (as a percentage of subunit peak area in the ungerminated grain [control]) were calculated and plotted versus germination time (Fig. 5). HMW subunit 10 was corrected for contaminating ω -gliadin, which coprecipitated with the HMW glutenin, as described previously (Marchylo et al 1989). The relative peak areas (as a percentage of the control) of all subunits extracted by propanol-DTT increased slightly during steeping and then gradually decreased during five days of germination. Subunits 10 and 9 exhibited the most rapid and greatest decrease such that after five days of germination, they had completely disap-

TABLE I
Total Integrated Chromatographic Areas^a
of Storage Proteins from Sequential Extracts of Neepawa Wheat
at Different Stages of Germination^b

Days of Germination	Area (Counts $\times 10^3$)		
	Propanol	Propanol-DTT ^c	Propanol-DTT-HAc ^d
Ungerminated	170.3 \pm 4.8	61.4 \pm 0.5	4.4 \pm 0.6
Steeped	177.2 \pm 11.4	77.0 \pm 11.5	5.6 \pm 0.2
1	164.9 \pm 15.8	77.2 \pm 12.1	7.8 \pm 2.0
2	173.2 \pm 5.9	59.2 \pm 10.3	8.3 \pm 1.5
3	164.6 \pm 7.4	27.1 \pm 1.4	7.9 \pm 0.1
4	148.7 \pm 9.6	18.4 \pm 1.2	4.9 \pm 1.5
5	109.2 \pm 0.9	8.5 \pm 0.5	5.0 \pm 0.4

^a Corrected to a per gram basis.

^b Analyses carried out in duplicate.

^c Dithiothreitol.

^d Acetic acid.

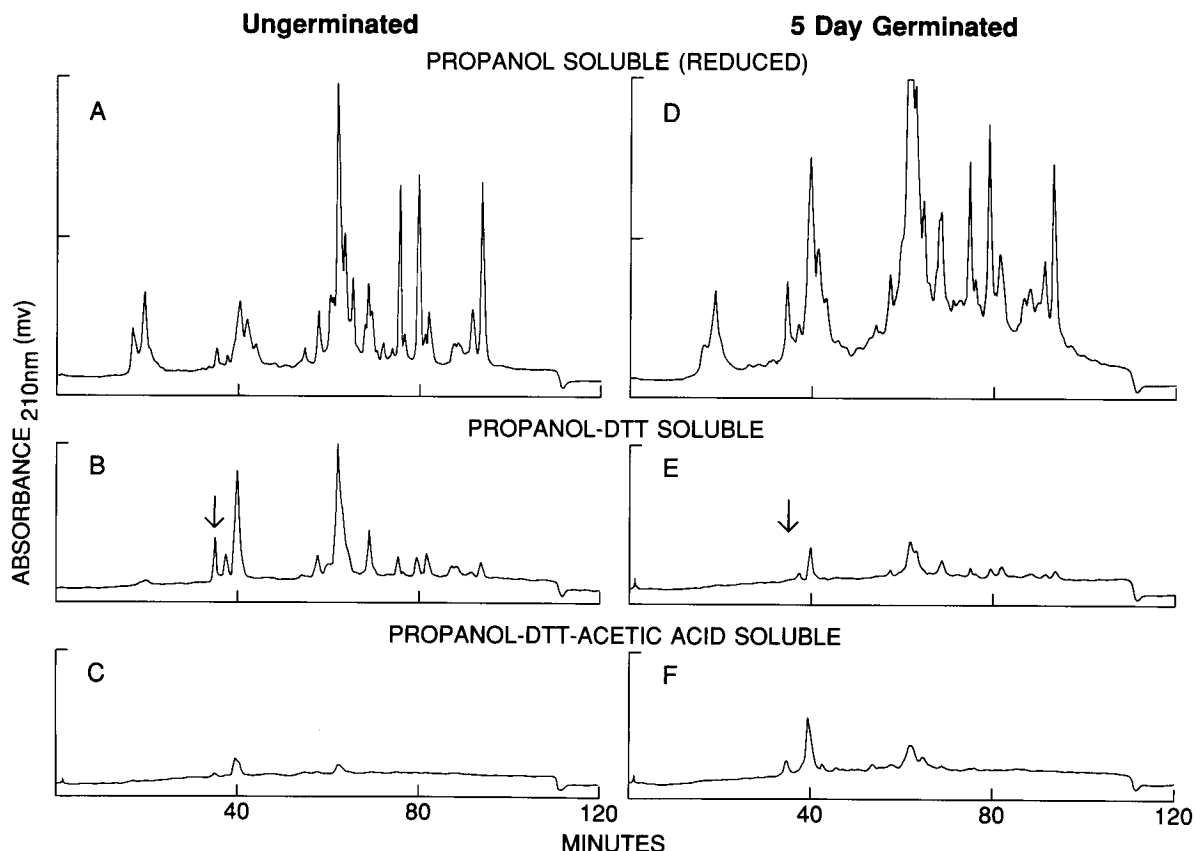


Fig. 2. Reversed-phase high-performance liquid chromatographic analysis of ungerminated and five-day germinated wheat proteins extracted sequentially with A and D, 50% 1-propanol; B and E, 50% 1-propanol containing 1% dithiothreitol; and C and F, 50% 1-propanol containing 4% dithiothreitol and 1% acetic acid. Injection volumes were 15 μ l for A and B; 30 μ l for C, D, and E; and 45 μ l for F. Full scale = 700 mV for A and D and 350 mV for B, C, E, and F. Arrow denotes peak lost during germination.

DISCUSSION

There is a progressive conversion of glutenin aggregates soluble in propanol-DTT to those which become soluble in propanol without the need for reductant during germination. A similar shift has been reported by Lukow and Bushuk (1984a,b) in which proteins increased in solubility in 70% aqueous ethanol and 0.05M

peared. Subunit 7 decreased somewhat less rapidly. The slowest decrease in relative peak area upon germination was found for subunits 5 and 2*. As seen in Figure 5, small amounts of these subunits were still present after five days of germination.

In contrast, HMW subunits extracted with propanol showed a more complex pattern of change during germination (Fig. 5). Each subunit decreased in relative peak area after one day of germination, increased to a maximum after between three and four days of germination, and then decreased up to five days germination. Subunit 5 showed the largest relative increase, to about 154%, but in all cases a larger amount of each subunit was extracted with propanol after three to four days of germination than that extracted from the ungerminated control.

RP-HPLC results were corroborated by SDS-PAGE analysis of sequential extracts, as illustrated in Figure 6 for extracts of ungerminated and five-day germinated wheat. It should be noted that precipitation of HMW glutenin subunits was highly quantitative, as indicated by the absence of any HMW subunit bands in the supernatants after precipitation from propanol (Fig. 6D) and propanol-DTT (Fig. 6G) extracts. Similarly, only small amounts of contaminating storage proteins (gliadins, LMW glutenins) coprecipitated along with the HMW subunits (Fig. 6E [propanol] and 6H [propanol-DTT]), thus facilitating quantitation of subunits by RP-HPLC.

Comparison of the HMW glutenin subunits in electrophoregrams of propanol (reduced) extracts (Fig. 6C) showed that all subunits were present even after five days of germination. Individual HMW glutenin subunits, however, were absent in electrophoregrams of unreduced propanol extracts (Fig. 6B, ungerminated and five-day germinated). The presence of protein at the origin, streaking, etc., was consistent with the presence of aggregates, presumably containing the HMW subunits, as previously discussed (Marchylo et al 1989). Electrophoregrams of propanol-DTT extracts (Fig. 6F) only contained HMW subunits 2* and 5 with trace levels of subunit 7 in five-day germinated extracts. The electrophoretic pattern of storage protein in the propanol DTT-HAc extracts was very similar. Confirming previous results (Kruger et al 1988), propanol-DTT-HAc extracts exhibited a subunit composition different from that of the propanol (reduced) and propanol-DTT extracts in that only subunits 7 and 9 were present in small amounts (Fig. 6I, ungerminated and five-day germinated). Thus, for all stages of the sequential extraction of wheat storage proteins, there was general agreement between electrophoretic and RP-HPLC analysis of the sequential extracts.

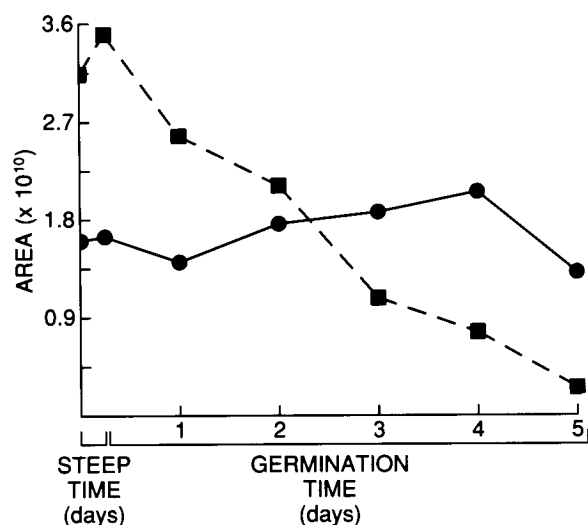


Fig. 3. Changes in distribution of total high molecular weight glutenin subunits during germination: soluble in 50% 1-propanol (●); soluble in 50% 1-propanol containing 1% dithiothreitol (■). Mean of duplicate determinations. Overall coefficient of variation = 11.6%.

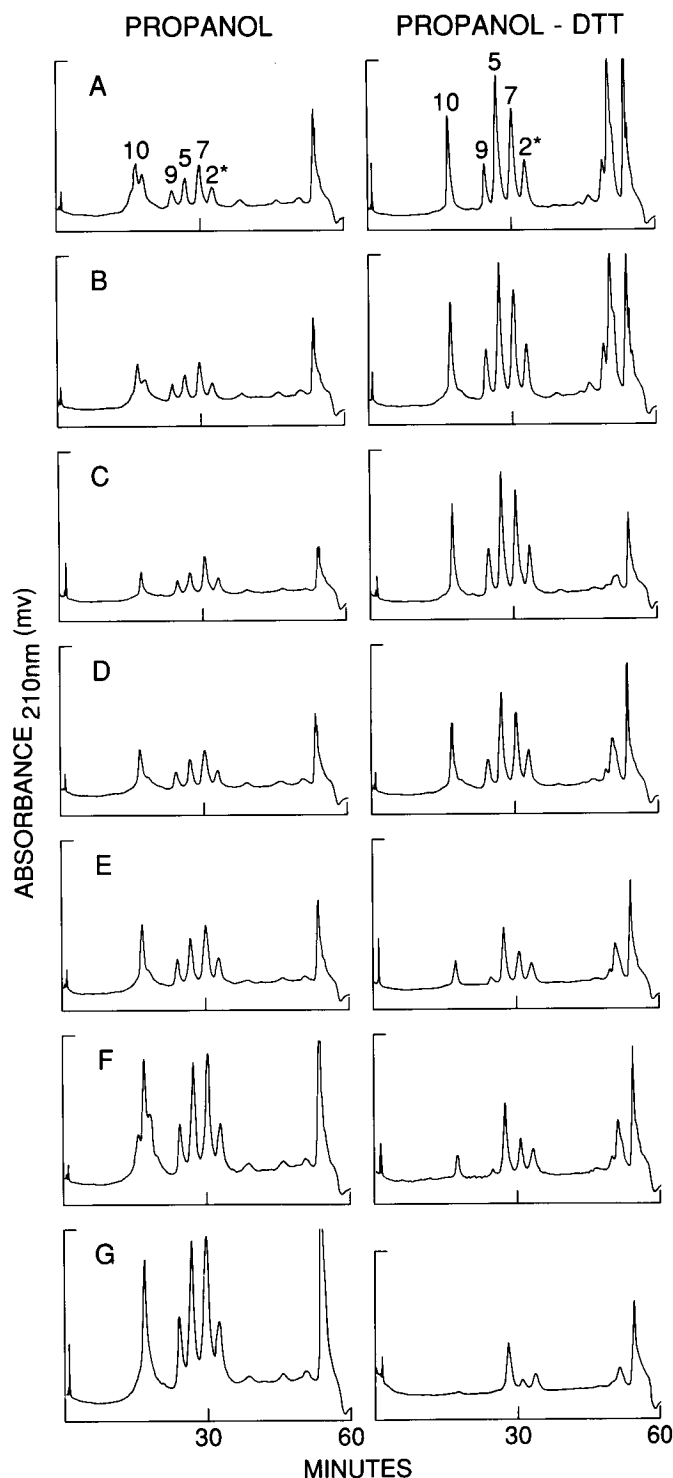


Fig. 4. Reversed-phase high-performance liquid chromatographic analysis of alkylated high molecular weight glutenin proteins present in extracts of 50% 1-propanol and 50% 1-propanol containing 1% dithiothreitol from A, ungerminated and B, steeped wheats, and wheats germinated for C, one day; D, two days; E, three days; F, four days; and G, five days. Identification of high molecular weight subunits 10, 9, 5, 7, and 2* is according to the nomenclature system of Payne and Lawrence (1983). Injection volume for A, B, C, D, E = 30 μ l and for F and G = 60 μ l. Full scale = 350 mV.

acetic acid at the expense of proteins insoluble in these solvents. In the present study, it was found that individual unaggregated HMW glutenin subunits were not present in the propanol extractants.

To speculate on the observed changes in solubility characteristics of the storage reserves upon germination, it is necessary to understand their structure in the ungerminated state. It is now generally accepted that the HMW glutenin subunits are present in the form of aggregates stabilized by covalent disulfide bonds (Bietz and Wall 1973, Miflin et al 1983, Field et al 1983, Graveland et al 1985, Shewry et al 1986) and these aggregates are probably present in a range of different sizes (Huebner and Wall 1980). Some of these aggregates are extracted by 50% 1-propanol as indicated by previous studies (Field et al 1983, Marchylo et al 1989) and as illustrated in this study by SDS-PAGE analysis of ungerminated and five-day germinated wheat extracts. Presumably, the HMW subunits remaining in the grist following

extraction with 50% 1-propanol are covalently linked in the form of larger insoluble aggregates that are only extracted upon addition of reducing agents. In the ungerminated grain, the HMW subunit composition of these large insoluble aggregates must differ from that of the more soluble aggregates extractable in propanol. This is shown by differences in the relative proportions of individual subunits in the propanol compared with the propanol-DTT extract.

Upon germination, the observed changes in the relative proportions of individual HMW subunits in both the propanol and propanol-DTT extracts suggests that aggregates with different HMW subunit compositions vary in their susceptibility to degradation during germination. Thus, as shown by the preferential loss of subunits 9 and 10 in the propanol-DTT extracts, aggregates containing these subunits must be more susceptible to degradation.

The increase during germination of HMW subunits in propanol extracts must result from the degradation of large insoluble aggre-

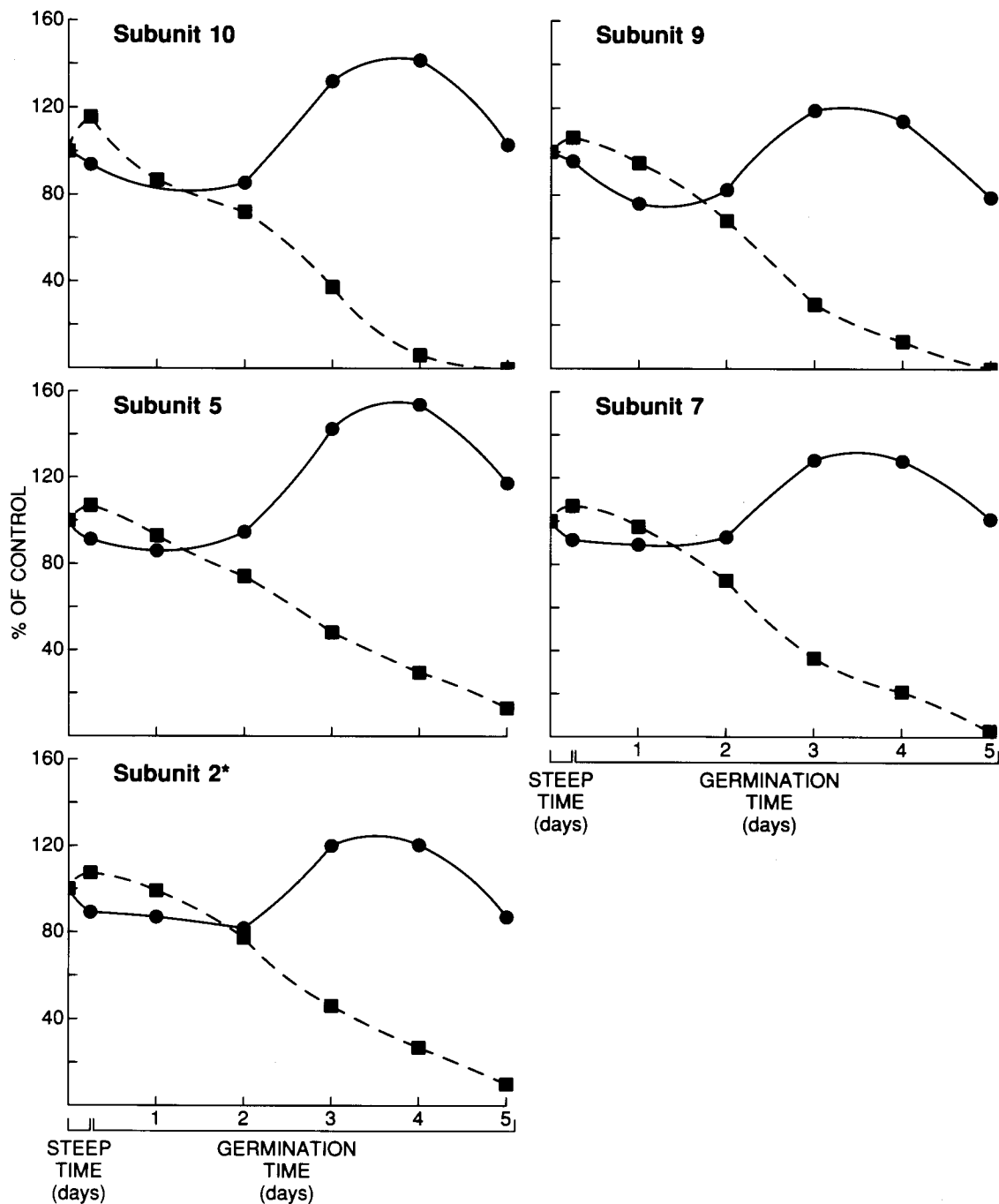


Fig. 5. Changes in percentage of individual high molecular weight glutenin subunits upon germination of wheat proteins sequentially extracted in 50% 1-propanol and 50% 1-propanol containing 1% dithiothreitol. Analyses carried out in duplicate. Overall coefficient of variation = 7.64%.

gates into smaller propanol-soluble aggregates. Differences in the relative increases of individual subunits may be reflective of variations in the subunit composition of new smaller aggregates released. Alternatively, since proteolytic degradation of individual subunits into small peptides and/or amino acids also must be occurring (i.e., the increase in glutenin extracted by propanol is less than the decrease in glutenin extracted by propanol-DTT) some subunits could be selectively or more rapidly degraded.

The enzyme reflecting the alteration in solubility characteristics of glutenin aggregates is unknown. If it were a disulfide reductase, it would require a highly restricted specificity. Alternatively, it

is possible that an endoprotease is synthesized during germination that has very high specificity. The present results could be explained if we assume that there is restricted accessibility of the responsible hydrolytic or oxidoreductase enzymes with their substrate. This would certainly be compatible with a model in which glutenin aggregates exist in clusters, perhaps similar to that proposed by Graveland et al (1985) and in which individual clusters had different subunit compositions. If such high molecular weight clusters were linked by intermolecular disulfide bonds, limited disulfide reductase or proteolytic activity action would have a large effect on the size and, hence, solubility characteristics of the resulting aggregates. Limited accessibility of the above enzyme to the released smaller aggregates would prevent further degradation to monomeric subunits. Subsequent degradation of the smaller aggregates, however, must take place by a combination of exo- and endoproteolytic activity, resulting in the formation of peptides and amino acids (Preston and Kruger 1976a,b, 1977, 1979; Preston et al 1978; Kruger 1984; Kruger and Marchylo 1985). Whatever enzyme is responsible for the initial degradation of glutenin aggregates must be extremely important and needs to be more clearly defined in the future.

The results of this study also provide some insight into how deterioration of quality may occur in wheat that has undergone preharvest sprouting. The fact that aggregates containing HMW subunits are rapidly changing in size, solubility, and composition during germination is probably related to quality since it has been reported that differences in glutenin aggregate molecular weight can affect breadmaking quality (Payne et al 1987). In addition, HMW glutenin subunit composition also is known to be very important from the standpoint of baking quality (Payne et al 1979, 1981, 1984). It is interesting to note that HMW glutenin subunit 5, which has been connected to good breadmaking quality in wheat, was the subunit found to be the most resistant to solubilization in the present study. Furthermore, the sequential extraction scheme used in this study, followed by RP-HPLC, was recently used to relate the relative extractabilities of various storage protein fractions to quality differences among wheat classes (Kruger et al 1988) and wheat backcross lines (Marchylo et al 1989). It was found that the ratio of HMW glutenin subunits in propanol extracts to those present in propanol-DTT and propanol-DTT-HAc extracts is inversely related to dough strength. In the present study, the relative proportion of HMW glutenin subunits in propanol extracts increased with germination, and, therefore, this could be an indicator of decreased dough strength that is known to occur in flours originating from sprout-damaged wheat.

ACKNOWLEDGMENTS

The authors would like to acknowledge the competent technical assistance of D. Hatcher, P. Funk, K. Handel, and D. Daniel.

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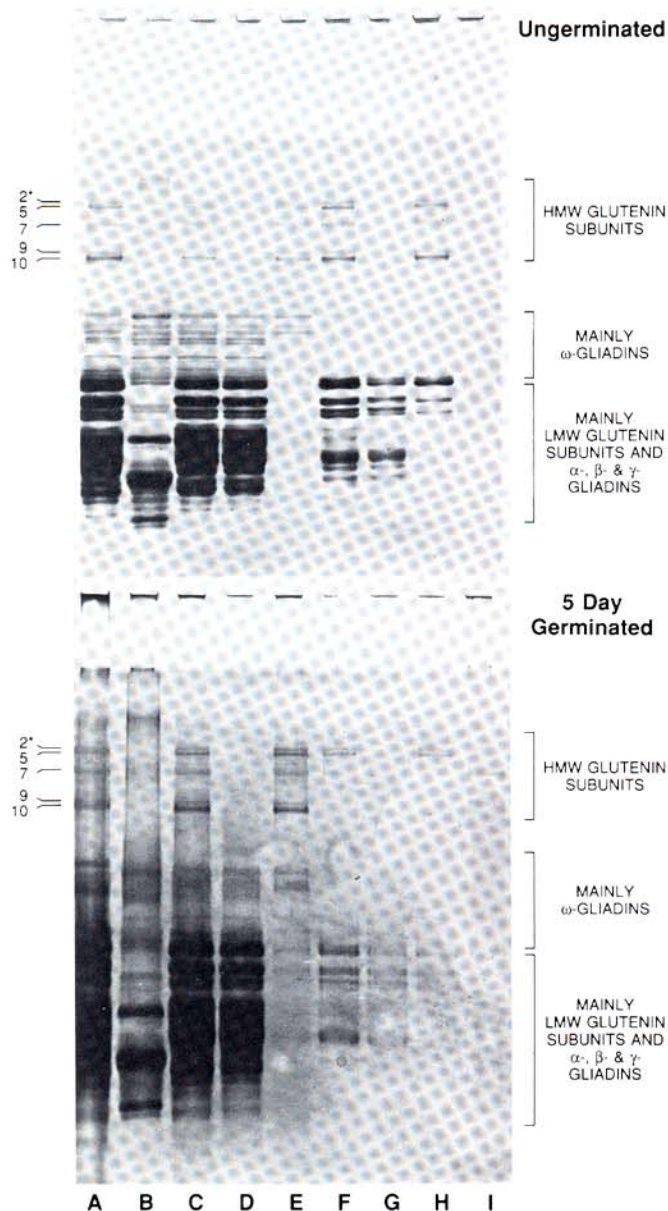


Fig. 6. Sodium dodecyl sulfate gradient polyacrylamide gel electrophoresis of ungerminated and five-day germinated wheat extracts. "Total" storage protein extract using 50% 1-propanol containing 4% dithiothreitol (DTT) and 1% acetic acid, after preextraction with 0.5M sodium chloride (A). Sequential extracts: propanol-soluble (B); propanol-soluble (reduced) (C); supernatant (D); resolubilized and alkylated precipitate from propanol extract (E); propanol-DTT soluble (F); supernatant from precipitation step of propanol-DTT extract (G); resolubilized and alkylated precipitate from propanol-DTT extract (H); propanol-DTT-acetic acid soluble proteins (I). Extract samples of 1 and 2 μ l were loaded onto the ungerminated and five-day germinated samples, respectively. Supernatants following precipitation (D and G) were included to show the effectiveness of the precipitation step in removing all of the high molecular weight glutenin subunits.

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[Received January 17, 1989. Revision received September 5, 1989. Accepted September 7, 1989.]