Molar fractions of high-molecular-weight glutenin subunits are stable when wheat is grown under various mineral nutrition and temperature regimens

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Abstract

Molar fractions of the high-molecular-weight glutenin subunits (HMW-GS) were determined for flour from bread wheat (\textit{Triticum aestivum} L. cv Butte86) produced under 13 different combinations of temperature, water and mineral nutrition. Albumins, globulins and gliadins were removed from the flour by extraction with 0.3 M NaI in 7.5% 1-propanol. Total HMW-GS were recovered by extracting the remaining protein with 2% SDS and 25 mM DTT. Individual HMW-GS were then separated and quantified by RP-HPLC. Constant molar fractions for the five HMW-GS were maintained under all environmental conditions, despite large differences in duration of grain fill, total protein per grain, flour protein percentage, and total HMW-GS per grain. Similar molar fractions were found for five other US wheat varieties. The Bx7 subunit accumulated to the highest level at 30% of total HMW-GS. The Dx and Dy subunits were present in smaller but nearly equal proportions, 22% and 23%, respectively, and the Ax and By subunits were the least abundant, 14% and 12%, respectively. Although the amounts of HMW-GS per unit of flour are strongly affected by environment, the different subunits respond so similarly to external conditions that their final proportions appear to be determined mainly by genetic factors.

Keywords: Glutenin; Environment; HPLC; Bread wheat; High-molecular-weight glutenin subunits; Wheat; Wheat flour

1. Introduction

Two important determinants of flour quality are the amount of protein, which is strongly influenced by environment, and the protein composition, which is determined by genetics and environment (reviewed in Dupont and Altenbach, 2003). Of particular importance to quality are the amount of glutenin polymer and the type of high- and low-molecular-weight glutenin subunits (HMW-GS and LMW-GS) that form the polymer (Gupta \textit{et al.}, 1992; Payne, 1987). Bread wheat has six genes for HMW-GS, encoding closely linked x and y subunits on the long arms of chromosome 1A, 1B, and 1D. Of these, four or five genes are expressed. The promoter regions of these genes differ somewhat, which might influence their level of expression and/or their response to environment (Gu \textit{et al.}, 2004; Kong \textit{et al.}, 2004). For example, two \textit{Glu-B1} alleles encode forms of HMW-GS B\textsubscript{x7} that differ by a six amino acid repeat in the coding region, and a 643 bp insertion in the DNA matrix attachment region of the promoter. The longer form of B\textsubscript{x7} is consistently expressed at a higher level than the shorter form (Butow \textit{et al.}, 2003).

Supply of N and temperature during grain fill are important determinants of total protein per unit of flour. N, S and temperature also influence the proportions of the different protein types in flour (Daniel and Triboi, 2000; DuPont \textit{et al.}, 2006\textit{a,b}; Fowler, 2003; Martre \textit{et al.}, 2003; Shewry \textit{et al.}, 2001; Wieser and Seilmeier, 1998;
Wieser et al., 2004; Wrigley et al., 1984). Triboi et al. (2000) suggested that as total HMW-GS per unit of flour increased, the proportions of individual HMW-GS changed, and Carceller and Aussenac (2001) proposed that the ratio of x to y-type HMW-GS increased during grain fill, indicating that this ratio might be susceptible to environmental effects. However, Martre et al. (2003), suggested that environment had little effect on the N-partitioning coefficient for gliadins or glutenins. Therefore, it is of some interest to determine to what extent environmental factors influence the proportions of the individual HMW-GS subunits.

In a study of the five HMW-GS from the variety Butte86, transcripts for the five genes accumulated with a similar time course during grain fill and had a similar response to temperature, indicating that transcription was coordinately regulated (Altenbach et al., 2002). Also, the five protein subunits had similar patterns of accumulation during grain fill, all increasing in response to addition of N under a cool temperature regimen, and accumulating at slower rates in the absence of N or under a temperature regimen of hot days and warm nights (DuPont et al., 2006a, b). In this study, we asked whether temperature and/or supply of N during grain fill influenced the final proportions of the individual HMW-GS in flour protein.

2. Experimental

2.1. Plant material and growth conditions

Plants of the US hard red spring wheat (HRSW) *Triticum aestivum* ‘Butte86’ were grown as described in Altenbach et al. (2003) and Dupont et al. (2006b). Briefly, the plants were grown in a climate-controlled greenhouse with 16 h days and 8 h nights, with maximum daytime temperature of 24 °C and minimum nighttime temperature of 17 °C (24/17 °C regimen). Plants were watered by drip irrigation with 0.6 g/l Plantex fertilizer (NPK 20:20:20), using two emitters and receiving a total of approximately 0.1 g each of N, P and K per pot per day (NPK treatment). For the minus NPK treatment, pots were flushed with 0.1 g each of N, P and K per pot per day (NPK treatment). For the minus NPK treatment, pots were flushed with irrigation with 0.6 g/l Plantex fertilizer (NPK 20:20:20), using two emitters and receiving a total of approximately 0.1 g each of N, P and K per pot per day (NPK treatment). For the minus NPK treatment, pots were flushed with irrigation with 0.1 g each of N, P and K per pot per day (NPK treatment). For the minus NPK treatment, pots were flushed with irrigation with 0.1 g each of N, P and K per pot per day (NPK treatment). For the minus NPK treatment, pots were flushed with irrigation with 0.1 g each of N, P and K per pot per day (NPK treatment).

Grain of Butte86 was produced in 15 separate experiments with growth conditions indicated in Table 1. For all experiments, mature grains from all pots in a treatment group were pooled. Average single kernel weight for mature grain was determined by sampling 100 grains from each pooled group. Samples of 100 g each were milled to flour. Flour N was determined by near infrared (NIR) and combustion analysis as detailed in DuPont et al. (2006a). Grain from the varieties Arapahoe, Bobwhite and Cheyenne was produced under the 24/17 °C regimen as described above. Flours from Anza and Chinese Spring were from field grown plants.

2.2. Preparation of glutenins

Proteins were sequentially extracted from flour by the method of DuPont et al. (2005). The advantage of this method is that glutenins are almost completely separated from the other flour proteins and are recovered in a single fraction. Briefly, flour samples were extracted twice with 1 ml of 0.3 M NaI, 7.5% l-propanol (NaI–propanol) per 100 mg of flour. The extracts were centrifuged for 10 min at 4500g to separate the albumins, globulins and gliadins from the starch and insoluble glutenin protein. The glutenins were then extracted from the starch pellet with 0.4 ml 2% SDS and 25 mM dithiothreitol (DTT) in 25 mM Tris, pH 8.0 per 100 mg of flour and centrifuged as above to separate them from the starch. The extraction was repeated and the extracts pooled. Glutenins were precipitated from the extract by addition of four volumes of 0.1 M ammonium acetate in 100% methanol. Following incubation at −20 °C overnight, the glutenins were pelleted by centrifugation as above. All protein fractions were freeze-dried and stored at −80 °C. Protein content of freeze-dried extracts was determined by combustion analysis of 10–15 mg samples.

2.3. Reversed-phase high-pressure liquid chromatography (RP-HPLC)

Freeze-dried proteins were dissolved at a concentration of 1 mg ml⁻¹ in 6 M guanidine HCl adjusted to pH 8.0 with Tris, plus 50 mM DTT, and were alkylated with vinyl pyridine prior to HPLC. Proteins were analyzed using a Hewlett Packard Series 1100 HPLC (Wilmington, DE). A total of 500 μl of the protein solution was applied to a Nucleosil (Ansys, Lake Forest, CA) C8 analytical column. Glutenins were eluted using a gradient with a 10 min delay followed by an increase from 10% to 65% acetonitrile, 0.05% trifluoroacetic acid (TFA) at 0.8 ml min⁻¹ for 60 min. Peptide bond absorbance was detected at 210 nm, and the HPLC peak areas were used to estimate the relative amount of HMW-GS in each peak. Each analysis was done in triplicate (DuPont et al., 2006a).

Molecular mass and number of amino acid peptide bonds per protein for each HMW-GS were based on derived protein sequences for genes from the variety Cheyenne. The molecular masses and number of amino acids for each of the processed proteins, without signal sequences, were Ax2* Accession #Q41553, 88,381, 815 amino acids; Bx7 Accession #JN0690, 84,883, 791 amino acids; By9 Accession #CAA43361.1, 75,620, 705 amino acids.
Absorbance at 210 nm is mainly due to the amino bond between the amino acids (Bietz, 2002), so it was assumed that absorbance per mole of a particular HMW-GS was proportional to the number of amino acids in that protein. The relative amount of each HMW-GS was calculated based on the peak area for that HMW-GS divided by a factor related to the number of amino peptide bonds per protein, using the factor of 1.0 for the largest protein, Dx5, and for Ax2* 0.96; Bx7 0.93; By9 0.83; and Dy10 0.76. The molar fraction of each HMW-GS was expressed as a fraction of total HMW-GS, with the total equal to 1.0.

Table 1
Effects of different post-anthesis regimens on grain weight, flour protein content and proportions of HMW-GS for Butte86

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Start date&lt;sup&gt;a&lt;/sup&gt; (dpa)</th>
<th>Environmental regimen</th>
<th>Single kernel weight&lt;sup&gt;e&lt;/sup&gt; (mg)</th>
<th>Flour protein&lt;sup&gt;f&lt;/sup&gt; (%)</th>
<th>HMW-GS molar fraction&lt;sup&gt;g&lt;/sup&gt;</th>
<th>Ax2*</th>
<th>Bx7</th>
<th>By9</th>
<th>Dx5</th>
<th>Dy10</th>
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<td>15.3</td>
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<td>0.31</td>
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</table>

**Expt** = Experiment; **Start date** = Time at which experimental treatment began; measured as days after anthesis. **Environmental regimen** = Day/night temperature regimen. **Fertilizer regimen** = Fertilizer regimen based on number of emitters per pot. Water was added as needed to maintain all pots at similar weights. +S indicates that S was added. **Water** = Pots were watered to 80% of capacity (+) or to 33% of capacity (−). **Single kernel weight** = Single kernel weight of mature grain. **Flour protein** = Flour protein percentage adjusted to 14% moisture. **HMW-GS molar fraction** = Molar fraction of each HMW-GS, determined by RP-HPLC peak area corrected for molecular mass of the different subunits. Data are the average of three measurements.
3. Results

3.1. Effect of environment on HMW-GS amounts for Butte86

Plants of Butte86 were exposed to various regimens of temperature, fertilizer and water from anthesis to maturity (Table 1). Grain fill (accumulation of dry weight) extended until 32–40 dpa (dpa—days post-anthesis) under the 24/17 °C regimen, 30 dpa under the 37/17 °C regimen, and 20–25 dpa under the 37/28 °C regimen. Average single kernel weight ranged from 34 to 59 mg grain⁻¹ under the 24/17 °C regimen, 29–41 mg grain⁻¹ under the 37/17 °C regimen and only 20–26 mg grain⁻¹ under the 37/28 °C regimen that began at anthesis. Under the 24/17 °C regimen protein percentage was 7.0–9.4% for flour produced without post-anthesis fertilizer, 12.2–14.5% for flour produced with half-strength post-anthesis fertilizer and 14.2–16.4% for flour produced with full-strength post-anthesis fertilizer. Under the 37/17 °C regimen, flour protein percentage was 9.4–11% for flour produced without post-anthesis fertilizer and increased to 14.6% or 16.5% for flour produced with half-strength post-anthesis fertilizer and 14.2–16.4 for flour produced with full-strength post-anthesis fertilizer. Under the 37/17 °C regimen, flour protein percentage was 16.3% for flour produced without post-anthesis fertilizer and increased to as high as 18.8% with fertilizer.

The glutenin proteins were extracted from flour, reduced, alkylated and separated by RP-HPLC (Fig. 1). HMW-GS Dy10, Bx7 and Ax2* were resolved as separate peaks. By9 was resolved as a shoulder on the Dx5 peak. The peak areas for the HMW-GS were converted into estimated molar fractions of the subunits (Table 1). For 32 flour samples from 12 separate growth experiments, the proportions of the HMW-GS types were extremely stable. For 1 mole of HMW-GS, the estimated amount of Ax2* ranged from 0.12 to 0.16 mole, of Bx7 from 0.26 to 0.32 mole, of By9 from 0.10 to 0.15 mole, of Dx5 from 0.19 to 0.25 mole and of Dy10 from 0.19 to 0.27 mole. The average values per mole for samples produced at 24/17 °C under three different fertilization regimens were Ax2*, 0.14 mole; Bx7, 0.30 mole; By9, 0.12 mole; Dx5, 0.22 mole and Dy10, 0.22 mole. Average values for samples produced at 37/17 or 37/28 °C under three different fertilization regimens were nearly identical to those for samples produced at 24/17 °C. Any variation in these numbers did not appear to be related to the treatment.

3.2. HMW-GS composition for flour from other wheat varieties

In similar experiments, glutenins were extracted from flour samples from five other varieties and the molar fractions of the HMW-GS were calculated (Table 2). The hard red winter wheat (HRWWs) Arapahoe and Cheyenne and the HWSW Bobwhite have the same complement of HMW-GS as Butte86, and the molar fractions were similar to those for Butte86. The varieties Anza and Chinese Spring lack an A subunit, which was compensated for by increased proportions of the x subunits, particularly Bx7. For Anza and Chinese Spring, the proportions of the two D subunits were similar to each other and the By-subunit was least abundant as for the other varieties.

We also evaluated published data from Wieser and Zimmermann (2000), converting their data on HMW-GS composition into molar ratios of HMW-GS. All varieties with the same complement of HMW-GS as Butte86 had molar fractions of HMW-GS subunits similar to those of Butte86. The varieties with Ax1 had a somewhat higher ratio of Dx5 to Dy10 than the varieties with Ax2* as did two of the varieties that had no A subunit. Varieties with no A subunit also compensated with a larger molar fraction of Bx7. Also, as reported by others, the Canadian extra strong variety Glenlea had a higher proportion of Bx7.

4. Discussion

Stability of flour composition and quality despite environmental variation is a sought-after trait in wheat. Environment generally has a significant influence on flour quality, exerted in part by effects on protein quantity, proportions of protein types, and glutenin polymerization (reviewed in DuPont and Altenbach, 2003). To
develop wheat varieties with increased environmental stability, it is important to understand which flour components are most susceptible to and which are unaffected by environment. Wieser and Zimmermann (2000) concluded that there was little variation in proportions of HMW-GS for varieties with the same subunit combination, and reported little effect of growing conditions on these proportions. In this study we evaluated HMW-GS from flour samples with a much wider range of protein concentrations than for the samples analyzed by Wieser and Zimmermann (2000). This larger range of protein concentrations was achieved by exposing wheat plants to very different temperature and fertilizer regimens after anthesis. Nonetheless, like Wieser and Zimmermann (2000) we found that the proportions of the HMW-GS subunits remained constant. HMW-GS proportions exhibit the desired trait of environmental stability. These findings do not support the suggestions of Carceller and Aussenac (2001) and Triboi et al. (2000) that the proportions of HMW-GS are affected by environment.

Environment does alter the rate and duration of accumulation of HMW-GS in the wheat grain. Temperature and N fertilization altered the ratio of HMW-GS to LMW-GS, the amount of HMW-GS per grain, and the proportion of HMW-GS per unit of flour protein. Raising the growing temperature increased the rate and shortened the duration of accumulation of HMW-GS (Altenbach et al., 2002; Dupont et al., 2006a). Supplying N after anthesis increased the rate of accumulation of HMW-GS, increased their amount per grain, and increased their relative amount compared to sulfur-rich gluten proteins such as LMW-GS (Dupont et al., 2006a, b; Wieser and Seilmeier, 1998). However, these environmental effects acted on each HMW-GS in a similar manner, so that in Butte86 the average molar proportions of approximately 30% Bx7: 22% Dx5: 22% Dy10: 15% Ax2*: 12% By9 were unaltered even with great differences in rate and duration of protein accumulation. Apparently, the coefficients for partitioning of N (Martre et al., 2003) among the HMW-GS were different, but were constant for each subunit under the different environments. The differences in partitioning coefficients may reflect differences in rates of transcription that are influenced by differences in the promoter regions of the HMW-GS genes, and/or by factors that influence transcript stability and rate of translation. The end result is that synthesis of the HMW-GS is coordinately regulated in such a manner that the proportional differences in the amount of each subunit are maintained under a range of growing conditions.

It is likely that differences in the promoter regions of the genes for the HMW-GS lead to differences in rate of transcription, as was proposed for the different Bx7 alleles. In several wheat varieties, Bx7 encoded by the GluB1a allele accounted for 39% or more of the total HMW-GS amount on a molar basis (Butow et al., 2003a, 2004). Varieties with the GluB1a allele were referred to as Bx7 overexpressers. Increased accumulation of this form of Bx7 was originally discussed by Marchylo et al. (1992) with respect to the variety Glenlea, an extra-strong Canadian wheat. Although it was proposed that the greater level of Bx7 protein in the extra-strong HRWW Red River was due to a gene duplication (D’Ovidio et al., 1997), it is now thought that GluB1a differs from other Bx7 alleles by having a 643 bp insertion in the DNA matrix attachment region that increases transcriptional efficiency (Butow et al., 2004). However, Bx7 tends to accumulate to a greater extent than the other HMW-GS, regardless of which Bx7 allele is present, so there must be other factors that contribute to this higher expression of Bx7 and to the lower levels for Ax2* and By9. The molar fraction of Bx7 also increased in the absence of the A subunit, indicating a greater sink strength for this HMW-GS subunit than for the others.

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References


