Effects of post-anthesis fertilizer on the protein composition of the gluten polymer in a US bread
wheat

Susan B. Altenbach*, Charlene K. Tanaka, Linda C. Whitehand and William H. Vensel
USDA-ARS Western Regional Research Center, 800 Buchanan Street, Albany, CA 94710

* Corresponding author

Email: susan.altenbach@ars.usda.gov

Phone: 510-559-5614

Fax: 510-559-5818

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24 **Abstract**

25 Both genetic and environmental factors influence the types and amounts of wheat proteins that
26 link together to form polymers essential for flour quality. To understand how plant growth
27 conditions might influence gluten polymer formation, protein fractions containing small and
28 large polymers were separated from flour from the US wheat Butte 86 grown in the absence or
29 presence of post-anthesis fertilizer. Proteins in the polymer fractions were analyzed by
30 quantitative two-dimensional gel electrophoresis (2-DE). The ratio of high molecular weight
31 glutenin subunits (HMW-GS) to low molecular weight glutenin subunits (LMW-GS) increased
32 in both fractions in response to fertilizer, due in part to small increases in the proportions of
33 individual HMW-GS. There were also changes within the LMW-GS. In particular, omega and
34 alpha chain terminators increased in proportion in both polymer fractions, but changes were
35 more pronounced in the large polymer fractions. Serpins also increased in both polymer
36 fractions. Additionally, the study revealed differences in the proportions of traditional LMW-GS
37 in small and large polymer fractions. LMW-s type proteins were more abundant in the large
38 polymers while LMW-i type proteins were more prevalent in the small polymers, suggesting that
39 these proteins may play different roles in the gluten polymer.

40

1. Introduction

The size of the gluten polymers and the ratio of polymeric to monomeric proteins in wheat flour are important in determining the viscoelastic properties that make it possible to produce a wide range of products from wheat flour. The gluten polymers range in size from about 500,000 to over 10 million MW (Wieser, 2007) and consist of two main types of proteins, the high molecular weight glutenin subunits (HMW-GS) and the low molecular weight glutenin subunits (LMW-GS), linked together by disulfide bonds. Despite their importance in wheat flour quality, there is relatively little information about gluten polymer structure. Most hexaploid wheat varieties contain three to five HMW-GS. These fall into two different groups, x-type HMW-GS with MWs from 83-88 kDa and y-type HMW-GS with MWs of 67-74 kDa. Both consist of central repetitive regions flanked by unique N- and C-terminal domains (Shewry and Tatham, 1997). HMW-GS contain between four and seven cysteine residues, mostly in the N- and C-terminal domains. Several y-type HMW-GS also contain a cysteine in the repetitive region. It has been proposed that the HMW-GS form the backbone of the gluten polymer (Shewry and Tatham, 1997).

The LMW-GS are a much more complex group of proteins, consisting of as many as 30 different proteins that range in size from about 32-45 kDa. Most traditional LMW-GS, referred to as B-type LMW-GS, consist of a short N-terminal region followed by a variable repetitive region and a conserved C-terminal region. The traditional LMW-GS are often classified as LMW-m, LMW-s and LMW-i types, on the basis of their N-terminal amino acids, either methionine, serine, or isoleucine, respectively (D'Ovidio and Masci, 2004). All contain eight cysteine residues, most in the C-terminal region, and the positions of seven are conserved between different LMW-GS. Six

cysteines are involved in intramolecular bonds, leaving two available for intermolecular linkages (Wieser, 2007). It is thought that traditional LMW-GS extend the polymer chain.

In addition, there are some proteins that have been associated with the gluten polymer that have sequences very similar to gliadins (Tao and Kasarda, 1989). While most gliadins are present in the flour as monomers, some contain an extra cysteine residue that allows them to be incorporated into the gluten polymer. It has been hypothesized that these proteins act as chain terminators of the gluten polymer and limit its size (D'Ovidio and Masci, 2004). Of the three types of gliadins in wheat flour, alpha gliadins contain six cysteine residues and gamma gliadins contain eight cysteine residues that are involved in intramolecular linkages while omega gliadins do not contain any cysteine. Alpha and gamma gliadins containing seven and nine cysteine residues, respectively, are sometimes referred to as alpha and gamma chain terminators or as C-type LMW-GS. Omega gliadins containing a single cysteine residue are sometimes referred to as omega chain terminators or as D-type LMW-GS.

It has been challenging to quantify the amounts of gluten polymer in wheat flour, decipher the protein composition of the gluten polymers, determine how individual proteins are linked together in the polymer and relate polymer size and structure to quality. One of the best methods to study gluten polymers to date was developed by Gupta et al. (1993). They used SDS to separate total flour protein into fractions that were extractable with SDS, referred to as extractable polymeric protein (EPP), and fractions that were extractable with SDS only after sonication, referred to as unextractable polymeric protein (UPP). Polymers and monomers from these fractions were further separated by size-exclusion chromatography (SEC). They found that

the ratio of HMW-GS to LMW-GS, determined by SDS-PAGE, was higher in polymer from the SDS unextractable fraction than the SDS extractable fraction. Additionally, the HMW-GS to LMW-GS ratio in the SDS unextractable fraction significantly correlated with dough strength (Gupta et al., 1995). Recently, Vensel et al. (2014) analyzed the protein composition of the different polymer fractions by quantitative 2-dimensional gel electrophoresis (2-DE) combined with tandem mass spectrometry (MS/MS) and found that the proportions of certain proteins, especially the chain terminators, varied among the small and large polymer fractions.

While genetic factors determine the complement of proteins that are available for polymer formation, the growth conditions of the plant also are important in influencing the amounts of particular flour proteins that might be incorporated into the polymer. Consequently, a number of studies have examined the effects of sulfur deficiency, nitrogen application and high temperatures on the percentage of extractable and unextractable polymers (Irmak et al., 2008; Johansson et al., 2005, 2013; MacRitchie and Gupta, 1993; Naeem et al., 2012). In a study that looked at the combined effects of temperature and nitrogen, Malik et al. (2013) concluded that nitrogen was one of the most important factors in determining protein polymerization during grain development. These studies have provided important information, however, none examined the protein compositions of the different polymer fractions in detail. Indeed it is challenging to relate the small but significant changes in flour protein composition that occur because of environmental factors (Hurkman et al., 2013) or agronomic inputs (Altenbach et al., 2011) during grain development to alterations in the gluten polymer and quality. To address this shortcoming, we have used quantitative 2-DE combined with MS/MS to examine the partitioning of proteins in polymers from the SDS extractable fraction (EPP, small polymers) and the

unextractable fraction (UPP, large polymers) in flour samples produced with and without post-anthesis fertilizer.

2. Material and methods

2.1 Flour samples

Flour was obtained from the US spring wheat Butte 86 that was grown in triplicate in a greenhouse under a 24/17°C temperature regimen with and without post-anthesis fertilizer supplied as 20:20:20 N:P:K as described in detail in Altenbach et al. (2011). As reported previously, average protein was 70 µg/mg for flour produced without fertilizer and 140 µg/mg for flour produced with fertilizer (Altenbach et al., 2011).

2.2 Preparation of protein fractions, SEC and 2-DE analysis

Each of three separate 10 mg flour samples produced without or with post-anthesis fertilizer was separated into proteins extractable and unextractable with 0.5% SDS using the method developed by Gupta et al. (1993) and described in detail by Vensel et al. (2014). Six hundred µl of each protein sample was then further fractionated by SEC using a Hewlett Packard Series 1100 high-pressure liquid chromatograph (Santa Clara, CA) fitted with a Phenomenex 00H-2147-P0 BioSep™ 5 µm SEC-s4000 500 Å column. Samples were loaded using an autosampler and column elution was at 2 ml/min. Three fractions were collected for each sample corresponding to Peak 1: 18.5 - 27.4 min, ~17.8 ml; Peak 2: 27.4 – 33.4 min, ~12 ml; Peak 3: 33.4 – 39 min, ~11.2 ml. Each fraction was vacuum dried and the amount of protein in the fraction was determined by the method of Lowry et al. (1951) as modified by Hurkman and Tanaka (2004). Proteins (15 µg) from the EPP Peak 1 and UPP Peak 1 fractions containing small and large polymers,

respectively, were separated by 2-DE in triplicate as described in detail by Vensel et al. (2014). Normalized spot volumes were determined for 447 spots in each 2-D gel. One hundred ninety-two of the most abundant spots that had been previously identified by MS/MS (Dupont et al., 2011) were analyzed further and are reported in this manuscript. The other 255 spots were of minor abundance with average volumes of ~20 and were not considered further. The total normalized volumes of identified spots were 8911 and 9130 for the EPP Peak 1 fractions and 10101 and 9612 for the UPP Peak 1 fractions from flour produced without and with fertilizer, respectively. Normalized volumes of spots with the same identities were summed. Spots were then grouped by protein type and the normalized volumes of all proteins of the same type were summed. Proteins in the EPP and UPP Peak 2 fractions were also separated by 2-DE (not shown). These gels were used only for MS/MS identifications of proteins.

2.3 Statistical Analysis

Mean normalized spot volumes for the three biological and three technical replicates for the EPP and UPP Peak 1 fractions were determined for each fertilizer regimen and subjected to analysis of variance for each spot using SAS software (SAS Institute Inc. 2013. SAS OnlineDoc 9.4, Cary, NC). For individual 2-DE spots, unique proteins and protein types, probabilities from an F-test comparing means of the two treatments were calculated, both assuming that the variance were similar enough to be pooled for both treatments (4 error degrees of freedom) or differed enough between treatments that pooling would not be appropriate (2 error degrees of freedom). An F-test was used to compare the two error terms to determine which mean comparison test was more appropriate. Spots with an F probability less than 0.02 were deemed to show significant changes. A Bonferroni adjustment to the F probability was also made to account for the

increased likelihood of chance significance in a large set of tests. Bonferroni adjustments are shown when below 0.10. All spot volume data, calculations and statistics are shown in Supplementary File 2.

2.4 Mass spectrometry

Protein spots from 2-D gels of the various protein fractions that weren't analyzed in the Vensel et al. (2014) study were excised and identified by MS/MS (Supplemental file 1). These included 24 spots from the EPP Peak 1 fraction, 22 spots from the UPP Peak 2 fraction and 18 spots from the EPP Peak 2 fraction. Excised spots were digested individually with one of three different enzymes, chymotrypsin, thermolysin or trypsin. Details of the mass spectrometry and data analysis were essentially as described in Vensel et al. (2014) and are as presented in brief below.

The excised spots were placed into 96 well plates that were transferred to a DigestPro (Intavis, Koeln, Germany) where the proteins in the gel spots were, reduced, alkylated and enzymatically digested with either chymotrypsin, thermolysin or trypsin. The resulting peptides were collected in 96 well sample plates and transferred to the autosampler of the nanoflow HPLC that was interfaced to the Orbitrap Elite mass spectrometer (Thermo Scientific, San Jose, CA). Peptide samples were automatically introduced into the mass spectrometer by way of a nanoflow electrospray source. Operation of the nanoflow HPLC and data acquisition were carried out under control of the mass spectrometer software (Thermo Scientific, San Jose, CA). Specific details of instrument parameters were as previously described (Vensel et al., 2014).

Following data acquisition, the instrument RAW files were converted to MGF files using software obtained from the ProteoWizard project [<http://proteowizard.sourceforge.net/downloads.shtml>]. The initial data search of the MS/MS spectra was carried out against the Super_Wheat database as described previously (Vensel et al., 2014). The results of that search were analyzed and visualized using Scaffold version 4.2.1. Subsequently, a 2nd pass search against a reversed concatenated database created from all hits obtained in the 1st pass search was carried out. MS/MS identifications are reported in Supplemental File 1. Spots identified from the UPP and EPP Peak 2 fractions are not addressed further in this study.

3. Results

The application of post-anthesis fertilizer doubled the protein content of the flour (Altenbach et al., 2011). About 74% of the protein from flour produced without post-anthesis fertilizer was extractable with 0.5% SDS (EPP) (Figure 1). The remaining 26% required sonication for extraction (UPP). When flour was produced with post-anthesis fertilizer, 67% of the protein was extractable (EPP) while the remaining 33% required sonication (UPP). Soluble (EPP) and insoluble (UPP) proteins were separated further by SEC (Figure 2). Three fractions were collected from each, referred to as Peaks 1, 2 and 3. Polymeric proteins, eluted in Peak 1, comprised less than one third of the total soluble protein (EPP), about 30 and 28% of flour produced without and with fertilizer, respectively (Figure 1). In comparison, polymeric protein comprised the bulk of the insoluble protein (UPP). A greater percentage of the insoluble protein (79%) was found in the first peak in flour produced without fertilizer than in flour produced with fertilizer (74%).

3.1 Quantitative 2-DE analysis of protein polymers in EPP and UPP Peak 1 fractions

Polymer proteins from the both the soluble and insoluble SDS fractions (Peak 1) were separated by 2-DE. At first glance the protein profiles from the different fractions and fertilizer treatments were very similar (Figure 3). However, a previous study revealed that spots with the same isoelectric points and molecular weights from different protein fractions sometimes yielded different identifications by MS/MS (Vensel et al., 2014). As a result, 24 protein spots from the 2-D gel of the EPP Peak 1 fraction that weren't analyzed in the Vensel et al. (2014) study were excised from gels and identified by MS/MS (Supplemental file 1). Spot 170 was identified as LMW-m type in UPP Peak 1 fraction in Vensel et al. (2014), but the same spot was identified as methionine synthase in the EPP Peak 1 fraction (Supplemental file 1). The predominant proteins in spots 314, 315, 316 and 322 in the UPP Peak 1 fraction were LMW-s types. In the EPP fraction, these spots were identified as LMW-m types. Spot 314 also contained an LMW-s type. Spots 317 and 319 contained both LMW-s and LMW-m types in the UPP Peak 1 fraction, but a greater number of spectra were obtained for the LMW-s than the LMW-m types. The only proteins identified in the corresponding spots in the EPP Peak 1 fraction were LMW-m types. Spot 472 was identified as an LMW-m type in the UPP Peak 1 fraction but as tritacin in the EPP Peak 1 fraction. These new identifications were taken into account in tabulating the normalized spot volumes for proteins and protein types for the EPP and UPP Peak 1 fractions (Table 1, Supplemental file 2).

3.2 HMW-GS

Total glutenin comprised about 74% of the UPP Peak 1 fraction, but only 61% of the EPP Peak 1 fraction (Table 1). Although post-anthesis fertilizer doubled the protein content of the flour, the proportions of glutenin in the two fractions did not change with the application of fertilizer. However, there were changes in the relative proportions of different glutenin proteins in the EPP and UPP Peak 1 fractions as a result of the fertilizer treatment. In the absence of fertilizer, HMW-GS accounted for 16.9% of protein in the EPP polymers and 24.5% of the protein in the UPP polymers. HMW-GS comprised a greater proportion of the protein in both the EPP and UPP polymers when plants were supplied with fertilizer, 18.1% and 27.5%, respectively. Of the HMW-GS, the x-type proteins were more abundant than the y-type proteins. Both the x-type and the y-type HMW-GS showed significant increases in response to fertilizer, but the increases were greater for the UPP Peak 1 fraction than the EPP Peak 1 fraction. The Bx7 subunit comprised the greatest proportion of the HMW-GS, about 28% of total HMW-GS in both the EPP and the UPP Peak 1 fractions regardless of the fertilizer regimen (Table 2). All HMW-GS but Dy10 showed statistically significant increases with fertilizer in the UPP polymers, while only the By9 and Ax2* showed significant increases in the EPP polymers (Supplemental file 2). The ratio of x-type HMW to y-type HMW-GS was 1.8 for the EPP Peak 1 fraction and 1.6 for the UPP Peak 1 fraction and did not change with fertilizer.

3.3 LMW-GS

LMW-GS made up 43.8 and 41.6% of the EPP Peak 1 fraction and 49.7 and 46.4% of the UPP Peak 1 fraction when plants were grown in the absence or presence of post-anthesis fertilizer, respectively (Table 1). Traditional LMW-GS, also referred to as B-type LMW-GS, accounted for

94.6 and 90.9% of the LMW-GS in the UPP polymers, but only 77.8 and 73.7% of the LMW-GS in the EPP polymers when plants were grown in the absence or presence of fertilizer, respectively (Table 3). Proteins beginning with an N-terminal methionine (LMW-m) were the most abundant LMW-GS in both fractions, comprising 39.5 to 40.5% of LMW-GS in the EPP polymers and 46.6 to 48.6% of the LMW-GS in the UPP polymers. Proteins beginning with an N-terminal serine (LMW-s) were more predominant in the UPP Peak 1 fractions where they comprised 38.5 to 40.7% of total LMW-GS as compared to the EPP Peak 1 fractions where they accounted for 24.5 to 26.7%. LMW-GS with an N-terminal isoleucine (LMW-i) were more abundant in the EPP Peak 1 fractions where they accounted for 9.8 to 10.5% of the LMW-GS compared to the UPP Peak 1 fractions where they accounted for only 5.3 to 5.7% of the total LMW-GS. Only one LMW-m type, Bu-8, increased 23% in response to fertilizer and only in the UPP polymers (Supplemental file 2).

As reported previously, the chain terminators, also referred to as C- and D-type LMW-GS, were more abundant in the EPP Peak 1 fractions, where they comprised 22.2 to 26.3% of the total LMW-GS, than in the UPP Peak 1 fractions, where they comprised between 5.4 and 9.1% of the total LMW-GS (Table 3). Gamma chain terminators were the most abundant type in the EPP polymers while omega chain terminators comprised a larger proportion of the chain terminators in the UPP polymers. In both fractions, there were statistically significant increases in the levels of alpha and omega chain terminators in response to post-anthesis fertilizer (Table 1). Alpha chain terminators increased 31.9 and 44.9% and omega chain terminators increased 87.8 and 118% in the EPP and UPP Peak 1 fractions, respectively. Gamma terminators did not show a significant change in either fraction in response to fertilizer, although there was a significant

increase in gamma terminator Bu-3 (39.0%) and a decrease in gamma terminator Bu-4 (20.6%) in the UPP polymers (Supplemental file 2).

3.4 Ratio of HMW-GS to LMW-GS

Including the chain-terminators, the ratio of HMW to LMW was 0.43 in the EPP Peak 1 fraction when plants were supplied with fertilizer and 0.38 when they did not receive fertilizer. The ratio of HMW to LMW was also higher in the UPP Peak 1 fraction when plants were supplied with fertilizer (0.59) as opposed to those that did not receive fertilizer (0.49).

3.5 Gliadins

Not surprisingly, gliadins made up only a small proportion of the polymer fractions, ranging from 10.7 to 11.2% in the EPP Peak 1 fractions and 8.7 to 9.6% in the UPP Peak 1 fractions, with and without fertilizer, respectively (Table 1). Significant increases were detected in the levels of alpha gliadins Bu-1 (26.5%) and Bu-5 (32.4%) in the UPP polymers and in Bu-14 (25.3%) in the UPP polymers in response to fertilizer. A decrease in the minor alpha gliadin BQ8062090 was also detected in the UPP polymers (Supplemental file 2). Among the gamma gliadins, Bu-2 increased 32.5 and 25.8% in the EPP and UPP Peak 1 fractions respectively, in response to fertilizer, while gamma gliadins Bu-6 and Bu-7 decreased 26.5 and 36.3% in the EPP Peak 1 fraction and 34.8 and 25.4% in the UPP Peak 1 fraction, respectively. Despite these changes in individual proteins, changes in the levels of specific classes of most gliadin proteins in response to the fertilizer treatment were either less than 20% (gamma gliadins) or not deemed to be significant (alpha and omega gliadins).

3.6 Non-gluten proteins

As reported in Vensel et al. (2014), serpins comprised a larger proportion of the EPP than the UPP Peak 1 fractions. Serpins also increased significantly in proportion in both fractions when plants were supplied with post-anthesis fertilizer, from 3.7% to 5.9% in the EPP polymers and from 2.6% to 3.6% in the UPP polymers. Triticins also comprised a greater proportion of the EPP Peak 1 fraction than the UPP Peak 1 fraction under both fertilizer regimens. While there was little change in the proportions of triticin in the EPP polymers, triticins increased significantly in proportion from 3.2% to 4.8% in the UPP polymers in response to fertilizer. The farinins comprised from 2.6 to 3.1% of the protein in the EPP polymers and 1.9 to 2.4% of the protein in the UPP polymers (Table 1). Small but significant decreases in the levels of farinin were observed with fertilizer. These were due solely to changes in the levels of farinin Bu-2 (Supplemental file 2).

4. Discussion

During wheat grain development, disulfide linkages are formed among certain proteins in the endosperm resulting in the creation of protein polymers that have been shown to be important for flour quality. Some of the smaller polymers can be solubilized with 0.5% SDS (EPP fraction) while sonication is required to solubilize the larger polymers (UPP fraction). Since the polymers represent a continuum of different sizes rather than distinct size classes, it is perhaps not surprising that the EPP and UPP polymers contain many of the same proteins. Quantifying the amounts of specific proteins in each fraction is complicated by the overall complexity of the wheat gluten proteins and the overlap between LMW-GS, alpha and gamma gliadins in 2-DE. Additionally, some 2-DE spots with the same pIs and MWs have been shown to yield different

MS/MS identities in the EPP and UPP fractions (Vensel et al., 2014). In a previous study, we demonstrated that the proportions of certain proteins differ in the EPP and UPP polymers, suggesting that there may be differences in the manner in which subunits are linked together (Vensel et al., 2014). The current study expands upon our previous report in that it provides MS/MS identifications for additional proteins that differ between the small and large polymer fractions and demonstrates that the proportions of proteins in these polymers also vary in response to post-anthesis fertilizer.

The study revealed that the largest changes in response to post-anthesis fertilizer in both the EPP and the UPP polymers were increases in the proportions of omega chain terminators, serpins and alpha chain terminators. A large increase in triticin was also observed in the UPP polymers. Changes in other protein groups with fertilizer were statistically significant, but most were less than 20%. In the EPP polymers, these included small increases (~10%) in the HMW-GS as well as a small decrease in the farinins. The HMW-GS also increased to a somewhat greater extent (~18%) in the UPP polymers in response to fertilizer. In addition, small but significant decreases in the LMW-s type proteins, gamma gliadins and farinins were also observed in the UPP polymers.

The total glutenin amounts were markedly higher in the large polymers than in the small polymers and did not change with the application of fertilizer. The ratio of HMW-GS to LMW-GS also was higher in the large polymer fraction than in the small polymer fraction, as also reported by Gupta et al. (1993). This ratio increased somewhat in both fractions in response to post-anthesis fertilizer. According to Larroque et al. (1997), the molecular weight of the polymer

increases as the HMW-GS to LMW-GS ratio increases, presumably because the HMW-GS form the backbone of the gluten polymer. While previous studies have underscored the importance of the ratio of HMW-GS to LMW-GS, the current study suggests that the composition of the LMW-GS fraction is also important, particularly the proportions of the different types of traditional LMW-GS and the chain terminators. MS/MS identification of LMW-GS from the EPP and UPP Peak 1 fractions coupled with careful quantification of individual LMW-GS proteins revealed differences in protein composition that could potentially influence polymer structure and size. Indeed, this is the first study to examine the partitioning of the different LMW-GS in the polymer fractions. It is interesting that the LMW-s types comprised a greater proportion of the large polymer fractions, while the LMW-i types were more abundant in the small polymer fractions. The LMW-s type proteins consist of a short N-terminal region, followed by a variable repetitive region and a conserved C-terminal region. There is a single cysteine residue in the repetitive region and seven cysteine residues in the C-terminal domain and the first and seventh cysteine residues are involved in intermolecular disulphide bonds (D'Ovidio and Masci, 2004). In comparison, the LMW-i type proteins are missing the N-terminal region and begin immediately with the repetitive domain. These proteins contain eight cysteine residues in the C-terminal domain, the third and seventh of which are involved in intermolecular disulfide bonds (D'Ovidio and Masci, 2004). Zhen et al. (2014) suggested that the secondary structures of LMW-i types are quite different from the LMW-m and LMW-s type subunits. The preferential accumulation of the LMW-i type proteins in the small polymer fraction and the LMW-s type proteins in the large polymer fraction suggests that these proteins may play different roles in the structure of the gluten polymer. The proportions of chain terminators also were strikingly different in the small and large polymer fractions, as reported previously (Vensel et al., 2014).

Although the gamma chain terminators were more abundant, it is interesting that only the alpha and the omega chain terminators increased in response to post-anthesis fertilizer. The fact that the increases were more pronounced in the large polymer fraction suggests that some of the largest polymers may be limited in size by the application of fertilizer.

Of the non-gluten proteins, both the serpins and triticins were more abundant in the small polymer fractions than the large polymer fractions. However, both increased with fertilizer, the serpins in both polymer fractions and the triticins only in the large polymers. Further work is necessary to determine whether these protein types are linked to the gluten polymer via disulfide bonds, form disulfide bonds among themselves or are simply trapped in the protein matrix.

The study revealed that the application of post-anthesis fertilizer might have positive effects on the overall size of the gluten polymer in that the ratio of HMW-GS to LMW-GS increased in both polymer fractions. However, there were also potentially negative effects of fertilizer on polymer size in that the proportions of chain terminators and several non-gluten proteins increased. This may partially explain why a slightly lower percentage of that protein was present as large polymers in flour produced with fertilizer despite a doubling of the protein content.

Chope et al. (2014) also have reported that the proportion of protein present in polymers decreased with increasing nitrogen levels in UK wheat cultivars. It would be interesting to relate the observed changes in the proportions of the different protein types to polymer size, but given the insolubility of these polymers, it is still extremely difficult to get meaningful size estimates. It would also be interesting to investigate whether the structures of the polymers differ in the two fractions and in response to fertilizer. Recent improvements in mass spectrometry may make it

feasible to identify specific disulfide linkages between individual proteins in the polymer fractions and thereby provide new information about polymer structure (Ferreira et al., 2014; Lutz et al., 2012). Further studies also are needed on the importance of specific flour proteins in the gluten polymer. In this respect, biotechnology approaches such as gene silencing could provide insight into the roles of the LMW-i and the LMW-s type proteins, the chain terminators and the serpins in flour quality.

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Abbreviations

2-DE, two-dimensional gel electrophoresis; EPP, extractable polymeric protein; HMW-GS, high molecular weight glutenin subunit; LMW-GS, low molecular weight glutenin subunit; MS/MS, tandem mass spectrometry; SEC, size-exclusion chromatography; UPP, unextractable polymeric protein

Appendix A. Supplementary Data

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Tables

Table 1. Summary of protein types found in EPP and UPP fractions from flour produced from plants grown without or with post-anthesis fertilizer.

Protein Type	Mean Normalized Volume ¹				% Total Normalized Spot Volume ²				% Total Normalized Spot Volume ²
	EPP	EPP	UPP	UPP	EPP	EPP	UPP	UPP	
	Peak 1	Peak 1	Peak 1	Peak 1	Peak 1	Peak 1	Peak 1	Peak 1	
	Minus ⁴	Plus ⁵	Minus ⁴	Plus ⁵	Minus ⁴	Plus ⁵	Minus ⁴	Plus ⁵	Peak 1
x-type HMW-GS	964.47	1067.46	1461.73	1716.52	10.8	11.7	15.2	17.0	10.8
y-type HMW-GS	537.28	582.64	893.14	1056.72	6.0	6.4	9.3	10.5	6.0
total HMW-GS	1501.75	1650.10	2354.87	2773.25	16.9	18.1	24.5	27.5	16.9
LMW-GS-m type	1580.81	1500.52	2321.53	2182.60	17.7	16.4	24.2	21.6	17.7
LMW-GS-s type	1043.23	929.69	1942.21	1805.57	11.7	10.2	20.2	17.9	11.7
LMW-GS-i type	411.51	372.05	251.96	268.65	4.6	4.1	2.6	2.7	4.6
total traditional LMW-GS	3035.54	2802.26	4515.70	4256.82	34.1	30.7	47.0	42.1	34.1
alpha chain terminators	193.37	255.14	74.79	108.39	2.2	2.8	0.8	1.1	2.2
gamma chain terminators	540.08	495.13	80.21	95.72	6.1	5.4	0.8	0.9	6.1
omega chain terminators	131.98	247.80	102.37	223.13	1.5	2.7	1.1	2.2	1.5
total chain terminators	865.42	998.07	257.37	427.24	9.7	10.9	2.7	4.2	9.7
total LMW-GS	3900.97	3800.33	4773.07	4684.06	43.8	41.6	49.7	46.4	43.8
total glutenin	5402.72	5450.43	7127.94	7457.31	60.6	59.7	74.2	73.8	60.6
alpha gliadins	269.54	292.02	426.54	456.90	3.0	3.2	4.4	4.5	3.0
gamma gliadins	375.70	313.83	226.35	183.10	4.2	3.4	2.4	1.8	4.2
omega gliadins	179.39	202.33	131.12	130.46	2.0	2.2	1.4	1.3	2.0
mixed gliadins	174.09	171.11	137.63	110.05	2.0	1.9	1.4	1.1	2.0
total gliadins	998.71	979.29	921.64	880.51	11.2	10.7	9.6	8.7	11.2
farinins	273.22	240.64	230.86	190.17	3.1	2.6	2.4	1.9	3.1

purinins	77.40	68.41	66.15	66.10	0.9	0.7	0.7	0.7	-1
globulins	188.22	169.67	78.43	78.37	2.1	1.9	0.8	0.8	-
triticin	680.19	694.82	309.25	485.98	7.6	7.6	3.2	4.8	
serpins	325.58	540.03	247.48	367.53	3.7	5.9	2.6	3.6	6
alpha-amylase inhibitors	127.43	121.05	102.58	91.02	1.4	1.3	1.1	0.9	-
beta-amylase	96.05	81.66	67.36	64.13	1.1	0.9	0.7	0.6	-1
other identified proteins	741.62	783.77	460.16	419.61	8.3	8.6	4.8	4.2	

¹ mean normalized volumes of all proteins within the group were summed.

² includes all spots identified by MS/MS.

³ due to the application of post-anthesis fertilizer. Statistically-significant changes ($p < 0.02$) in each polymer fraction that occur with the application of post-anthesis fertilizer are shown in bold.

⁴ plants were grown without post-anthesis fertilizer.

⁵ plants were grown with post-anthesis fertilizer.

Table 2. HMW-GS composition of protein polymer fractions from plants grown in the absence and presence of post-anthesis fertilizer.

Protein	% Total HMW-GS			
	EPP Peak 1	EPP Peak 1	UPP Peak 1	UPP Peak 1
	Minus ¹	Plus ²	Minus ¹	Plus ²
HMW-GS By9	14.0	16.0	14.5	16.1
HMW-GS Ax2*	20.2	20.9	16.0	16.2
HMW-GS Bx7	28.7	28.9	28.0	27.8
HMW-GS Dx5	15.4	14.9	18.1	17.8
HMW-GS Dy10	21.7	19.3	23.5	22.0
x-type HMW-GS	64.2	64.7	62.1	61.9
y-type HMW-GS	35.8	35.3	37.9	38.1

¹ plants were grown without post-anthesis fertilizer.

² plants were grown with post-anthesis fertilizer.

Table 3. LMW-GS composition of protein polymer fractions from plants grown in the absence and presence of post-anthesis fertilizer.

Protein	% Total LMW-GS			
	EPP Peak 1	EPP Peak 1	UPP Peak 1	UPP Peak 1
	Minus ¹	Plus ²	Minus ¹	Plus ²
LMW-m type	40.5	39.5	48.6	46.6
LMW-s type	26.7	24.5	40.7	38.5
LMW-i type	10.5	9.8	5.3	5.7
alpha chain terminators	5.0	6.7	1.6	2.3
gamma chain terminators	13.8	13.0	1.7	2.0
omega chain terminators	3.4	6.5	2.1	4.8
total traditional LMW-GS	77.8	73.7	94.6	90.9
total chain terminators	22.2	26.3	5.4	9.1

¹ plants were grown without post-anthesis fertilizer.

² plants were grown with post-anthesis fertilizer.

Figure Captions

Figure 1. Extraction of soluble (EPP) and insoluble (UPP) protein polymers from flour. The percentage of protein that partitioned into the EPP and UPP fractions and into SEC peaks 1, 2 and 3 is indicated for flour produced without post-anthesis fertilizer (black) and with post-anthesis fertilizer (red). Proteins in Peak 1 from EPP and UPP fractions were analyzed by quantitative 2-DE.

Figure 2. Superimposed SEC traces of EPP (blue, green) and UPP (red, magenta) fractions from flour produced with (red, blue) and without (magenta, green) post-anthesis fertilizer.

Figure 3. 2-DE of proteins from peak 1 fractions of extractable polymeric protein (EPP) (A,B) and unextractable polymeric protein (UPP) (C,D). Protein was from flour produced without (A,C) or with (B,D) post-anthesis fertilizer.