Journal of Cereal Science 68 (2016) 66–73

Contents lists available at ScienceDirect

Journal of Cereal Science

journal homepage: www.elsevier.com/locate/jcs

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

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ARTICLE INFO

Article history:
Received 30 September 2015
Received in revised form
4 December 2015
Accepted 25 December 2015
Available online 29 December 2015

Keywords:
Chain terminators
Flour quality
Gluten polymer
Glutenins

ABSTRACT

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

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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 to 88 kDa and y-type HMW-GS with MWs of 67 to 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 to 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

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.

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http://dx.doi.org/10.1016/j.jcs.2015.12.002
0733-5210/Published by Elsevier Ltd.

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 10,101 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 variances 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