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A reassessment of the electrophoretic mobility of high molecular weight glutenin subunits of wheat

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ABSTRACT

The high molecular weight subunits of wheat glutenin (HMW-GS) are important for bread-making quality. Their composition is routinely identified by Tris-glycine SDS-PAGE after reduction of glutenin disulfide bonds. However, the relation between their molecular weight and, hence, their primary structure, and their mobility in Tris-glycine SDS-PAGE has proven to be ambiguous. We demonstrate a Bis-Tris SDS-PAGE procedure with a neutral, instead of alkaline, pH in the gel and running buffers. In this method commonly occurring HMW-GS from wheat migrated in the order $5 > 2 \approx 3 > 1 > 6 \approx 2^* > 7 > 8 > 9 > 12 > 10$, which is different from the order obtained in the Tris-glycine system. HMW-GS were identified by N-terminal sequencing after isolation with RP-HPLC. Protein sequences of HMW-GS were further confirmed by LC-MS/MS analyses of chymotryptic peptides after comparing the MS data to amino acid sequences in protein databases. The numbers of amino acids of HMW-GS reflected well the mobility order in Bis-Tris SDS-PAGE. The results indicate that Bis-Tris SDS-PAGE may not only be used to identify HMW-GS, but also to estimate the length of their polypeptide chain, as such avoiding previously observed anomalies in migration order.

1. Introduction

The unique properties of hexaploid common wheat (*Triticum aestivum* L. genomes AABBDD) mainly reside in its gluten-forming storage proteins, *i.e.* gliadins and glutenins. Their intrinsic viscoelastic behavior is responsible for the characteristics of different wheat-based foods. The most common use and well-known effect of gluten-forming proteins is in bread making, where they determine dough rheological and gas-holding properties and the bread loaf quality (Delcour et al., 2012). Although end-use quality of common wheat is influenced by both growing conditions and genotype, the composition of glutenin, the polymeric part of gluten consisting of disulfide linked glutenin subunits (GS), is responsible for the major part of the variability in bread-making quality. Structurally, they are grouped into low molecular weight (LMW-) and high molecular weight (HMW-) GS. The composition of the HMW-GS alone may account for up to 60% variation in the quality of bread flour (Payne et al., 1987). This underlines the importance of a correct and reliable detection of the HMW-GS composition of a given wheat cultivar.

HMW-GS are coded by genes at three genetically unlinked loci, *Glu-A1*, *Glu-B1* and *Glu-D1*, which occur on the long arms of chromosomes 1A, 1B and 1D respectively (Payne et al., 1984). Each locus consists of two genes encoding a higher MW x-type subunit and a lower MW y-type subunit. The *Glu-1* loci exhibit extensive allelic variation and the allelic composition of HMW-GS (mostly three to five compounds per cultivar) can be distinguished by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), which usually separates proteins based on their apparent molecular weight (Payne et al., 1981). SDS-PAGE led to the current HMW-GS nomenclature, in which individual subunits are numbered in order of increasing mobility on the gel. Even more, this HMW-GS

Abbreviations: ACN, acetonitrile; Bis-Tris, bis (2-hydroxyethyl) imino-tris(hydroxymethyl) methane-HCl; CID, collision-induced dissociation; cv., cultivar; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; ESI, electrospray ionization; HMW-GS, high molecular weight-glutenin subunits; LC-MS, liquid chromatography-mass spectrometry; LMW-GS, low molecular weight-glutenin subunits; MOPS, 3-(N-morpholino)propanesulfonic acid; MS/MS, tandem mass spectrometry; MW, molecular weight; PVDF, polyvinylidene difluoride; RP-HPLC, reversed-phase high-performance liquid chromatography; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SPE, solid phase extraction; TFA, trifluoroacetic acid; Tris, tris (hydroxymethyl)-aminomethane.

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polymorphism led to a Glu-1 quality score developed by Payne (1987), which provides a basis for the correlation of specific alleles with differences in bread-making performance. With the use of other high resolution methods, such as reversed-phase high-performance liquid chromatography (RP-HPLC) and proteomics with mass spectrometry (MS), further polymorphism of HMW-GS has been reported even in single cultivars (Anjum et al., 2007). The increasing number of HMW-GS alleles makes the current HMW-GS nomenclature based on SDS-PAGE mobilities sometimes problematic (Cunsolo et al., 2012).

HMW-GS have MWs ranging from 65,000 to 90,000. However, on SDS-PAGE they show anomalously high MW ranging from 80,000 to 120,000 (Veraverbeke and Delcour, 2002). In addition to their overestimated MW, the relative mobilities of individual subunits are not always directly related to their MW. For example, subunits Dx5 and Dy12 have higher MW than the allelic subunits Dx2 and Dy10, respectively, but migrate faster, when using the widespread Tris-glycine SDS-PAGE method of Laemmli (1970). Also, subunit Ax1 has a lower MW than Dx5, but migrates slower (Shewry et al., 1992). The anomalous mobilities of Dy10 and Dy12 have been ascribed to structure differences leading to a different sensitivity to detergents and chaotropic agents (Goldsbrough et al., 1989). It has been assumed, but never confirmed, that similar small differences in amino acid sequences may account for the other observed anomalous subunit mobilities (Shewry et al., 1992). Regardless of the anomalous mobilities, the Tris-glycine SDS-PAGE system, and the classification system and score derived therefrom by Payne et al. (1987) have been extensively used by researchers, geneticists and plant breeders to analyze wheat proteins. Even nowadays, Tris-glycine SDS-PAGE is still commonly used, often in combination with other methods, such as capillary electrophoresis, RP-HPLC and MS, as accentuated in different research and review papers on wheat protein composition and quality (Anjum et al., 2007; Gao et al., 2010; Guo et al., 2011).

However, after pioneering work of Shapiro et al. (1967), the introduction of the popular discontinuous Tris-glycine buffer system by Laemmli (1970) and the use of this Tris-glycine SDS-PAGE system to identify wheat proteins (Bietz and Wall, 1972; Payne et al., 1981), various SDS-PAGE procedures have been developed to improve convenience, safety and resolution. An important improvement was the use of neutral pH conditions (pH 7.0) during electrophoresis, instead of the alkaline pH (pH 8.2–8.5) of the resolving gel from the Tris-glycine system, leading to an improved protein stability (Moos et al., 1988). As such, a discontinuous Bis-Tris electrophoresis system with a neutral operating pH led to an excellent resolution after electrophoretic separation of the HMW-GS under investigation (Kasarda et al., 1998). Furthermore, based on the band intensities in these Bis-Tris gels, it was observed that subunits Ax1 and Dx5 and Ax2* and Dx5, respectively, switched position in comparison to their positions in the Tris-glycine system at pH ca. 8.5. This remarkable switch in migration order was confirmed for subunits 5 and 2* (Blechl et al., 2007; DuPont et al., 2005). Apparently, an operating pH around neutrality not only improved resolution, but also changed the migration order of (some) subunits. However, why these and, possibly also, other subunits change position, has never been investigated.

Given the importance of subunit analysis with regard to wheat quality determination and the common use of precast Bis-Tris gels, the aim of this study was to identify commonly occurring HMW-GS and to determine their migration order in Bis-Tris SDS-PAGE. This was done by comparing the migration order in homogeneous 10% Bis-Tris gels of HMW-GS from wheat cultivars with known subunit composition and by establishing the migration order of purified HMW-GS. Furthermore, the migration order of HMW-GS was related to their structural properties.

2. Materials and methods

2.1. Wheat samples

The following common wheat cultivars (cvs.) with known HMW-GS composition and allelic classification according to Payne and Lawrence (1983), at the *Glu-A1*, *Glu-B1*, and *Glu-D1* loci respectively, were selected: Akteur (subunits Ax1, Bx7, By9, Dx5, Dy10; alleles a, c, d), Apache (Ax2*, Bx7, By9, Dx3, Dy12; alleles b, c, b), Atlantis (Ax1, Bx6, By8, Dx2, Dy12; alleles a, d, a), Contra (Bx6, By8, Dx2, Dy12; alleles c, d, a), Cubus (Bx7, Dx5, Dy10; alleles c, a, d), Enorm (Ax1, Bx7, Dx5, Dy10; alleles a, a, d), which are cvs. from homogeneous lines, and cv. Magnus from a heterogeneous line with subunits Bx6, By8, Dx5, Dy10 and alleles c, d, d. Kernels from cvs. Atlantis, Cubus, Enorm and Magnus were directly milled into whole meal flour by means of a laboratory mill (IKA, Staufen, Germany). For subunit isolation, wheat kernels of the cvs. Akteur (harvest 2009) and Apache (harvest 2011) were milled into white flour with a Bühler Mill (Bühler, Uzwill, Switzerland). Wheat kernels of the cv. 'Contra' (harvest 2009) were milled into white flour with a Quadrumat Junior Mill (Brabender, Duisburg, Germany). All flour samples were sifted through 0.2 mm screen.

2.2. Chemicals

The quality of all chemicals was "pro analysi" or stated otherwise. Acetonitrile (LiChrosolv), ammonia solution (25%, w/w), Coomassie Brillant Blue R-250, formic acid (98–100%), glacial acetic acid, hydrochloric acid (32%, w/w), methanol (LiChrosolv), 1-propanol (LiChrosolv), sodium azide, sodium dodecylsulfate (SDS), trichloroacetic acid, and tris (hydroxymethyl)-aminomethane (Tris) were from Merck (Darmstadt, Germany). Calcium chloride hexahydrate, α-chymotrypsin (from bovine pancreas, 40 units/mg protein), sodium hydroxide (≥98%), and trifluoroacetic acid (TFA, ≥ 98%) were from Sigma–Aldrich (Steinheim, Germany). 3-(N-morpholino)propanesulfonic acid (MOPS) was from AppliChem (Darmstadt, Germany). Dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), phenol red, and serva blue G250 were from Serva (Heidelberg, Germany). Water was deionized by a Millipore-O Milli-Q purification system.

2.3. SDS - polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out according to Kasarda et al. (1998) with some modifications including a homogeneous NuPAGE 10% polyacrylamide – Bis-Tris [bis (2-hydroxyethyl) imino-tris(hydroxymethyl) methane-HCl] gel at pH 6.4, 1.0 mm × 10 well (Invitrogen, Carlsbad, CA, USA) and a MOPS-Tris (50 mmol/L MOPS, 50 mmol/L Tris, 3.5 mmol/L SDS, 1 mmol/L EDTA, pH 7.7) running buffer containing DTT (5 mmol/L) as reducing agent added to the inside chamber. Flour (20 mg) was extracted with 1 mL of extraction buffer (293.3 mmol/L sucrose, 246.4 mmol/L Tris, 69.4 mmol/L SDS, 0.51 mmol/L EDTA, 0.22 mmol/L serva blue G250, 0.177 mmol/L phenol red, 0.105 mmol/L HCl, pH 8.5) for 24 h under reducing conditions (DTT, 50 mmol/L). The flour suspension was then shaken for 10 min at 60 °C and centrifuged at 5000 *g* for 5 min at 20 °C. Seven proteins with different MW (myosin: 200 k; β-galactosidase: 116 k; bovine serum albumin: 66 k; ovalbumin: 43 k; carbonic anhydrase: 29 k; trypsin inhibitor from soybean: 20 k; lysozyme from chicken egg white: 14 k) were used as MW markers. The sample volumes on the gel were between 5 and 10 μL per slot. Running time was 40 min at 200 V and 115 mA. After the run, proteins were fixed for 30 min in 12% trichloroacetic acid. Proteins were stained for 30 min with Coomassie Brilliant Blue R-250 according to Neuhoff et al. (1988). Gels were first destained twice