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8 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 visco-elastic 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 polymorphism led to a Glu-1 quality score developed by Payne and co-workers (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

90 resolution. An important improvement was the use of neutral pH conditions (pH 7.0) during
91 electrophoresis, instead of the alkaline pH (pH 8.2 to 8.5) of the resolving gel from the Tris-
92 glycine system, leading to an improved protein stability (Moos et al., 1988). As such, a
93 discontinuous Bis-Tris electrophoresis system with a neutral operating pH led to an excellent
94 resolution after electrophoretic separation of the HMW-GS under investigation (Kasarda et
95 al., 1998). Furthermore, based on the band intensities in these Bis-Tris gels, it was observed
96 that subunits Ax1 and Dx5 and Ax2* and Dx5, respectively, switched position in comparison
97 to their positions in the Tris-glycine system at pH ca. 8.5. This remarkable switch in
98 migration order was confirmed for subunits 5 and 2* (Blechl et al., 2007; DuPont et al.,
99 2005). Apparently, an operating pH around neutrality not only improved resolution, but also
100 changed the migration order of (some) subunits. However, why these and, possibly also, other
101 subunits change position, has never been investigated.

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

109 **2. Materials and Methods**

110 **2.1. Wheat samples**

112 The following common wheat cultivars (cvs.) with known HMW-GS composition and
113 allelic classification according to Payne and Lawrence (1983), at the *Glu-A1*, *Glu-B1*, and
114 *Glu-D1* loci respectively, were selected: Akteur (subunits Ax1, Bx7, By9, Dx5, Dy10; alleles
115 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, Uzwil, 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 Brilliant 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 and co-workers (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 x 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 to 10 μ L 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 with methanol/water/acetic acid (50/40/10, v/v/v) for 15 min and then overnight with water/methanol/acetic acid (80/10/10, v/v/v) (Kasarda et al., 1998).

2.4. Isolation of individual HMW-GS by preparative RP-HPLC

HMW-GS were extracted from flour (33 g) for 30 min at 60°C with 200 mL 50% 1-propanol containing 1.0% DTT. After centrifugation (20 min at 4600 g), the pellet was extracted with 100 mL of the same solvent in the same way and centrifuged (20 min at 4600 g). The combined supernatants were filtered over paper. HMW-GS were then selectively precipitated at room temperature by increasing the 1-propanol concentration of the supernatant to 60.0% (v/v) (Marchylo et al., 1989). After centrifugation (20 min at 4600 g) the isolated HMW-GS were washed twice with 50 mL water and freeze-dried.

Freeze-dried HMW-GS samples (5.0 mg protein) were solubilized (20 min, 60 °C) in 1.0 mL 50% 1-propanol containing 1.0% DTT and preparative RP-HPLC was conducted using a Jasco LC system (Jasco, Easton, MD, USA). After filtration over polyethersulfone (0.45 μ m), protein solutions were repetitively loaded (300 μ L) on a Nucleosil 300-5 C₈ column

(Macherey-Nagel, Düren, Germany). The elution was with 0.1% TFA (A) and 0.1% TFA in ACN (B). The following gradient was used: (i) isocratic at 30% B for 10 min; (ii) linear from 30 to 35% B over 15 min; (iii) linear from 35 to 90% B over 5 min; (iv) isocratic at 90% B for 10 min; (iv) linear from 90 to 30% B in 0.1 min; (v) isocratic at 30% B for 7.9 min. Proteins were eluted (60 °C, flow rate: 1.0 mL/min) and detected at 210 nm.

Individual peaks were collected and ACN was removed by rotary evaporation. An aliquot (2.0 mL) of each peak fraction was kept for N-terminal sequencing. The remainder was freeze-dried and kept for further analysis.

2.5. N-terminal sequence analysis

Peak fractions (2 mL) were applied onto the polyvinylidene difluoride (PVDF) membrane of a ProsorbTM dot blotting device (Applied Biosystems, Foster City, CA, USA). Sequence analysis was then carried out by automated Edman degradation on a protein sequencer Procise 492 (Applied Biosystems) running in the pulsed liquid mode as described by Koehler (2003).

2.6. Chymotryptic digestion of purified HMW-GS

Purified HMW-GS (1.0 mg) were dissolved at 60°C in 1.0 mL 50% 1-propanol containing 1.0% DTT. To remove excess DTT prior to chymotryptic digestion, the protein solution was concentrated using a Vivaspin Concentrator (molecular weight cut-off 10 k, Sartorius Stedim Biotech, Goettingen, Germany) and redissolved in 1.0 mL 50% propanol. The samples were evaporated to dryness and suspended in 1.0 mL of a 100 mmol/L Tris/HCl buffer at pH 7.6 containing 20 µg chymotrypsin. The ratio of enzyme to protein was 1:50 (w/w). Samples were shaken at 37 °C, and after 24 h the reaction was stopped by lowering the pH to 1.4 by adding 3.0 µL TFA.

2.7. Solid phase extraction (SPE)

After chymotryptic digestion, peptides were purified by solid phase extraction (SPE) on Strata-X-C devices (Phenomenex, Aschaffenburg, Germany). SPE cartridges were

conditioned with methanol (1.0 mL) and equilibrated with 1.0 mL 0.1% TFA. Then the digest (1.0 mL) was loaded and the cartridge was washed with 50% methanol (5×1.0 mL). Peptides were eluted with 1.0 mL 2.0 mol/L ammonia. After evaporation of the ammonia solution, peptides were dissolved in 1.0 mL 0.1% formic acid.

2.8. Liquid chromatography-mass spectrometry (LC-MS) of glutenin peptides

LC-MS experiments were performed on an HCT-Ultra PTM ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany) with collision-induced dissociation (CID) detection coupled with an UltiMate 3000 HPLC (Dionex, Idstein, Germany) system equipped with an Aeris PEPTIDE 3.6u XB-C18 column (2.10×150 mm; Phenomenex). The mass spectrometer contained a spherical ion trap with an electrospray ionization (ESI) interface running in the positive mode (capillary voltage: -4000 V; capillary exit voltage: 158.5 V; skimmer voltage: 40 V). Nitrogen was used as drying (8.0 L/min, 325 °C) and nebulizing gas (0.2 MPa). The mobile phase for LC separation was (A) 0.1% (v/v) formic acid and (B) 0.1% (v/v) formic acid in ACN. The following gradient was used: (i) linear from 0 to 32% B over 80 min; (ii) linear from 32 to 90% B over 5 min; (iii) isocratic at 90% B for 9 min; (iv) linear from 90 to 0% B in 1 min; (v) isocratic at 0% B for 10 min. The flow rate was 0.2 mL/min, injection volume was 5 μ L, and column temperature was 30 °C. The scan range was m/z 200 to $2,000$ (smart target value: $200,000$; maximum acquisition time: 100 ms), and CID-MS² scan steps were performed on precursor ions using the AutoMS/MS mode (fragmentation amplitude: 1.0 V; collision gas: helium). For a general search of HMW-GS peptides, MS/MS data were shown in a Mascot Generic File (*.mgf) using the Bruker Daltonics Data Analysis 3.4 and analyzed using the MS/MS ions search module of Mascot software (Matrix Science, London, UK) based on the SwissProt database 2012 (taxonomic category: *Viridiplantae*; peptide and MS/MS tolerance: ± 0.5 ; peptide charges: 1^+ , 2^+ and 3^+ ; monoisotopic ions). Oxidation of methionine and one missed cleavage by chymotrypsin were allowed. Individual ion scores obtained by the Mascot search engine based on peptide mass fingerprints were

calculated as $-10 \times \log(p)$, where a random was presented as the probability (p) of the observed match. All ion scores with a threshold value >66 were considered to indicate identity or extensive homology ($p < 0.0001$) as defined by the Mascot probability analysis. For the search of peptides from HMW-GS with sequences that are not available in the SwissProt database, MS/MS data (*.mgf) were compared with a peptide list generated from HMW-GS sequences from the literature and/or the NCBI database (National Library of Medicine, Bethesda, MD, USA) using the SequenceEditor module of the Bruker Biotools software 3.2 (m/z range: 200-3,000; peptide and MS/MS tolerance: ± 0.5 ; peptide charges: 1^+ , 2^+ and 3^+ ; monoisotopic ions; one missed cleavage of chymotrypsin was allowed. Subunit MWs were also determined with the SequenceEditor using the calculated average mass for a given protein sequence.

3. Results and Discussion

3.1. SDS-PAGE of protein extracts from different wheat cultivars

Figure 1 shows the SDS-PAGE patterns of the storage proteins from different wheat cultivars analyzed in this study, using a homogeneous 10% polyacrylamide gel in Bis-Tris-HCl buffer (pH 6.4) and a MOPS-Tris running buffer (pH 7.7) resulting in an operating pH of 7.0 during electrophoresis. This SDS-PAGE system is further referred to as the Bis-Tris system. Based on the positions of the subunits from cv. Cubus in lane 2 (5, 7, 10) and cv. Enorm in lane 3 (5, 1, 7, 10), subunit 1 was located. By comparing the migration orders (from slow to fast) of cv. Enorm to cv. Akteur subunits (lane 4; subunits 5, 1, 7, 9, 10), and those of cv. Cubus to those of cv. Magnus (lane 6; subunits 5, 6, 8, 10), subunits 9 and 7, respectively, were located. Since HMW-GS 5 and 10 are both coded for by the D-genome, they were consistently co-expressed. Thus, the positions of these subunits on the Bis-Tris SDS-PAGE gel could only be revealed by loading them as single (isolated) HMW-GS. To that end, the HMW-GS from cv. Akteur were isolated from flour and separated using

preparative RP-HPLC. The isolated subunits were then identified by N-terminal sequencing and separated by SDS-PAGE. Based on the N-terminal sequence of the first 12 amino acids, HMW-GS 1, 5 and 7 could be directly identified (Table 1). Subunit 5 was the slowest migrating HMW-GS (Figure 2A). Based on the positions of HMW-GS 5, 1, 7 and 9 in the gel, subunit 10 was identified as the fastest migrating subunit (Figure 2A). In conclusion, the migration order of cv. Akteur HMW-GS on the Bis-Tris system is 5, 1, 7, 9, 10. This confirms the observation of Kasarda et al. (1998), who used a Bis-Tris gradient gel and a neutral 3-(N-morpholino)ethanesulfonic acid (MES)-Tris buffer, that, in a Bis-Tris system, subunits 1 and 5 have a different migration order than that reported by Payne et al. (1987) using the Tris-glycine system.

Comparison of the migration orders of HMW-GS from cv. Magnus to the ones of HMW-GS from cv. Contra (Figure 1, lane 7; subunits 2, 6, 8, 12), allowed locating the allelic subunit pairs 2+12 and 6+8. To determine the migration order of the individual subunits, HMW-GS from cv. Contra were isolated using preparative RP-HPLC. N-terminal sequencing of the first 12 amino acids of the collected HMW-GS allowed a direct identification of HMW-GS 2 and 6 (Table 1). Based on the positions of HMW-GS 2 and 6 in the Bis-Tris gel, the positions of HMW-GS 8 and 12 were revealed (Figure 2B). The subunits of cv. Contra migrated in the order 2, 6, 8 and 12 when using the Bis-Tris system, which is the same as in the Tris-glycine system.

A similar approach was followed for cv. Apache, which contains HMW-GS 2* and 3 in addition to subunits 7, 9 and 12. HMW-GS 2* and 3 were isolated from Apache flour using preparative RP-HPLC. Subunit 3 was identified by N-terminal sequencing (Table 1), and was the slowest migrating Apache subunit (Figure 2C) with a similar mobility than subunit 2 from cv. Contra (Figure 1). Because the positions of all other Apache subunits were known, subunit 2* could be identified as having a mobility intermediate between that of subunits 3 and 7. This was further confirmed by N-terminal sequencing (Table 1). From the comparison

between cv. Apache and cv. Contra it could also be observed that subunits 2* and 6 migrated to the same position (Figure 1). The migration order of HMW-GS from cv. Atlantis (Figure 1, Lane 8, HMW-GS 2, 1, 6, 8, 12) further indicated a faster migration of subunit 1 than 2 on the Bis-Tris gel.

In summary, the migration order (from slow to fast) of HMW-GS in Bis-Tris SDS-PAGE is $5 > 2 \approx 3 > 1 > 6 \approx 2^* > 7 > 8 > 9 > 12 > 10$ (Figure 1). A different migration order is obtained when using the Tris-glycine SDS-PAGE method developed by Laemmli (1970). In addition to the opposite position of subunit 5 and subunits 1 or 2*, opposite positions of subunits 10 and 12 were also observed. Moreover, subunits 2 and 2*, which were reported to migrate to the same position in the Tris-glycine system, could be distinguished by conducting Bis-Tris SDS-PAGE.

3.2. Sequence and molecular weight determination of individual HMW-GS

To investigate the reason for the migration order of HMW-GS in Bis-Tris SDS-PAGE, the primary structure of HMW-GS was determined using LC-MS/MS. To that end, isolated and reduced HMW-GS were partially hydrolyzed with chymotrypsin, which preferentially cleaves peptide bonds after tyrosine, tryptophan, or phenylalanine (but never before proline) and to a lesser extent after leucine, histidine and methionine. Given the low levels of lysine and arginine in gluten, chymotrypsin results in smaller gluten peptides than trypsin and is therefore better suited for HMW-GS primary structure determination. After ESI-MS/MS, a peptide list was generated for each subunit and checked against the Swiss Protein database using the MS/MS ion search module of Mascot software. Based on peptide matches with amino acid sequences of HMW-GS in the database, the sequences of subunits 2, 5, 10 and 12 were confirmed with high scores (Table 2). The peptide lists of other HMW-GS were checked against peptide lists generated using the Bruker SequenceEditor and based on HMW-GS sequences reported in literature and the NCBI protein database. As such, sequences of subunits 1 (Halford et al., 1992), 2* (Anderson and Greene, 1989), 7 [based on Anderson and

Greene (1989) with database updates] and 9 (Halford et al., 1987) were confirmed (Table 2). The peptide list derived from the MS/MS data of subunit 8 agreed well with a protein sequence for subunit 8 in the NCBI protein database (Table 2). The NCBI protein database contained no sequences for *Triticum aestivum* subunits 3 and 6. A protein sequence was found in the database for a Bx subunit from *Triticum turgidum* (durum wheat) containing 803 amino acids (Kong et al., 2004), and which had a calculated MW of 86,393 (Table 2). Comparison of the peptide list derived from the MS/MS data of subunit 6 with that of the sequence from the database resulted in an MS/MS sequence coverage of 68.4%, which strongly suggests a sequence match between subunit 6 and the Bx sequence from durum wheat (Table 2). Since no gene or protein sequence was found for subunit 3, in Table 2 we used the subunit 3 MALDI-TOF MW determined by Gao et al. (2010), which was determined from the sequence of subunit 4¹ (815 amino acids) from *Aegilops tauschii* based on their good MW agreement (Wang et al., 2012). Also here the identified peptide list derived from the MS/MS data of subunit 3 at least suggested a strong sequence homology between subunits 3 and 4¹ (Table 2).

3.3. Comparison between structural properties and SDS-PAGE migration order of HMW-GS

The migration order of all HMW-GS on the Bis-Tris SDS-PAGE system was in good agreement with the number of amino acids in the sequence (Table 2). Apparently, Bis-Tris SDS-PAGE performed at neutral pH conditions resulted in a good separation of HMW-GS based on their amino acid chain length. This is in accordance with the known correlation between electrophoretic mobility and the effective molecular radius of a protein (Wiltfang et al., 1991). The length of the primary structure is mostly reflected in the protein's MW, although, for example, subunit 1 has a higher MW than subunit 2, but contains fewer amino acids (Table 2). The agreement between protein chain length and its migration on the gel also implies that HMW-GS with the same or similar chain lengths, such as subunits 2 and 3

migrate to the same position in the Bis-Tris gel. In addition, there is no clear difference between the electrophoretic mobilities of subunits 2* and 6, which have different chain lengths, but similar molecular weights (Table 2).

The most remarkable difference between the Bis-Tris SDS-PAGE method and the Tris-glycine system was the lack of anomalies between the elution order of HMW-GS and the number of amino acids for the former. As such, subunit 5 (and not 1) was the slowest migrating subunit on a Bis-Tris SDS-PAGE gel (Table 2). This also implied that at neutral pH subunit 5 migrated slower than subunit 2 (Figure 3A). The clear separation between subunits 2 and 2* (Figure 3B) and the observation that subunit 12 migrated slower than 10 (Figure 3C) were also in line with the amino acid chain lengths of the respective subunits (Table 2).

We can only speculate on the reason for the anomalous mobilities observed in the Tris-glycine system. Anomalies in SDS-PAGE are observed when there is no hydrodynamic homology between the proteins or atypical SDS-binding to (parts of) the protein, resulting in no constant net charge per mass unit of the SDS-protein complex. This can be due to differences in conformation, a variable content of hydrophobic or hydrophilic amino acids, or differences in intrinsic charge of the proteins (Wiltfang et al., 1991). Table 3 shows the number of acidic and basic amino acids of the subunits with reported anomalous mobilities. There is a noticeable difference in the number of amino acids with charged side chains when comparing the Dx to the Ax subunits (Table 3). It is assumed that at neutral pH the ratio of positively and negatively charged amino acids and the SDS-binding is similar for both subunit types. Higher pH values result in a net negative charge of the proteins. A higher negative charge density and, as a result thereof, atypical SDS-binding of the Ax subunits in the Tris-glycine system probably results in their anomalous slow mobility. However, different levels of charged amino acids or different hydrophobicity cannot explain the apparent anomalous mobility of Dx5 versus the other Dx subunits in the Tris-glycine system, nor the mobilities of Dy10 and Dy12. The latter was explained by amino acid differences in the

central repetitive domain with an effect on the β -spiral structure. As such, subunit 10 is assumed to have longer stretches of amino acids in a continuous β -spiral conformation giving the protein an apparent higher MW in the Tris-glycine system (Goldsbrough et al., 1989). However, this structural feature did not affect the mobility of subunit 10 in the Bis-Tris SDS-PAGE, suggesting that the alkaline pH values used in the Tris-glycine system have an impact on HMW-GS secondary structure and/or hydrodynamic volume. A similar reasoning can be made when comparing subunits 5 and 2, which only differ little in number of amino acids. It is most likely that also for these subunits a difference in conformation results in their anomalous mobilities in the Tris-glycine system.

4. Conclusion

Results in this paper show that the Bis-Tris SDS-PAGE system separates HMW-GS according to their amino acid chain length. This is consistent with the overall aim of SDS-PAGE and avoids the anomalous mobilities that occur when using the widespread Tris-glycine system. The good separation and resolution on the Bis-Tris system had yet been reported for Bis-Tris gradient gels and was now confirmed for the 10% homogeneous gels. However, attention should be paid to the discrepancy between the electrophoretic mobilities of HMW-GS when using the Bis-Tris system and their original nomenclature. The use of different conditions may not only alter resolution or mobility of gluten proteins, but also the protein migration order, especially when different operating pH values are used. The present results, together with the increasing knowledge of the primary structure of different HMW-GS, which show strong sequence similarities, stress the need for an upgrade of their naming system, which could be based entirely on sequence similarities rather than electrophoretic mobilities.

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Abbreviations used

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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Figure captions

Figure 1: Bis-Tris SDS-PAGE of protein extracts from flours of the wheat cultivars (cvs.) Cubus, Enorm, Akteur, Apache, Magnus, Contra and Atlantis. The first lane shows the molecular weight markers.

Figure 2 Bis-Tris SDS-PAGE of the HMW-GS of single wheat cvs. and their individual purified subunits. A. cv. Akteur, B. cv. Contra, C. cv. Apache. The first lane shows the molecular weight markers.

Figure 3: . Bis-Tris SDS-PAGE of the subunits with anomalous mobilities in the Laemmli system. A. HMW-GS Dx5 and Dx2. B. HMW-GS Dx2 and Ax2*. C. HMW-GS Dy12 and Dy10. The first lane shows the molecular weight markers.

501 **Figure 3**

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503

504 **Table 1.** Overview of the unique N-terminal sequences that allowed discriminating purified
505 HMW-GS in the wheat cultivars Akteur, Apache and Contra.

Wheat cultivar	Subunit	<i>N-terminal sequence</i> ¹ (12 amino acid residues)
Akteur	Ax1	EGEAS G QLQCER
	Dx5	EGEASEQLQCER
	Bx7	EGEAS G QLQCE H
Contra	Dx2	EGEASEQLQCER
	Bx6	EGEAS G QLQCER
Apache	Ax2*	EGEAS G QLQCER
	Dx3	EGEASEQLQCER
	Bx7	EGEAS G QLQCE H

506 ¹One-letter code of amino acids

Table 2 Overview of the migration order of HMW-GS in Bis-Tris SDS-PAGE and their sequence identification with LC-MS/MS after chymotryptic digestion.

HMW-GS	GI ¹	Amino acid residues	Calculated MW	Score ²	Sequence coverage MS/MS (%)	Matched peptides ³
Dx5	288860106	827	88,126	278	64.8	14
Dx2	736319	817	87,007	301	72.5	16
Dx3	-	815 ⁴	86,666 ⁴	-	70.3	12
Ax1	21743	809	87,678	-	78.4	16
Bx6	37575359	803 ⁵	86,393 ⁵	-	68.4	11
Ax2*	170743	794	86,335	-	70.3	5
Bx7	21749	769	82,526	-	62.1	9
By8	359500291	699	75,131	-	66.0	11
By9	22090	684	73,517	-	70.8	14
Dy12	121452	639	68,713	190	68.4	11
Dy10	21751	627	67,474	192	63.8	16

¹Protein accession number in the NCBI database

²Mascot score for protein recognition in the SwissProt database

³Number of peptides identified with a score > 66

⁴Based on MW similarities between 1Dx3 and 1Dx4^t as demonstrated by Gao et al. (2010)

⁵Based on a Bx protein sequence from *Triticum turgidum* (Kong et al., 2004)

515 **Table 3** Counts of acidic and basic amino acids in HMW-GS that showed anomalous
 516 mobilities in Tris-glycine SDS-PAGE.

	Ax1	Ax2*	Dx2	Dx5	Dy10	Dy12
Asp + Glu	30	30	20	19	21	21
Arg + His + Lys	28	29	19	20	33	33

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