

# Rapid Identification of HMW Glutenin Subunits from Different Hexaploid Wheat Species by Acidic Capillary Electrophoresis

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## ABSTRACT

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High molecular weight glutenin subunits (HMW-GS) from three hexaploid wheat species (AABBDD,  $2n=6x=42$ , *Triticum aestivum* L., *T. spelta* L., and *T. compactum* L.) were separated and identified by acidic capillary electrophoresis (A-CE) with phosphate-glycine buffer (pH 2.5) in uncoated fused-silica capillaries (50  $\mu\text{m}$ , i.d.  $\times$  25.5 cm) at 12.5 kV and 40°C. The rapid separations (<15 min) of HMW-GS with good repeatability (RSD < 2%) were obtained using a fast capillary rising protocol. All 17 HMW-GS analyzed could be well separated and their relative migration orders were ranked. In particular, the good quality subunit pair

5+10 could be differentiated from poor quality subunit pair 2+12. In addition, the other three allelic pairs of 13+16, 17+18, and 7+8 subunits that were considered to have positive effects on dough properties, as well as three pairs of novel subunits 13+22\*, 13\*+19\*, and 6.1+22.1 detected from spelt and club wheat, can also be readily separated and identified. An additional protein subunit presented in Chinese bread wheat cultivar Jing 411 and club wheat TRI 4445/75, respectively, was detected by both A-CE and 2-D gel electrophoresis (A-PAGE  $\times$  SDS-PAGE), for which further identification is needed.

The main storage proteins in the endosperm of wheat (*Triticum aestivum* L.) include high molecular weight glutenin subunits (HMW-GS, 80–140 kDa), low molecular weight glutenin subunits (LMW-GS, 30–50 kDa), and gliadins (30–80 kDa). Although HMW-GS represent  $\approx$ 10% of the total seed storage proteins, they play an important role in breadmaking quality (Shewry et al 1992). Through intermolecular disulfide bonds, glutenins as well as some gliadins polymerize during dough formation to develop the functional gluten (Wrigley 1996). The genes coding for HMW-GS, located on the long arms of chromosomes 1A, 1B, and 1D were three complex loci *Glu-1A*, *Glu-1B*, and *Glu-1D* (Payne 1987). By far, more than 30 allelic forms of these subunits have been detected in bread wheat cultivars. Among these subunits, it is well documented that the allelic pair of 1Dx5+1Dy10, as well as 1Bx17+1By18, 1Bx13+1By16, and 1Bx7+1By8, have positive effects on dough properties, while 1Dx2+1Dy12 has negative effects on dough quality (Rodriguez-Quijano et al 1990; Gianibelli et al 2001).

It is highly important to develop powerful techniques for fast differentiation between good quality and poor quality HMW-GS in wheat end use, germplasm screening, and quality improvement. SDS-PAGE has been the most popular method for detecting the allelic compositions of wheat cultivars and for identifying desirable HMW-GS in quality improvement programs based on relative mobility (Payne et al 1979). In addition, reversed-phase HPLC was also used for HMW-GS analysis (Bietz 1983; Courcoux et al 1992). Because some HMW-GS showed very similar mobilities, it is difficult to correctly identify these subunits by routine SDS-PAGE. Furthermore, gel electrophoresis is relatively slow and labor-intensive, and the resolution and reproducibility of SDS-PAGE are generally not satisfactory. Also, the quantitation of traditional gel electrophoresis is especially difficult (Bean et al 1998). Although HPLC overcame many of these difficulties, separation for some HMW-GS is still limited. For example, subunits 10, 12, 2, and 5 cannot be separated by RP-HPLC (Sutton and Bietz 1997).

Capillary electrophoresis (CE) is a powerful tool for storage protein analysis (Bean and Lookhart 2001). It provides very rapid, high-resolution, automated separations of cereal proteins (Lookhart and Bean 1995, 1996, 2000; Yan et al 1997, 1999a, 2003a,b; Yan and Liu 1999; Bean et al 2000). The narrow internal diameter capillaries are intrinsically anticonvective, thus avoiding the use of gels to obtain high-resolution separations (Werner et al 1994). Until now, some investigations on the separation and characterization of HMW-GS by CE have been conducted (Werner et al 1994; Bean and Lookhart 1997, 1998; Sutton and Bietz 1997; Zhu and Khan 2001). However, most of these studies focused on SDS-CE with commercial basic buffers such as the SDS-protein analysis kit produced by the Applied Biosystems Division of Perkin-Elmer (Werner et al 1994; Weegels et al 1995; Sutton and Bietz 1997) and other buffers with different polymers (Bean and Lookhart 1999; Zhu and Khan 2001). In general, the separation mechanism of SDS-CE is similar to traditional SDS-PAGE and, in some cases, subunits still cannot be well separated (Zhu and Khan 2001).

By far, investigations on HMW-GS separation and identification by CE with acid buffers are still very limited. Recently, we focused on acidic capillary electrophoresis (A-CE) of HMW-GS from bread wheat and related species, and it appears to be a powerful tool for HMW-GS analyses (Yan et al 2003a–c). In this work, we further use the low-conductive phosphate-glycine buffer with acetonitrile (ACN) additive to rapidly separate and identify some good quality subunits, especially for differentiating allelic subunit pairs of 1Dx5+1Dy10 and 1Dx2+1Dy12 as well as 1Ax1, 1Bx13+1By16, 1Bx17+1By18, and some novel subunits (1Bx13+1By22\*, 1Bx13\*+1By19\*, etc.) detected in European spelt wheat and club wheat with the acidic CE method.

## MATERIALS AND METHODS

### Wheat Samples

The materials studied included three hexaploid wheat species ( $2n=6x=42$ , AABBDD): bread wheats (*T. aestivum* L.) Hope (USA), Karl (USA), Jing 411 (China), and Kontrast (Germany); spelt wheats (*T. spelta* L.) Baulander Spelz (Germany), TRI 5648/92 (Germany), Rouguin (Belgium), and Spelt 1086 (Spain); and club wheats (*Triticum compactum* L.) PI330540 (UK), TRI 4445/75 (Germany), and Club 20 (Germany).

### Extraction of HMW-GS

The HMW-GS extraction for SDS-PAGE, A-PAGE, and 2-D gel electrophoresis was based on the methods of Singh et al

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(1991) and Morel (1994). The sample preparation for CE analysis was mainly based on the method of Bean and Lookhart (1998). The half kernel (part of the nonembryo) was weighed and crushed into flour. The monomeric gliadins were first removed by continuous 4× extractions with 50% 1-propanol for 30 min at 65°C, and then glutenins were reduced and extracted with 0.25 mL of 50% (v/v) propanol + 1% (m/v) DTT for 30 min at 65°C with regular stirring. After centrifuging for 10 min at 13,000 × *g*, HMW-GS were precipitated from glutenin extracts with acetone added to a final concentration of 40% (v/v). Precipitated HMW-GS were redissolved in 0.2 mL of 25% (v/v) ACN + 0.1% (v/v) trifluoroacetic acid (w/v) and centrifuged for 10 min at 13,000 × *g*. All samples were used for CE analysis within 24 hr of extraction.

### 1-D and 2-D PAGE

One-dimensional SDS-PAGE of HMW-GS was conducted on a Hoeffer vertical electrophoresis unit according to Jackson et al (1996). A-PAGE of HMW-GS was performed according to Yan et al (1999b) using the same electrophoretic apparatus described above. Two-dimensional A-PAGE × SDS-PAGE was conducted according to Yan et al (2003c) with some modifications. The 2-D electrophoresis was performed in the same apparatus as above. After the 1-D A-PAGE, the gels were cut into single strips and incubated for 30 min at room temperature in the equilibration solution containing 10% (v/v) glycerol, 2% (m/v) SDS, and 0.0625M Tris-HCl at pH 6.8. The equilibrated gel strips were placed on top of the 2-D SDS-PAGE gel (12.5%, m/v) prepared as described above. Electrophoresis was performed at 14 mA for 1 hr followed by 25 mA for 15 hr at a constant temperature of 10°C, and then gels were stained as described above.

### Acidic Capillary Electrophoresis (A-CE)

A BioFocus 3000 instrument was used for capillary electrophoretic separations of the HMW-GS according to Yan et al

(2003b) with minor modifications. A-CE buffer according to Bean and Lookhart (1998) consisted of 0.1M phosphate-glycine (pH 2.5), containing 20% (v/v) acetonitrile (ACN) and 0.05% (m/v) hydroxypropylmethylcellulose (HPMC). Uncoated fused silica capillaries (25.5 cm long [20 cm to detector] and 50 μm, i.d.) were used. CE was performed at 12.5 kV and 40°C. All samples were injected at 10 kV for 8 sec and analyzed within 24 hr. HMW-GS were detected by UV absorbance at 200 nm.

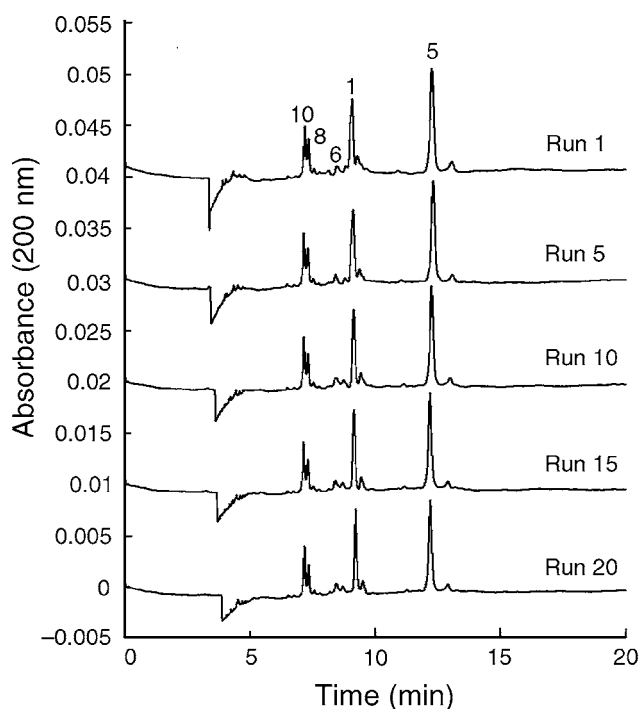
### HMW-GS Identification and Nomenclature

The nomenclature of HMW-GS was based on the classification of Payne and Lawrence (1983). HMW-GS identification by A-CE was achieved by comparing migration times and peak heights of HMW-GS from single and mixed samples according to cultivar standards and the results obtained from 1-D and 2-D gel electrophoresis as well as the previous reports (Bean and Lookhart 1997, 1998; Yan et al 2003b,c). The novel subunits identified in this study were designated as in previous studies (Yan et al 2003c).

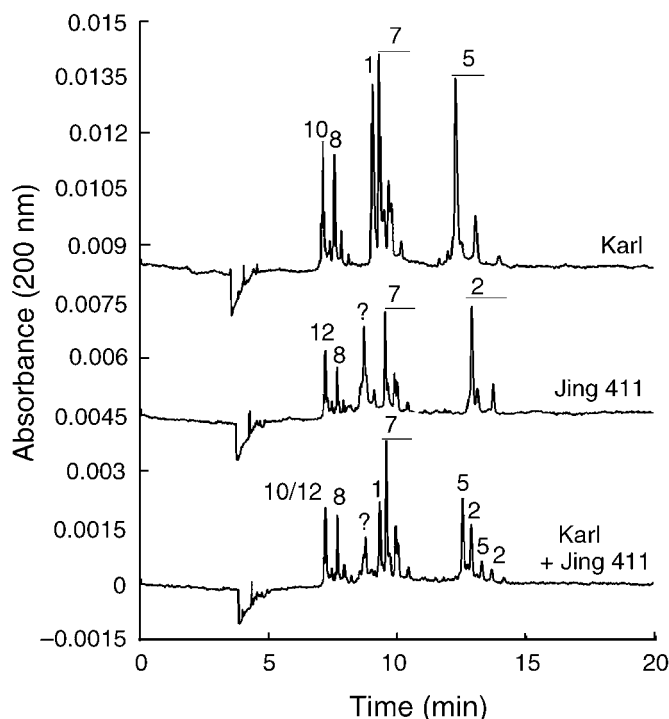
## RESULTS AND DISCUSSION

### Gel Electrophoresis Analysis of HMW-GS

HMW-GS compositions of all wheat cultivars studied were separated and identified by three gel electrophoretic methods (SDS-PAGE, A-PAGE, and 2-D PAGE). We confirmed that 11 hexaploid wheat cultivars possessed the following HMW-GS compositions: Hope (1, 6+8, 5+10), Karl (1, 7+8, 5+10), Jing 411 (N, 7+8, 2+12), Kontrast (N, 17+18, 5+10), Bauland Spelz (1, 13+16, 2+12), TRI 5648/92 (1, 7, 2+12), Rouguin (N, 6.1+22.1, 5+10), Spelt 1086 (1, 13+22\*, 2+12), PI330540 (N, 17+18, 2+12), TRI 4445/75 (N, 13\*+19\*, 2+12), and Club 20 (N, 17+18, 2+12). Several novel allelic pairs of HMW subunits at *Glu-B1* locus in spelt and club wheat were detected that were not found in bread wheat in the previous studies. The electrophoretic mobility



**Fig. 1.** Repeatability of 20 consecutive separations of HMW-GS from wheat cultivar Hope. The 1st, 5th, 10th, 15th, and 20th runs and HMW-GS (1, 6+8, 5+10) are indicated. Acidic CE separation buffer was 0.1M phosphate-glycine (pH 2.5) containing 20% ACN and 0.05% HPMC. CE conducted at 12.5kV and 40°C with uncoated fused-silica capillaries (25 μm, i.d. × 25.5 cm).



**Fig. 2.** Acidic CE separation of HMW-GS from bread wheat cultivars Karl (1, 7+8, 5+10) and Jing 411 (N, 7+8, 2+12) and mixed samples (1:1, v/v). HMW-GS and an additional protein peak (question mark) in Jing 411 are indicated. Subunits 10 and 12 had the same migration time, so they cannot be distinguished. A-CE separation conditions as described in Fig. 1.

of subunit 1Bx6.1 is a little faster than that of subunit 1Bx6, while subunit 1By22.1 is between 1By8 and 1By18. Subunit 1By22\* moves faster than subunit 1By22.1 and is located between 1By22.1 and 1By8. Club wheat TRI4445/75 contained another pair of new subunits at *Glu-B1* locus (1Bx13\*+1By19\*) that moved slower and faster, respectively, than 1Bx13. According to our recent survey (Yan et al 2003c), the central European spelt wheat cultivars contained 1Bx6.1+1By22.1, 1Bx13+1By22\*, and 1Bx13\*+1By19\* subunits with frequencies of 32.34, 5.11, and 2.98%, respectively. These subunits were also present in cultivated emmer wheat (*T. dicoccum* Schrank), so we speculated that club wheat and cultivated emmer were the progenitors of central European spelt wheat. These novel protein subunits may have important roles in breadmaking quality, therefore they are expected to widen the genetic base of bread wheat in quality improvement.

#### A-CE Separation of HMW-GS and Run-to-Run Repeatability

According to our recent study, HMW-GS can be well separated by the acidic buffer and the CE conditions as described above. In general, one sample separation can be finished in <15 min. At the same time, the resolution of CE separation of HMW-GS was high. In the present work, all protein subunits analyzed appeared to be well separated and characterized. Results showed that good repeatability of CE separation of HMW-GS can be obtained when the following capillary rinsing protocol was used: new capillaries were rinsed with 0.1M phosphate buffer (pH 2.5) for 10 min, 1M phosphoric acid for 10 min, and then separation buffer (0.1M phosphate-glycine buffer [pH 2.5] containing 20% ACN and 0.05% HPMC) for 30 min. After each separation, capillaries were rinsed with 1M phosphoric acid for 2 min, and then with separation buffer for 2 min.

Five electrophoregrams including runs 1, 5, 10, 15, and 20 of a series of 20 CE separations of HMW-GS from cultivar Hope are shown in Fig. 1. The HMW-GS 1, 6+8, and 5+10 can be readily identified when compared with SDS-PAGE patterns and our previous results (Yan et al 2003b). It is obvious that the repeatability of migration times as well as peak height was good, and the relative standard deviation (RSD) of five HMW-GS was <2%. According to our results, if the fresh running buffer was used at each run, the repeatability would be higher than consecutive injections of the same running buffer, normally RSD < 1%. It could be concluded that the capillary rinsing protocol used is suitable for A-CE separation of HMW-GS. In general, this method can remove peak tailing and rising baselines during consecutive analyses of wheat samples. Previous studies have also confirmed that when capillaries were rinsed with harsh chemicals (such as 1M phosphoric acid), there was no protein binding to capillary walls (Bean and Lookhart 1998; Yan et al 2003b).

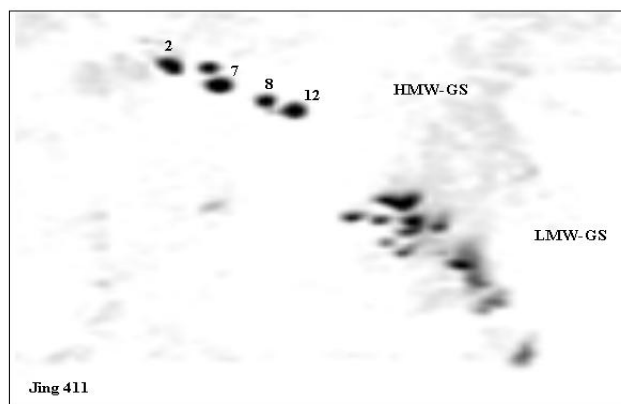
We also determined the day-to-day and capillary-to-capillary reproducibility. In general, run-to-run reproducibility was not effective (generally RSD >3%). Because the samples prepared should be analyzed within 24 hr, we focused on investigating the run-to-run reproducibility. To positively identify HMW-GS, we needed to use a set of standard cultivars with known HMW-GS.

#### Characterization of HMW-GS in Different Hexaploid Wheats

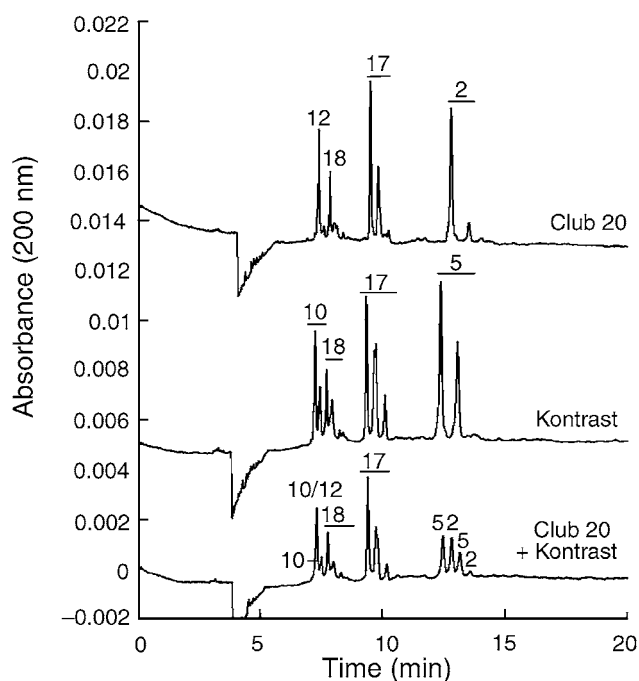
In this work, we focused on differentiating two important allelic pairs, 1Dx5+1Dy10 and 1Dx2+1Dy12, as well as some other HMW subunits with high quality scores and several pairs of novel subunits detected from spelt and club wheat. To distinguish different HMW-GS, single and mixed (1:1, v/v) samples were used for A-CE analysis. Figure 2 shows the A-CE patterns of HMW-GS from bread wheat cultivars Karl (1, 7+8, 5+10) and Jing 411 (N, 7+8, 2+12) and mixed samples. It is easy to see that all HMW subunits presented in Karl and Jing 411 can be well separated. The mixed samples of different single subunits 1, 5, and 2 presented in Karl and Jing 411, respectively, displayed about half peak height when compared with single samples. Because

both cultivars possess subunits 7+8, they showed the mean peak heights of two single samples in the mixed samples. Apparently, subunit 5 could be readily differentiated from subunit 12; however, subunits 10 and 12 cannot be separated in the mixed samples because of their similar migration time. Both subunits showed the same single peak with the mean peak height of two single samples.

As previously reported (Yan et al 2003b), Jing 411 displayed an apparent additional peak at ≈9 min in single sample. Furthermore, the mixed samples of Jing 411 and Karl also showed this peak. However, no additional bands were found with SDS-PAGE (results not shown). To confirm the presence of this novel subunit, Jing 411 was further separated with 2-D gel electrophoresis (A-PAGE × SDS-PAGE) (Fig. 3). The single subunit 7 on 1-D SDS-PAGE was separated into two apparent protein components. The biochemical and genetic characteristics of this novel subunit are not known. It is possible that this protein subunit is encoded by unknown loci other than *Glu-B1*, but other possible causes may exist. Further identification is underway in our laboratory.

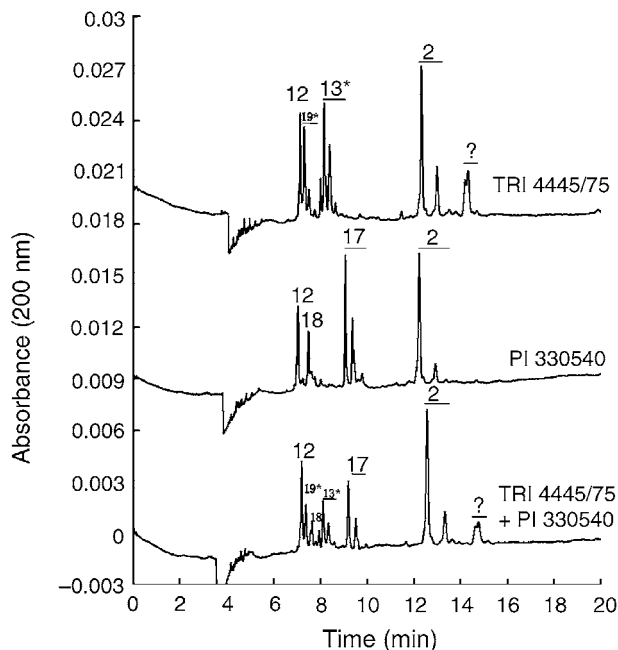


**Fig. 3.** 2-D Gel electrophoresis (A-PAGE × SDS-PAGE) pattern of HMW-GS in bread wheat cultivar Jing 411. Subunit 7 separated into two apparent protein components.

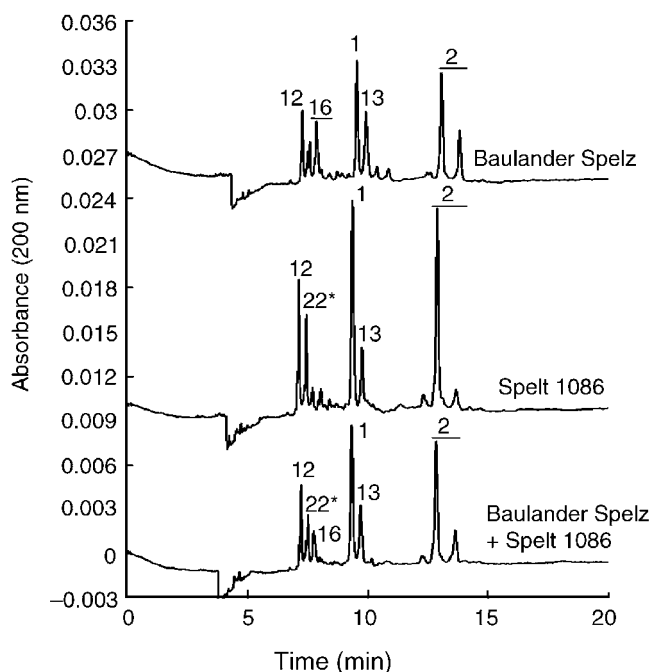


**Fig. 4.** Separation of HMW-GS from club wheat Club 20 (N, 17+18, 2+12) and bread wheat cultivar Kontrast (N, 17+18, 5+10) and mixed samples (1:1, v/v). Different peaks of HMW-GS and minor peak of subunit 10 are indicated. A-CE separation conditions as described in Fig. 1.

As shown in Fig. 2, as well as all other figures of this work, HMW-GS were generally separated into two to three multiple peaks. In most cases, they exhibited a high peak and a minor peak, although all HMW-GS analyzed were separated only into a single band by SDS-PAGE. According to previous surveys (Bean and Lookhart 1997, 1998; Yan et al 2003a-c), when low pH phosphate buffer was used, HMW-GS normally displayed isoforms on CE patterns. Similar results were also obtained by 2-D RP-HPLC



**Fig. 5.** Separation of HMW-GS from club wheats TRI 4445/75 (N, 13\*+19\*, 2+12) and PI 330540 (N, 17+18, 2+12) and mixed samples (1:1, v/v). Allelic pairs 17+18 and 13\*+19\* as well as subunits 2+12 were readily differentiated. An additional minor peak of TRI4445/75 is indicated with question mark. A-CE separation conditions as described in Fig. 1.

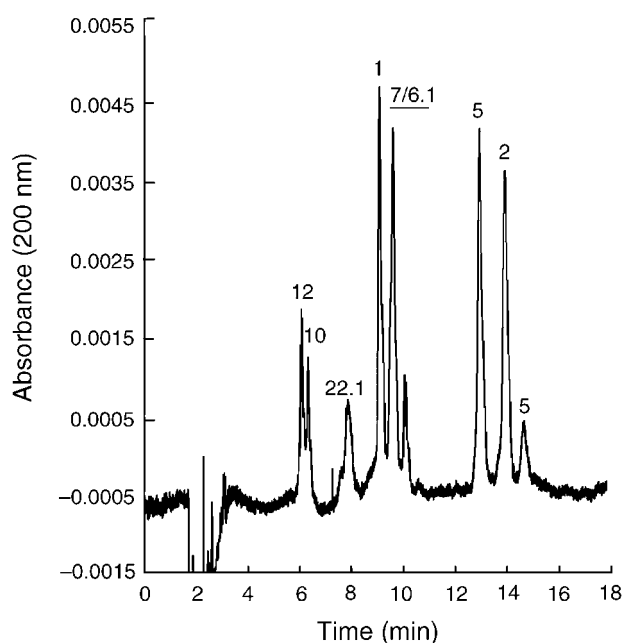


**Fig. 6.** Separation of HMW-GS from spelt wheats Baulander Spelz (1, 13+16, 2+12) and Spelt 1086 (1, 13+22\*, 2+12) and mixed samples (1:1, v/v). Allelic pairs 13+16 and 13+22\* as well as subunits 1, 2+12 were readily differentiated. A-CE separation conditions as described in Fig. 1.

plus FZCE (Bean and Lookhart 1997). In these studies, the contamination by additional proteins (LMW-GS and gliadins) was also excluded. According to our studies, only a few HMW-GS showed big and small components on A-PAGE and 2-D patterns (results not shown). Thus, it is most likely that these multiple peaks result from different types of posttranslational modifications (Bean and Lookhart 1997; Yan et al 2003b).

The HMW-GS patterns of club wheat Club 20 (N, 17+18, 2+12) and bread wheat Kontrast (N, 17+18, 5+10) and mixed samples are shown in Fig. 4. It is obvious that subunits 5 and 2 showed the same patterns as those in Fig. 2. Both of them contained a high peak with a minor peak that can be readily differentiated. The main protein components of subunits 10 and 12 still had the same migration times, so they overlapped. However, they can be distinguished by the minor peak height of subunit 10 (Fig. 4). Subunits 17 and 18 both contained a main peak and two minor peaks, but the peak height of subunit 18 was lower than that of subunit 17 because the former had low protein (identified by SDS-PAGE). Club wheat TRI 4445/75 contained a pair of novel subunits 13\*+19\* that could be differentiated from subunits 17+18 (Fig. 5). Subunit 19\* was slightly slower than subunit 12, while subunit 13\* eluted earlier than subunit 18. The subunits 2+12 and 17+18 showed the same CE patterns in migration time, peak height, and peak number as described above. In addition, an unknown component located at  $\approx 14.8$  min was present in TRI 4445/75 was found in both single and mixed samples (shown as a question mark). Two-dimensional electrophoresis also showed an additional protein spot (results not shown). These minor protein components require further identification.

The subunit pair 1Bx13+1By16 with good quality characteristics is frequently present in European spelt wheat (Rodriguez-Quijano et al 1990). Recently, we found another allelic pair of 1Bx13+1By22\* subunits present in both spelt and cultivated emmer wheat (Yan et al 2003c). The CE patterns of these two pairs of HMW-GS are shown in Fig. 6. Subunit 22\* eluted earlier than subunit 16, while subunit 13 eluted later than subunit 1. The remaining subunits 1 and 2+12 showed the same CE patterns as those of other cultivars described above. The mixed samples of



**Fig. 7.** Separation of HMW-GS from mixed samples (1:1, v/v) of spelt wheat cultivars TRI 5648/91 (N, 7, 2+12) and Rougiun (1, 6.1+22.1, 5+10). Allelic pairs 5+10 and 2+12 as well as subunits 1 and 22.1 were well separated and differentiated. Subunits 7 and 6.1 with the same migration times cannot be separated. A-CE separation conditions as in Fig. 1.

spelt wheat cultivars TRI 5648/91 (N, 7, 2+12) and Rougiun (1, 6.1+22.1, 5+10) are shown in Fig. 7. It is evident that subunits 2 and 5, as well as subunits 10 and 12, can be well separated and differentiated. According to our investigations of 15 mixed samples including subunits 5+10 and 2+12, the 10 and 12 subunits in only two samples could not be differentiated using the acidic buffer system. Therefore, they could be identified in the mixed samples, providing the strict rinsing protocol used in this study is performed between runs. However, subunits 1Bx7 and 1Bx 6.1 showed the same migration times, so they cannot be distinguished in the mixed samples. Subunit 1By22.1 located between subunits 1Dy10 and 1Ax1 were readily identified (Fig. 7).

Although some HMW-GS had close migration times, the primary relative migration orders of the HMW-GS were 12/10→10→19\*→22\*→8→18→16→13\*→22.1→6→1→7/6.1→17→13→5→2 based on migration times (from low to high). It is apparent that the y-type subunit in any allelic pair eluted earlier than the x-type subunit and showed lower peak height because of its relatively low protein. This is in accordance with SDS-PAGE patterns. However, some HMW-GS separated by A-CE and SDS-PAGE exhibited different migration orders, especially for subunit 1Ax1 that moved the most slowly among all subunits analyzed by SDS-PAGE. But subunit 1Ax1 migrated earlier than subunits 2, 5, 13, 7, and 6.1. Subunits 10 and 12 were not well separated when they were mixed. However, they can be distinguished by the minor peak in some mixed samples, suggesting that the HMW-GS content may affect A-CE patterns. Therefore, the same subunits in different cultivars may display various peak heights on CE patterns. Though the migration times of some HMW-GS are so close (10 and 12, 7 and 6.1, etc.), it is still possible to better separate and differentiate these subunits by improving buffer compositions, electrophoresis parameters, and rinsing protocols. Thus, A-CE is expected to become a powerful, more widely applied tool in the separation and identification of HMW-GS. Further investigations are underway in our programs.

## CONCLUSIONS

A-CE is capable of separating wheat HMW-GS with high efficiency, as well as simplicity, speed, high-resolution, good repeatability, and small sample requirements. These features are well suited for storage protein analysis, especially for rapid identification of the desirable glutenin subunits in both end use products and early hybrid generations of wheat quality improvement. In this work, 17 HMW-GS from bread wheat, spelt wheat, and club wheat were separated and identified by A-CE (0.1M phosphate-glycine buffer [pH 2.5] containing 20% ACN and 0.05% HPMC). Several important allelic pairs of HMW-GS can be rapidly separated and identified on the basis of their relative migration times and peak heights. In particular, subunits 5+10 with good breadmaking quality characteristics could be differentiated from the 2+12 subunits with poor breadmaking quality characteristics. Several pairs of other good quality subunits encoded by *Glu-B1* locus, such as 7+8, 17+18, and 13+16, as well as the novel subunit pairs 13\*+19\*, 13+22\*, and 6.1+22.1 from spelt and club wheat can also be readily distinguished. Thus, A-CE could provide wheat breeders with an additional approach to rapidly separate and identify HMW-GS composition in quality improvement programs.

## ACKNOWLEDGMENTS

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