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High throughput microchip-based separation and quantitation of high-molecular-weight glutenin subunits

L. Rhazi [a,*], A.-L. Bodard [a], B. Fathollahi [b], T. Aussenac [a]

[a] Institut Polytechnique LaSalle Beauvais, 19 rue Pierre Waguet, 60026 Beauvais, France
[b] Caliper LifeSciences, 605 Fairchild Drive, Mountain View, 94043-2234 CA, USA

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ABSTRACT

Knowledge of glutenin subunit composition is important for the prediction of the genetic potential of breeding lines as these proteins are known to be responsible for the main differences in bread-making quality. In this study, a commercial high throughput microchip capillary electrophoresis-sodium dodecyl sulfate (microchip CE) platform, LabChip 90, was evaluated for qualitative and quantitative analyses of HMW-GS. 130 French common wheat varieties of known composition were analyzed for rapid identification and the allocation of individual HMW-GS. In addition, the HMW-GS were individually quantified and the ratio of HMW-GS to LMW-GS was determined for genotype comparison. The microchip CE analysis provides comparable resolution and sensitivity to conventional RP-HPLC for identification of the HMW-GS but at a time scale of approximately 100 times faster (45 s per sample analysis versus 80 min for RP-HPLC). The results show that the high throughput microchip CE method can be used for routine identification and quantitation of glutenin subunits, in particular for screening wheat quality and wheat cultivar development activities where large numbers of samples are to be evaluated.

1. Introduction

Wheat endosperm proteins are divided into two main fractions, monomeric and polymeric proteins. The monomeric fraction includes gliadins, albumins, and globulins. Albumins and globulins are considered functional proteins, whereas gliadins are referred to as storage proteins. Polymeric fraction mainly consists of glutenin polymers composed of high-molecular-weight glutenin subunits (HMW-GS) and low-molecular-weight glutenin subunits (LMW-GS) associated together by intermolecular disulfide bonds.

The high-molecular-weight glutenin subunits (HMW-GS) are the most intensively studied protein fraction because their viscoelastic properties enable wheat dough to be made into bread, pasta, biscuit and other food products (Payne, 1987; Shewry et al., 1992). The HMW-GS are encoded by genes at *Glu-1* loci on the long arm of the homoeologous group one chromosomes of the A, B and D-genomes (Payne et al., 1981; Payne, 1987). Each homeologous contains two closely linked genes encoding subunits of lower and higher molecular weight called *x*-type and *y*-type respectively (Forde et al., 1985; Harberb et al., 1986; Payne et al., 1981; Payne and Lawrence, 1983). Hexaploid bread wheat consists of six HMW-GS genes. However, cultivars contain 3–5 HMW-GS: zero or one encoded by *Glu-A1*, one or two by *Glu-B1* and two by *Glu-D1* (Lawrence and Payne, 1983). The variation in the number of expressed subunits is due to gene silencing. Considerable investigations have shown that allelic variation is closely related to bread-making quality of wheat flour (Shewry et al., 1992). However, the subunits of 1Ax1, 1Ay2*, 1Bx17 + 1By18 and 1Dx5 + 1Dy10 are associated with stronger dough and thus improve bread-making quality. The subunit pair 1Dx2 + 1Dy12 is associated with weaker dough (Gupta et al., 1996; Payne et al., 1979; Radovanovie et al., 2002) that results in poor dough quality.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) has been widely used for HMW-GS polymorphism investigation (Briggle and Curtis, 1987; Gupta and Shepherd, 1990; Gupta et al., 1991). SDS-PAGE requires multiple manual steps some of which could be hazardous and not practical for high throughput screening. In addition, achieving sufficient resolution, reproducibility, and quantitation by SDS-PAGE is difficult. SDS-PAGE has been supplanted by capillary electrophoresis (CE) separations using SDS and soluble linear polymer (Bean and Lookhart, 1998, 1999; Sutton and Bietz, 1997; Werner et al., 1994; Zhu and Khan, 2001). CE separates the SDS coated HMW-GS based on size differences similar to SDS-PAGE and the proteins are detected by UV

Abbreviations: CE, capillary electrophoresis; CV, coefficient of variation; DTT, dithiothreitol; HMW-GS, high-molecular-weight glutenin subunits; LMW-GS, low-molecular-weight glutenin subunits; pDMA, polydimethyl methacrylate; RP-HPLC, reversed-phase high-performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

* Corresponding author. Tel.: +33 3 44 06 75 52; fax: +33 3 44 25 26.
E-mail address: larbi.rhazi@lasalle-beauvais.fr (L. Rhazi).

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absorbance which permits direct quantitation. Others (Huebner and Bietz, 1985; Sutton, 1991) have shown that reversed-phase high-performance liquid chromatography (RP-HPLC) is a useful analytical method for showing variability in pyridylethylated HMW-GS and determining the expression levels of HMW-GS (Marchylo et al., 1989). Naeem and Sapirstein (2007) recently used a new superficially porous silica-based column to develop a simple and fast (≈13 min) quantitative analysis by RP-HPLC.

Separations of HMW-GS by CE-SDS and RP-HPLC typically require 30 and 80 min per sample, respectively. The CE method often requires additional time between injections for rinsing and reloading of the polymer solution. The number of samples that can be analyzed per instrument in a 24-h day by these methods is less than 30. A cost effective high throughput analytical method is required in order to meet the demands of increasing number of samples that need to be analyzed at early stages of breeding.

Lab-on-a-chip technology, which emerged in the early 1990's (Harrison et al., 1993; Jacobson et al., 1994; Manz et al., 1992; Woolley and Mathies, 1994) has demonstrated great potential to increase throughput for CE-based separations by reducing the analysis time from minutes to seconds. Rapid separation in microchip devices is achieved from the ability to inject a very small sample plug in the order of 10–100 μm in length. The small plug size is created by controlling the voltages and/or currents at four terminal wells of a cross-section of microfluidic channels. Recently, rapid separation and quantitation of glutenin subunits have been achieved using a planar microfluidic chip-based electrophoresis. Uthayakumaran et al. (2005, 2006) used a commercial microchip CE (Agilent 2100; Agilent Technologies) platform to rapidly separate and quantitate the HMW-GS. However, the planar chips used in these studies are limited to 10 samples per chip where each sample is added to individual chip wells. The time required to analyze 10 samples is 30 min. As stated by the authors, the platform is not suitable for high throughput analysis (>150 samples per 8-h day) not only because of the limited number of samples per chip but the requirement of manual sample pipetting for each chip preparation. With increasing number of breeding lines comes greater demand for a high throughput platform that rapidly and continuously analyzes large number of samples (>400 samples per 8-h day).

In this paper, we report on the use of a high throughput microchip CE platform for analysis of HMW-GS in a large collection of French common wheat varieties. The accuracy in identification, quantitation of HMW-GS, as well as relative quantitation of HMW-GS to LMW-GS ratio, and the overall reproducibility of the platform were investigated.

2. Experimental

2.1. Wheat samples

130 French common wheat cultivars representing a wide range of allelic combinations, were used for this investigation. These cultivars were grown under conventional conditions (with fertilization and full fungicide protection) in 2004 at INRA (Station d'amélioration des plantes, Clermont-Ferrand). At maturity (53 DAA), grains were collected and ground in a Brabender Senior laboratory mill.

2.2. Glutenin subunits extraction for the microchip analysis

The purification and extraction of glutenin follows the protocol of Fu and Sapirstein (1996) with some modifications. Flour samples (30 mg) were stirred for 15 min at room temperature with 1 ml of 0.08 M Tris–HCl buffer (pH 7.5) containing 50% (v/v) propan-1-ol. Extraction was followed by centrifugation at 15,900 *g* for 10 min at 15 °C. The supernatant containing monomeric proteins (albumins, globulins and gliadins) was discarded. 600 μl of the Tris–HCl containing 2% (w/v) of SDS and 1% (w/v) of DTT was added to the pellet containing mainly polymeric glutenins and dispersed by sonication with amplitude of 30% for 15 s, which was performed using a stepped microtip probe of 3 mm diameter (Ultrasonic Processor, Sonics, model 75038). The mixture was maintained at a constant temperature of 60 °C for 30 min and then centrifuged at 12,500 *g* for 10 min at 20 °C.

Some optimization of the sample denaturing condition was required in order to obtain consistent and reliable separation and detection of the HMW-GS on LabChip 90. 5 μl of the supernatants was mixed with 4 μl of manufacturer's sample buffer containing 1 M DTT. The mixture was heat denatured at 95 °C for 5 min and afterwards 36 μl of ultra high quality water were added to each sample well. The final percentage of SDS in the sieving matrix was also increased from 0.2% (w/w) to 0.27% (w/w) in order to consistently stain hundreds of samples with single preparation of the microchip. This protocol deviates from the sample preparation protocol given by the manufacturer.

2.3. Glutenin extraction for the RP-HPLC analysis

Procedure of purification and extraction of glutenin used here was achieved according to Fu and Kovacs (1999) with some modifications. 500 mg of flour samples was stirred for 30 min at room temperature with 2 ml of 0.3 M NaI, 7.5% propan-1-ol buffer to separate the albumins, globulins and gliadins from the starch and insoluble glutenin proteins. After centrifugation for 10 min at 15,900 *g* at 15 °C, the glutenins contained within the pellets were extracted with 1 ml of 0.08 M Tris–HCl buffer (pH 7.5) containing 50% (v/v) propan-1-ol and 1% (w/v) DTT. To disintegrate the pellets and improve protein extraction rate, the samples were sonicated (Ultrasonic Processor, Sonics, model 75038), followed by incubation for 30 min at 60 °C. The supernatant obtained after centrifugation at 15,900 *g* for 30 min at 15 °C was filtered through a 0.45 μm nylon filter. An aliquot (600 μl) was then alkylated with 40 μl of 70% (v/v) 4-vinylpyridine in 50% (v/v) propan-1-ol buffered with 80 mM Tris–HCl (pH 7.5) at 60 °C for 20 min just prior to RP-HPLC analysis. Proteins were analyzed using a Surveyor system coupled to a UV detector set at 214 nm (Thermo Electron Corporation, Courtaboeuf, France), and were separated in a Zorbax 300-SB-C8 analytical column, 150 × 4.6 mm, particle size 5 μm (Supelco Inc., Bellefont, PA, USA) preceded by a pre-column with the same characteristics. The column was maintained at 50 °C during the run. Injection volumes were 30 μl in conjunction with a multiple 5 μl injection technique (Marchylo et al., 1989). Separation of glutenin subunits was carried out as described previously by Carceller and Aussenac (2001).

2.4. Separation of glutenin subunits by LabChip 90

High throughput analysis of the glutenin subunits was performed on LabChip 90 platform. Protein sizing and quantitation on a microchip is achieved by integration of the sample loading, staining, separation, destaining, and detection (Bousse et al., 2001; Chow, 2006). A schematic diagram of the microchannel layout of the protein chip is illustrated in Fig. 1. Unlike the planar chip, the denatured protein samples are introduced onto the chip directly from a 96- or 384-well microtiter plate through a sipper by applying vacuum pressure at well 1. Approximately 250 nl of the sample is introduced onto the chip and then mixed with lower marker from well 4 during the short time that the vacuum is applied. Sample and marker mixture are then electrokinetically loaded across the injection cross-section by applying voltage gradient across wells 3 and 6. A small volume of sample, 10–20 pl, is then injected into a separation channel 14 mm in length and 31 μm