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Charge-based characterisation of high-molecular-weight glutenin subunits from common wheat by capillary isoelectric focusing

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ABSTRACT

In this study, the capillary isoelectric focusing (CIEF) method for the separation and charge characterisation of the heterogeneity of high molecular-weight-glutenin subunits (HMW-GS) in common wheat (*Triticum aestivum* L.) using linear polyacrylamide (LPA) and polyvinyl alcohol (PVA) coated capillaries was developed. Particularly good repeatability and well-resolved charge isoform profiles were obtained by introducing a mixture of carrier ampholytes (pH 3–10 and pH 5–8), a high concentration of urea (6 M) and SB3-12 as detergent in a sample solution during separation in a PVA-coated capillary. One major and one or two minor isoforms were observed for the individual HMW-GS. These isoforms were satisfactorily separated using a pH gradient into two groups: y-type isoforms and x-type isoforms encoded by the *Glu-B1* locus with shorter migration times and remaining x-type isoforms with longer times. The method produced from eight to twelve isoforms of wheat HMW-GS with p*I* points in the range of 4.72–6.98. Generally, the minor isoforms were more acidic compared with the major isoform. The y-type subunits had an approximately neutral character (p*I* 6.70–6.98); however, x-types showed a weakly acidic character (p*I* 4.72–5.23), with the exception of subunits encoded by the *Glu-B1* locus. The isoelectric point peak profiles were compared with capillary zone electrophoresis (CZE) electropherograms. Generally, the number of detected isoforms for the particular HMW-GS detected using both methods were similar.

1. Introduction

Bread wheat (*Triticum aestivum* L) possesses unique dough viscoelastic properties that are primarily conferred by two groups of seed storage proteins: glutenins and gliadins. According to their mobility on SDS-gel electrophoresis (SDS-PAGE), glutenins are classified into high-molecular-weight glutenin subunits (HMW-GS) and low-molecular-weight glutenin subunits (LMW-GS) [1–2]. HMW-GS are a major class of proteins that are primarily responsible for a variety of grain and flour characteristics. These subunits are determined by genes at the *Glu-1* loci with multiple alleles localised in the A, B and D wheat genomes on the large arms of the homologous group-one chromosomes (*Glu-A1*, *Glu-B1* and *Glu-D1* loci) [2–3]. Each locus consists of two types of single-copy genes, encoding x-type and y-type subunits, although the *Glu-1 A* y-type gene is usually not expressed. In total, the bread wheat genotypes contain 3–5 HMW-GS, which differ considerably in size (65–90 kDa), charge and structure [4]. Relationships among the presence of specific subunits, their proportions and end-use quality have been described by many researchers [3,5–10]. Qualitative and quantitative changes of particular HMW-GS have been repeatedly determined via one- and two-dimensional polyacrylamide gel electrophoresis (A-PAGE, SDS-PAGE and IEF), reversed-phase (RP) high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE) [3,5–13]. The capillary zone electrophoresis (CZE) study indicated that the previously distinguished HMW glutenin subunits are heterogeneous. In most cases, a major peak and a minor peak for particular HMW-SS were observed [10,13–15]. According to data in the previous literature [13–16], these multiple peaks present various posttranslational modifications of these subunits.

Capillary isoelectric focusing (CIEF) is a special mode of capillary electrophoresis that is used for the separation and charge-based characterisation of amphoteric compounds of protein and monoclonal antibodies isoforms and for the determination of their isoelectric points (p*I*s) [17–22]. With this method, carrier ampholytes are added to the protein sample to create a stable pH gradient inside a capillary. Faster, more reproducible, quantitative results are obtained in the capillary mode, and the amount of ampholytes required for the analysis is less than in the slab gel format. In comparative proteomics, the p*I* of a protein is a key property that often permits protein expression and different post-translational modification states in multiple systems to be distinguished [16]. A stable capillary coating to eliminate or

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suppress EOF and protein adsorption to the inner capillary wall are the two primary obstacles inhibiting complete focusing in the CIEF method. CIEF experiments are most frequently performed in capillaries coated with a neutral and hydrophilic polymer using a dynamic or permanent coating [23,24]. A dynamic coating using additives such as methyl cellulose, hydroxypropylmethylcellulose (HPMC), polyethylene glycol and poly(ethylene oxide) (PEO) [20,25–27] is easy to prepare but lacks coating stability. When applying the above-mentioned capillaries, a deterioration of the charge isoform resolution with multiple types of proteins after a few consecutive injections was reported [19,25–28]. As an alternative, for the modification of the inner capillary wall in CIEF and the suppression of EOF, commercially available permanent coating capillaries (among others) with linear polyacrylamide (LPA), polyvinyl alcohol (PVA), dimethylpolysiloxane (DB-1) and fluorocarbon (FC) [19–20,28–32] have been used.

The aim of this work was to evaluate a two-step CIEF technique for the characterisation of charge heterogeneity of HMW-GS from bread wheat grains. With regard to the specific properties of HMW-GS, which are indissoluble in water and salts and have tendencies towards aggregation and precipitation, separations were performed with different concentrations of urea, ampholytes, detergent (lauryl sulphobetaine) and other IEF-specific optimisations. Major and minor x-type and y-type HMW-GS isoforms were determined first according to the author's knowledge of p*I*. Furthermore, the obtained CIEF profiles of wheat HMW-GS were compared with the CZE electropherograms of these subunits.

2. Experimental

2.1. Reagents and materials

Iminodiacetic acid (IDA), sodium hydroxide, phosphoric acid (85%), HPMC, poly(vinylpyrrolidone) (Mr ~360,000, PVP), acetonitrile (AcN), dithiothreitol (DTT), urea, thiourea and N-dodecyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonate (lauryl sulphobetaine, SB3-12) were purchased from Sigma (St. Louis, MO, US), and arginine, glacial acetic acid, PEO were acquired from Aldrich (Milwaukee, WI, USA). Sodium phosphate monobasic and dibasic and potassium phosphate monobasic were the products of J. T. Baker (Phillipsburg, NJ). All chemicals were of electrophoresis or analytical grade.

The high resolution ampholytes (Pharmalytes pH 3–10 and pH 5–8) were purchased from GE Healthcare (Piscataway, NJ, USA). CIEF gel polymer solution [a mixture of ethylene glycol and PEO in water], "capillary revival solution" (a viscous formamide containing proprietary reagent) and p*I* Marker Kit (synthetic peptide markers with p*I* values of 10.0, 9.5, 7.0, 5.5 and 4.1) were obtained from Beckman Coulter (Brea, CA, USA). All solutions were filtered through a 0.5 μm Millipore (Bedford, MA) membrane filter before being injected into the capillary. All solutions were prepared in deionized 18 MΩ × m water using a Milli-Q system (Millipore, Bedford, MA).

2.2. Plant material

Grain samples of 6 bread wheat (*T. aestivum* L.) cultivars used as standard obtained from the Australian Winter Cereals Collection (Calala, Australia) and 10 Polish cultivars with different HMW glutenin subunit compositions were analysed. The list of analysed wheat samples and their HMW glutenin subunit compositions is presented in Table 1.

2.3. Extraction of HMW-GS from wheat flour

HMW glutenin extractions were performed according to the method described by Wieser et al. [7] with some modifications.

Table 1
Wheat HMW glutenin subunit compositions for analysed cultivars determined by SDS-PAGE.

Cultivars	*Glu-1*		
	Glu-A1	*Glu-B1*	*Glu-D1*
Banti	1	7+9	2+12
Finezja	N	7+8	5+10
Forkida	N	7+9	2+12
Global*	2*	6+8	5+10
Oxal*	1	17+18	3+12
Katepwa*	2*	7+9	5+10
Legenda	N	7+9	5+10
Ludwig	N	6+8	5+10
Millewa	N	17+18	2+12
Mobela	1	7+9	2+12
Nateja	2*	7+8	2+12
Rysa	N	7+9	5+10
Rywalka	N	7+9	5+10
Sakwa	2*	6+8	2+12
Sukces	N	7+9	2+12
Sunco*	1	7+8	2+12
Tasman*	2*	7+8	2+12
Thatcher*	2*	7+9	5+10

* Seeds from the Australian Winter Cereals Collection.

In short, the flour from the endosperm portion of single seeds was pre-extracted twice with a solution containing 0.4 M NaCl and 0.067 M $HKNaPO_4$ (pH 7.6) at room temperature to remove albumins and globulins. The gliadins were then removed via extraction with 70% (v/v) ethanol and then twice from each kernel residue with 50% (v/v) propan-1-ol. The polymeric glutenins were extracted twice with 0.05 M Tris buffer (titrated by HCl to pH 7.5) containing 50% (v/v) propan-1-ol, 2 M urea and 1% (m/v) DTT, at 60 °C under nitrogen and the mixtures were centrifuged (10 min, 16,000*g*). The joint supernatants were transferred into a new tube, the reduced total HMW proteins were precipitated via the addition of 1-propanol to a final concentration of 62% (v/v), and the samples were stored at 4 °C overnight. Precipitated HMW-GS were collected after centrifugation for 10 min at 13,000*g* and then redissolved in 40 μL of a water solution containing 40% (v/v) AcN, 2 M urea and 1% (m/v) DTT, followed by vortexing for 15 min at 40 °C and centrifuged again for 10 min at 16,000*g*. Three protein separations were performed for each analytical assay. The particular HMW-GS were identified through comparisons of SDS-PAGE, CIEF and CZE patterns obtained for single and mixture samples using standard wheat cultivars.

2.4. Separation via SDS-PAGE

HMW glutenin subunits were separated using SDS-PAGE on a Protean II xi cell (Bio-Rad Laboratories, Hercules, CA) electrophoresis unit using the discontinuous buffer system of Laemmli [33]. Ten-microliter volumes of HMW-GS supernatants were loaded onto the upper 4.5% gel, and the proteins were separated on 11.5% (m/v) polyacrylamide (C=1.35%) in separation buffer at 240 V for 4.5 h. The gels were stained overnight with Coomassie Brilliant Blue. HMW-GS were classified using the nomenclature of McIntosh et al. [34].

2.5. CIEF and CZE instrumentation and procedures

All CIEF and CZE separations were performed using a Beckman-Coulter P/ACE MDQ CE capillary electrophoresis system (Fullerton, CA, USA) equipped with either a UV detector for CIEF or with a photodiode array detector for CZE. Data treatment and integration were performed using 32 Karat software (version 8.1; Beckman

Coulter Inc.). Neutral-coated LPA and PVA capillaries were purchased from Beckman Coulter Inc. (Brea, CA, USA). Bare fused silica capillaries for CZE were obtained from Polymicro Technologies (Phoenix, AZ, USA). The separations were carried out using 50 μm internal diameter capillaries (31.2 cm in total length) with a detection window at 21 cm from the capillary inlet. The cartridge aperture was 100 μm × 200 μm, and data rate was 2 Hz.

For CIEF, both types of capillaries were conditioned prior to each day's analysis by hydrodynamically rinsing the capillary at 275.7 kPa for 5 min with a "capillary revival solution" followed by a 275.7 kPa 2 min rinse with water according to the method described by Bonn et al. [18] and Mack et al. [19]. The anolyte consisted of 200 mM phosphoric acid and 300 mM NaOH was applied as a catholyte. Regarding the chemical mobiliser, 350 mM acetic acid was used. To minimize the effect of cathodic and anodic drift, 0.5 M arginine (Arg) and 0.2 M iminodiacetic acid (IDA) solutions as cathodic stabiliser and anodic stabiliser, respectively, were used. A 4–8 M concentration of urea was optimised to improve of HMW-GS dissolubility and increase the resolution. Samples contained 20 μL of 0.5 M Arg, 2 μL of 0.2 M IDA, 2 μL of each p*I* marker, and 2–5% (v/v) of the carrier ampholytes (CA) [Pharmalyte pH 3–10 or mixture pH 3–10 and pH 5–8 (3:1, v/v)] and 10 μL of HMW-GS. Samples were diluted to a final volume of 250 μL using a cIEF gel polymer solution containing 6 M urea and 0.26 mM SB3-12 as detergent. The protein/CA/gel was centrifuged at 13,500*g* for 5 min to remove air bubbles and particulate matter. The entire capillary was filled with the sample solution via an injection for 90 s at 207 kPa. Focusing was performed via the application of 25 kV for 15 min under normal polarity, with anolyte and catholyte both pressurized at 275.7 kPa. Subsequent protein mobilisation with 350 mM acetic acid as the chemical mobiliser was performed at 30 kV with the application of 1.38 kPa pressure at the anolyte. The temperature was maintained at 20 °C and protein detection was performed at 280 nm. At the end of each run, the capillary was rinsed with water at 275.7 kPa for 2 min. Between successive runs, the capillary was rinsed using "capillary revival solution" for 5 min followed by water for 2 min, and then with the polymer gel for 5 min (all at 275.7 kPa). All reagent vials were exchanged after six runs.

To test the run-to-run repeatability of separations 24 consecutive injections (four series of six runs) were performed. Five major HMW-GS isoform peaks from two wheat cultivars (Tasman and Nateja) were selected to assess correct the p*I* assessment with internal standards and correct the peak area.

CZE separation was performed according to the method described by Salmanowicz [15] in a bare fused-silica capillary. The separation buffer consisted of 0.1 M IDA, 0.15% PEO with molecular weight 8,000,000, 26 mM SB3-12 and 20% (v/v) AcN. As the polymer solution for dynamic coating of the capillary wall, a buffer containing of 0.1 M IDA, 0.2% (m/v) PVP with Mr 360,000, 0.05% (m/v) HPMC, and 20% (v/v) AcN was used. Conditioning comprised a rinsing sequence of 1.0 M NaOH, ultrapure water, 0.1 M HCl, and buffer (all at 275.7 kPa). Samples were injected hydrodynamically under low pressure at 34.5 kPa for 6 s into the anodic end. All solutions and buffers were filtered through a 0.20 μm pore size syringe filters. The temperature of the capillary was maintained at 40 °C and the run current was held constant at 12.5 μA. Detection of the proteins was performed at 200 nm.

2.6. Data processing

The apparent isoelectric points were calculated via a linear regression analysis of marker p*I* versus migration time. The resolution was calculated using the half-width method. The mean differences were compared using an unpaired Student's *T*-test. The statistical analyses were performed using the Statistica software (version 8.0 PL, StatSoft Polska). A p value of 0.05 was considered significant.

3. Results and discussion

3.1. SDS-PAGE analysis

Reduced HMW extracts from 18 wheat varieties were separated via one-dimensional SDS-PAGE. Fig. 1 shows the electrophoretic pattern of certain wheat varieties that differ in their alleles at three *Glu-1A*, *Glu-B1* and *Glu-D1* loci. All analysed accessions have four or five HMW-GS and represent two subunits (Ax1 and Ax2*) encoded by genes of the *Glu-A1* locus as well as six x-type (Bx6, Bx7, Bx17, Dx2, Dx3 and Dx5), and five y-type subunits (By8, By9, By18, Dy2 and Dy12) encoded by genes of the *Glu-B1* and *Glu-D1* loci (Table 1).

3.2. CIEF optimisation

A two-step capillary isoelectric focusing method for the separation of wheat HMW-GS using neutral (LPA) and PVA-coated fused silica capillaries was developed. HMW-GS make up ca. 10% of the major storage proteins in wheat grains and consist of a specific complex of proteins. Native glutenin and glutenin subunits are not soluble in water or salt solutions. An HMW glutenin fraction with a 2*/7+8/2+12 composition from the Tasman wheat cultivar was used as a model of complex proteins for the developed CIEF separation method. The resolution of individual protein peaks using both capillaries for the first three sample injections enabled the identification of charge heterogeneities between subunits. Experimental p*I* values of individual HMW-GS were calculated from the curve of the linear relationship between the detection time and theoretical p*I* values of the utilised peptide p*I* markers. All "cathodic peaks" during the focusing stage of 0–15 min were visible, and the mobilisation stage from 15–60 min shows p*I* peptide markers and resolved isoform peaks of HMW-GS in the range of p*I* 4.7–7.0. Attempts to use LPA-coated capillary was unsatisfactory both because of longer time of sample mobilization and lack of repeatability in repeated runs. Thus, further optimisation of the separation efficiency and the resolution of HMW-GS were performed using only PVA-coated capillary.

For the potential improvement of performance robustness and to improve the repeatability of separated HMW-GS via PVA-coated capillary, modifications of method parameters were assessed. Increasing the carrier ampholyte Pharmalyte pH 3–10 concentration in the range of 2%–5% (v/v) in the sample solution led to insignificant decreases in the mobilisation times of proteins from

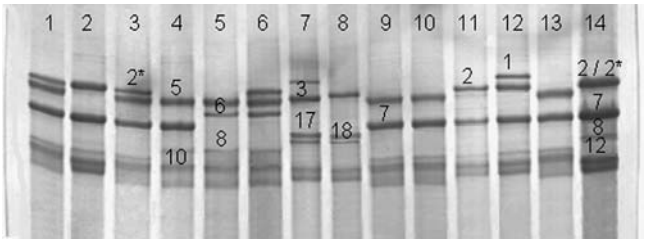

Fig. 1. SDS-PAGE patterns of high-molecular-weight glutenin subunits (HMW-GS) in analysed wheat cultivars with characteristic subunit sets at the *Glu-A1*, *Glu-B1* and *Glu-D1* loci. The bands are marked according to McIntosh nomenclature [34]. Lanes: 1 – Katepwa 2*/7+9/5+10, 2 – Sukces N/7+9/2+12, 3 – Thatcher 2*/7+9/5+10, 4 – Finezja N/7+8/5+10, 5 – Ludwig N/6+8/5+10, 6 – Global 2*/6+8/5+10, 7 – Oxal 1/17+18/3+12, 8 – Millewa N/17+18/2+12, 9 – Legenda N/7+9/5+10, 10 – Rywalka N/7+9/5+10, 11 – Forkida N/7+9/2+12, 12 – Banti 1/7+9/2+12, 13 – Rysa N/7+9/5+10, 14 – Tasman 2*/7+8/2+12 (HMW-GS standard cultivar).

the capillary but no improvement in protein resolution. To achieve better resolution of the HMW-GS, which frequently possess very close p*I*s (difference in p*I*s on the order of 0.3–0.4 units), 4% ampholyte pH 3–10 was added at a concentration of 1% (v/v) of narrow-range ampholyte Pharmalyte pH 5–8 (results not shown). Further optimisation of the separation efficiency and the resolution was performed by decreasing the sample aggregation during focusing due to the limited solubility of HMW-GS near their isoelectric points. A different concentrations of urea (1.5–7.5 M) were added to the sample solution to disrupt the non-covalent bonding leadings to protein aggregation and preparation without sacrificing detection sensitivity. The increased concentration of urea in the sample solution extended the time of mobilization of HMW-GS, significantly increasing the isoform peak signals and improving the resolution. The beneficial effect of urea between 3.0 and 6.0 M is illustrated in Fig. 2. Above 6.0 M urea, the HMW-GS peak profiles became less resolved, and the peak irreparability split. The addition of zwitterionic detergent SB3-12 at its critical micelle concentration (26 mM) in cases of the CIEF separation of wheat HMW-GS additionally improved the run-to-run repeatability. This compound was previously recommended by Lookhart and Bean [11] as a factor for improving the resolution of cereal storage proteins in CE techniques and by Mechin et al. [35] and Rabilloud et al. [36] for plant proteins focused on immobilised pH gradients.

To test the run-to-run repeatability of HMW-GS separations 24 consecutive injections (four series of six runs) were performed. Five major HMW-GS isoform peaks from the Tasman and Nateja wheat cultivars were selected to assess correct the p*I* assessment with internal standards and correct the peak area (Supplementary materials Fig. S1). A quantitative analysis of the run-to-run study shows that the assays were reproducible. The standard deviations for p*I* values representing major peaks of corresponding subunits were very low (0.02–0.03). The relative standard deviation (RSD) values for p*I* values of these subunits ranged from 0.34% to 0.86%. Additionally, the corrected peak area repeatability was satisfactory; the RSD values for the main peaks ranged from 3.84% to 4.66%. These results are in close agreement with results obtained with different capillary coatings [18,19,32].

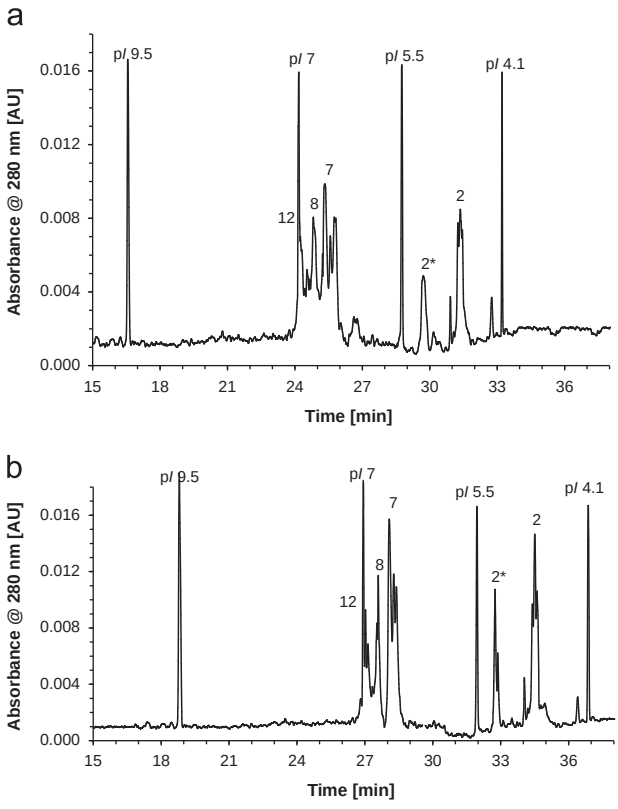

Fig. 2. Effects of urea concentration (**a**, 3 M) and (**b**, 6 M) on CIEF profile of HMW-GS isoforms from the Tasman wheat cultivar. The sample solutions contained 10 μL of redisolved HMW-GS, 200 μL cIEF gel, 9 μL Pharmalyte 3–10, 3 μL Pharmalyte 5–8, 20 μL cathodic stabiliser, 2 μL anodic stabiliser and 2 μL of each pI marker. The samples were focused for 15 min at 25 kV with 200 mM H_3PO_4 and 300 mM NaOH and chemically mobilised at 30 kV with 200 mM H_3PO_4 and 350 mM CH_3COOH.

3.3. Detection of wheat HMW-GS heterogeneity via CIEF

Electrophoretic patterns obtained on the basis of charge-based CIEF separation of wheat HMW-GS show a satisfactory resolution of particular isoforms for all subunits. In analysed wheat cultivars, in total eight x-type and five y-type subunits were distinguished. The samples with the same HMW-GS composition give CIEF profiles with the same number of isoforms for particular subunits, although insignificant differences in quantity (differences of peak areas) of particular isoforms were observed. Three selected CIEF profiles of HMW-GS with different subunit compositions in wheat genotypes [(a) 2*/6+8/2+12, (b) N/7+8/5+10 and (c) 1/7+9/2+12)], which were determined earlier by the SDS-PAGE analysis, are shown in Fig. 3. In CIEF profiles of the analysed samples four or five HMW subunits were distinguished from which each of them consisted from one major and one or two minor isoforms. The isoforms of the individual HMW-GS were separated via pH gradient in two groups, namely, y-type subunits with shorter mobilisation times (26.8–27.7 min) and x-type subunits with longer mobilisation times (28.1–34.6 min). In most cases, the relative migration orders of the wheat HMW glutenin subunits separated in PVA-coated capillary and SDS-PAGE gel were different. Using the CIEF method, the relative mobilisation orders of y-type HMW-GS encoded by *Glu-A1*, *–B1* and *–D1* loci from the PVA-coated capillary were Dy12 < Dy10 < Dy9 < Dy8 < Dy18; however, x-type subunits migrate in the order of Bx7 < Bx17 < Bx6 < Ax2* < Ax1 < Dx5 < Dx3 < Dx2 (from the fastest to the slowest).

One major and two minor isoforms were observed in subunits Bx7 and Dx2, and the remaining subunits had one major and one minor isoform. Generally, the minor isoforms were later mobilised from the capillary (as more acidic forms) compared with the major isoform of individual subunits. For comparison, in Fig. 4a–c are presented CZE electropherograms of HMW-GS obtained for the same wheat samples as were analysed by the CIEF method (Fig. 3a–c). Generally, using both methods similar number of major and minor isoforms for the individual HMW-GS was detected. However, the relative mobilisation orders of particular subunits in capillaries using both methods differ partly. Among other things, the x-type subunits (Ax1, Ax2*) encoded by *Glu-A1* locus migrated via pH gradient slowest than by CZE method. Also, the CIEF distinct from CZE enabled the accurate identification of the y-type subunits encoded by *Glu-D1* locus (Dy10 and Dy12 subunits). According to previous literature data [13–16], detected multiple peaks for the particular HMW-GS via CIEF and CZE methods present different variants of posttranslational modifications of these subunits. An acid shift on p*I* by introducing negative charge(s) for some minor HMW-GS isoforms may indicate the phosphorylation or glycosylation of these proteins [37–39]. However, the glycoprotein nature of HMW-GS should be exclude, because earlier conducted investigations revealed that these subunits are not glycosylated [40–41].

Regardless of qualitative differences (presence or absence and number of subunit peaks), considerable quantitative differences (values of peak areas or peaks height) during the analyses of individual cultivars were also observed. The relative quantities of individual HMW-GS derived from CIEF-corrected areas for isoforms of y-type subunits are considerably lower than those for the x-type isoform subunits. The subunits Ax1, Ax2*, and Dx5 with *pI*

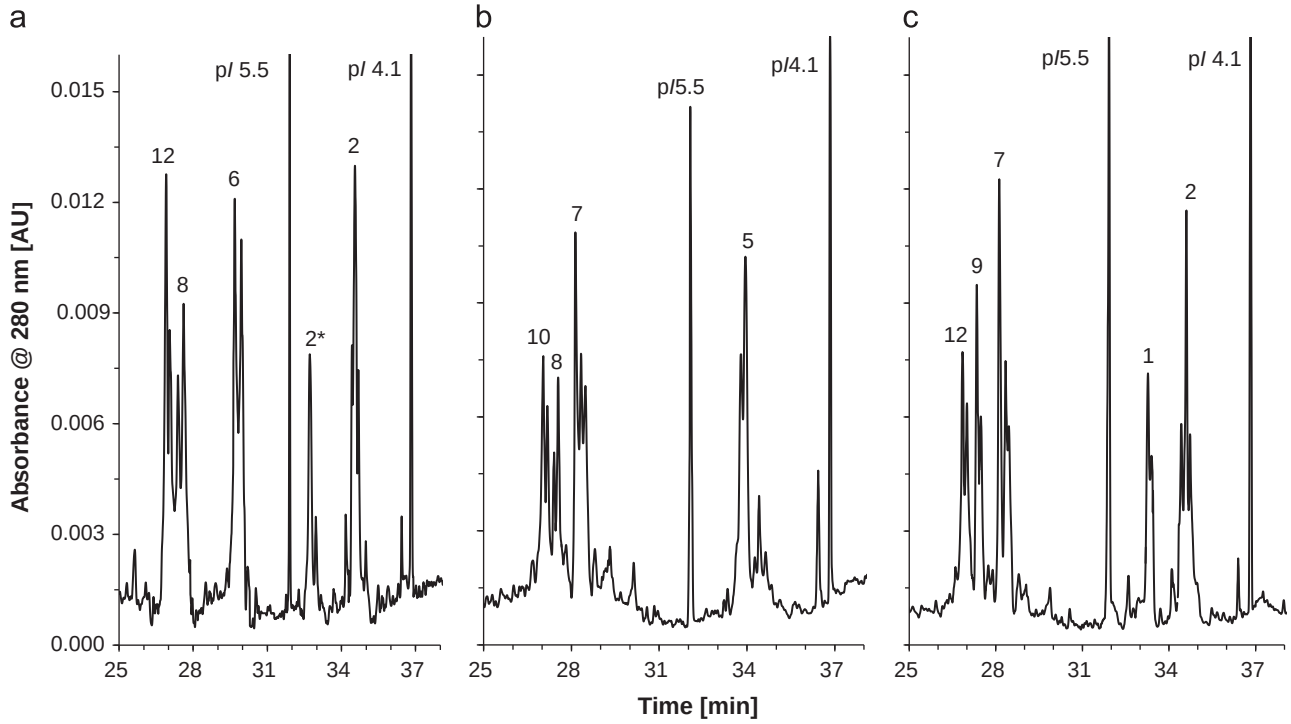

Fig. 3. CIEF profiles of HMW-GS isoforms from the wheat cultivars with various compositions. ((**a**) 2*/6+8/2+12; cv. Sakwa, (**b**) N/7+8/5+10; cv. Finezja and (**c**) 1/7+9/2+12); cv. Banti. Separation conditions are as in Fig. 2 b. HMW-GS are numbered according to McIntosh nomenclature [34].

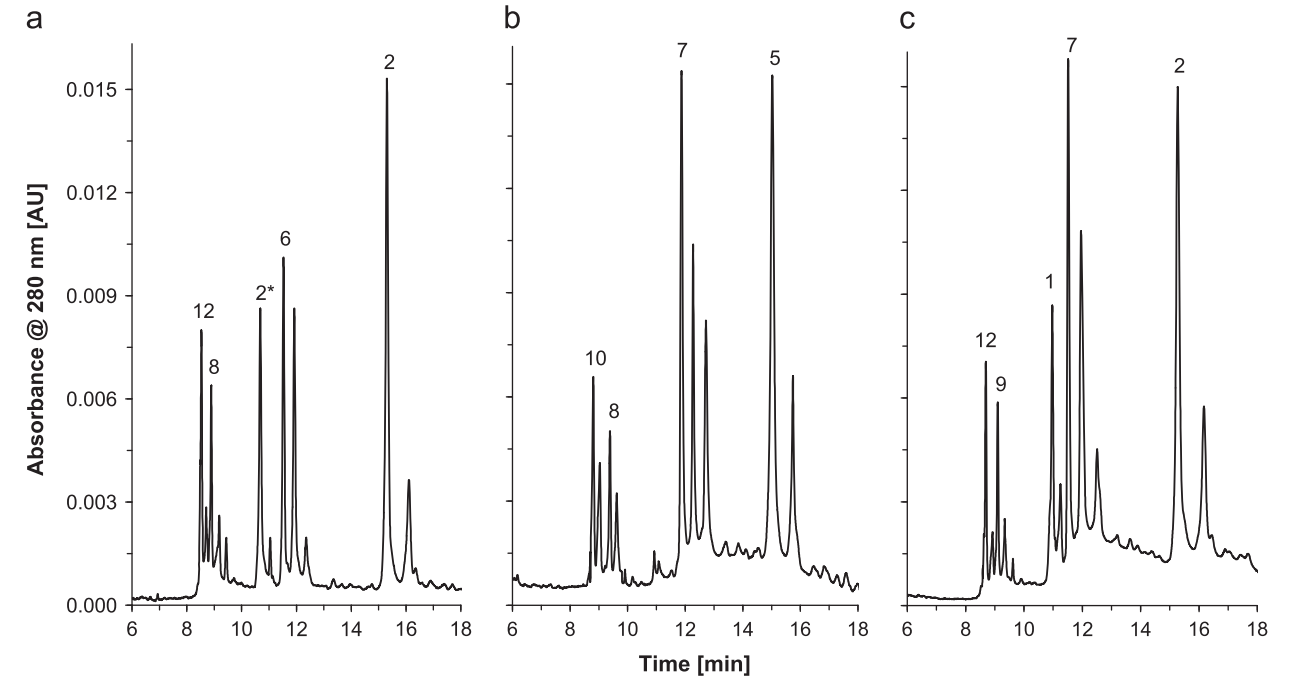

Fig. 4. CZE electropherograms of HMW glutenin analyzed in Fig. 3. Proteins were separated at 10.5 kV and 40 °C with a 100 mM isoelectric IDA buffer, containing 0.15% (m/v) PEO with M_r 8,000,000, 26 mM SB3-12, and 20% (v/v) AcN. Prior to the separation, the capillary was rinsed with 0.1 M IDA solution containing 0.2% (m/v) PVP, 0.05% (m/v) HPMC, and 20% (v/v) AcN. HMW-GS are numbered according to McIntosh nomenclature [34].

below 5.1 and high peak area values have particularly positive influence on bread-making properties of wheat dough [4,7,10].

3.4. Determination of pI values for HMW-GS via CIEF

For the HMW-GS fraction from 18 wheat cultivars with different subunit compositions (Table 1), we determined the experimental p*I* values of all isoforms belonging to individual HMW-GS, which were calculated from curves of linear relationship between the detection time and the theoretical p*I* values of the utilised synthetic peptide p*I* markers with p*I* values of 10.0, 9.5, 5.5 and 4.1. The average calculated p*I* values of HMW-GS isoforms for analysed wheat varieties differing in their alleles at three loci (*Glu- A1*, *Glu-B1* and *Glu-D1*) are presented in Table 2. The HMW-GS isoforms have p*I* values in the range of 4.72–6.98. The y-type subunit isoforms and subunits encoded by the *Glu-B1* locus have p*I* values close to pH neutral (p*I* 6.10–6.98);

Table 2
The average calculated p*I* values of x- and y-type HMW-GS isoforms for wheat varieties differing in alleles at three *Glu-1* loci.

HMW-GS	*Glu-A1*		*Glu-B1*			*Glu-D1*		
x-subunit	Ax1	Ax2*	Bx6	Bx7	Bx17	Dx2	Dx3	Dx5
p*I*	**5.08***; 5.06	**5.23;** 5.21	**6.19;** 6.10	**6.62;** 6.57; 6.52	**6.48;** 6.45	4,8; **4.76;** 4.72	**4.91;** 4.86	**5.01;** 4.97
y-subunit			By8	By9	By18	Dy12	Dy10	
p*I*			6.82; **6.76**	**6.86,** 6.83	**6.73;** 6.70	**6.98;** 6.95	6.94; **6.90**	

* Bold digits are marked major HMW-GS isoforms.

however, p*I* values of the remaining x-type subunit isoforms encoded by *Glu-A1* and *Glu-D1* loci are in the range of 4.72–5.23 (weakly acidic). Obtained results show unambiguously, that the introduced CIEF method makes possible accurate determination of p*I* values of wheat HMW-GS. Until now, p*I* values of HMW-GS can will only initially estimate on the basis of separation via very time-consuming two-dimensional SDS-PAGE–IEF method [1,4].

4. Conclusions

The presented CIEF method using PVA-coated capillary enabled the separation and charge characterisation of wheat HMW glutenin subunits with good repeatability and separation efficiency. The use of the mixture of carrier ampholytes (pH 3–10 and pH 5–8), high concentration of urea (6 M) and SB3-12 as a detergent in the sample solution improved the separation resolution between consecutive isoform peaks of HMW-GS. All isoforms of wheat HMW-GS in wheat cultivars can be identified and characterised on the basis of their relative migration times and peak areas. Both qualitative and quantitative characterisation of the heterogeneity of HMW-GS subunits was performed in the capillary mode more exactly in comparison with SDS–PAGE–IEF slab gel format. The obtained data indicate that using CIEF and CZE methods the similar number of isoforms for the individual HMW-GS was affirmed. The particularly significant advantage of presented CIEF method is possibility to detect the exact p*I* values for all HMW-GS isoforms, The knowledge of p*I* value is a crucial property that permits different post-translational states and protein expression in wheat gluten composition to be distinguished [1,4,16]. All y-type HMW-GS isoforms and x-type subunits encoded by the *Glu-B1* locus possess near a neutral pH; however, remaining x-type subunits are weakly acidic. Like CZE, the CIEF method also enables the accurate quantification of individual subunits. This advantage is of great importance for HMW-GS because both the quality and quantity of individual subunits greatly influence the dough functionality and bread-making quality of wheat cultivars.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2014.04.055.

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