Preparative Fractionation of Gliadins by Electrophoresis at pH 3.1 (A-PAGE)

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Purification of gliadin subclasses has been difficult since they share many biochemical and physicochemical properties. In this report, the optimization of a preparative electrophoretic method to fractionate gliadins is described. Separation was performed in preparative 7% polyacrylamide gels at pH 3.1. The separation performance was tested using analytical electrophoresis at pH 3.1 and capillary electrophoresis. Preparative gels of different lengths were employed. Using 5-cm preparative gels, several fractions of α-, *β*-, and *γ*-gliadins and fast-mobility and slow-mobility *ω*-gliadins were collected in 40 h of separation. Resolution was maintained at a protein load of up to 30 mg in each run. The highest efficiency of recovery was achieved using aluminum lactate as the collecting buffer. Fractionation with 10 cm in length gels improved resolution but increased operation times. Gels of 2 cm in length did not separate α/*β*- and *γ*-gliadins efficiently but were useful to separate the two main fractions of *ω*-gliadins in shorter times. In conclusion, preparative electrophoresis at low pH allowed the separation of α-, *β*-, *γ*-, and *ω*-gliadin fractions from crude material under nondenaturing conditions.

Keywords: *Gliadin; electrophoresis; A-PAGE*

INTRODUCTION

Gliadins and glutenins are the main wheat storage proteins. They are encoded by a complex cluster of genes sharing a common ancestor, and they present great similarities in sequence and structure and a high number of allelic variations (Shewry et al., 1986; Shewry and Tatham, 1990). Therefore, wheat storage proteins are a complex mixture of many highly polymorphic proteins that share common biochemical properties. Such complexity and the similarities among these proteins make their isolation as pure components difficult.

Electrophoretic techniques have been widely employed to analyze wheat storage proteins (Bietz and Simpson, 1992). Among the different electrophoretic procedures, electrophoresis at acid pH (A-PAGE) has been traditionally used to analyze and characterize gliadins (Bietz and Simpson, 1992; Lafiandra and Kasarda, 1985). The gliadin fraction was divided into four groups according to their mobility (Woychik et al., 1961; Bushuk and Zillman, 1978). Although electrophoretic techniques are mainly used as analytical tools, recently preparative electrophoretic methods have been reported (Curioni et al., 1988). Regarding the purification of wheat storage proteins, Curioni et al. (1989) described the use of an electroendosmotic preparative electrophoretic method to separate high molecular weight (HMW) glutenins, performing the separation under denaturing conditions at pH 8.3 in the presence of sodium dodecylsulfate (SDS). Curioni et al. (1995) described a method for separation of HMW and LMW glutenins using preparative electrophoresis at acid pH.

The objective of the present work is to evaluate the use of a preparative electrophoretic system in gliadin fractionation at acid pH, under nondenaturing conditions.

MATERIALS AND METHODS

Samples and Reagents. All reagents were purchased from Sigma Chemicals (St. Louis, MO) unless otherwise stated. The wheat flour employed was from *Triticum aestivum* L. (hard red spring wheat) ProINTA Oasis, kindly provided by Dr. Chidichimo from the Cátedra de Cerealicultura, Facultad de Ciencias, Agrarias, Universidad Nacional de La Plata. Flour was extracted following the method described by Sapirstein and Bushuk (1985) with slight modifications. Briefly, extraction was performed using 70% aqueous ethanol (10 mL/g) under gentle shaking for 1 h at room temperature. The gliadin fraction was obtained as a clear supernatant after centrifugation in a Sorvall centrifuge for 15 min, 10000*g*, at 8 °C.

Protein Quantification. The protein content of ethanol extracts and fractions was determined by the method described by Lowry et al. (1951). As a standard, a gliadin ethanol solution quantified by Kjeldahl's method was used as previously described (Chirdo et al., 1994).

Preparative Electrophoretic Procedure. Electrophoresis was performed using a PrepCell Model 491 (equipped with the 37 mm i.d. gel tube) from Bio Rad (Hercules, CA). Gels of 7% acrylamide concentration and the lactate buffers system,

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as described in the literature (Lafiandra and Kasarda, 1985), were used. Briefly, two different lactate buffers were employed. Buffer A was employed as electrode buffer, consisting of 0.028 M lactic acid/aluminum lactate pH 3.1 (prepared by dissolving aluminum lactate and adjusting the pH with lactic acid to pH 3.1. A stock buffer 50-fold concentrated was prepared and diluted immediately before use). Buffer B consisted of a 0.040 M lactic acid/potassium lactate buffer, prepared by dissolving KOH and adjusting the pH with lactic acid (it was prepared as stock buffer 50-fold concentrated and diluted immediately before use). Buffer B was used for preparation of the gel and prerun. All buffers and solutions employed were filtered by 0.22 μm pore diameter nylon membranes (MSI separations, Westboro, MA) and degassed immediately before use, employing a vacuum pump. A preparative gel was prepared to produce a 7% (w/v) polyacrylamide gel with 0.3% (w/v) bisacrylamide. Polymerization was achieved using 0.17 g/L silver nitrate in the polymerization mixture and by adding ammonium persulfate to a final concentration of 0.115 g/L. Once the solution was poured into the preparative tube, 2 mL of water was gently layered on the solution surface to exclude air and provide a smooth surface. Gels were polymerized in 1−2 h. After polymerization, water and any unpolymerized material at the top of the gel were carefully removed using a syringe and the top of the gel was rinsed several times with buffer B. Preparative gels were run without stacking gel. A prerun to remove silver and persulfate residues and to balance the buffer within the gel was performed using buffer B at a constant voltage of 160 V (h/cm of gel). After that, the buffer system was replaced and gliadin fractionation was performed using buffer A. The resolution power of gels of different lengths was analyzed. Gels 10, 5, and 2 cm in length were employed (80, 40, or 18 mL of 7% polyacrylamide, respectively). In each experiment, a 4 mL of sample (3 mL of ethanolic extract + 1 mL of saturated sucrose in running buffer), containing approximately 10 mg of protein, was loaded. The separation performance with variable amounts of samples was tested. It was carried out at a constant voltage (250 V). The current decreased from 40 mA at the beginning of the electrophoresis to 30−35 mA at the end. Elution of the separated protein bands outflowing from the bottom of the gel was obtained by use of a peristaltic pump. To this end a peristaltic pump Miniplus 3 from Gilson (Villiers le Bel, France) was employed. Samples were collected at 3 mL/h flow, in aliquots of 1 mL, employing a fraction collector Microcol TDC 80 from Gilson (Villiers le Bel, France). For longer runs, the collecting flow was reduced to 1 mL/h after the first 20 h of run. The use of either 0.01 M acetic acid or running buffer as the collecting buffer was also tested.

Analytical Electrophoresis. Fractions collected from the preparative system were analyzed by analytical A-PAGE. Electrophoresis was performed as described in the literature (Lafiandra and Kasarda, 1985) using a Mini Protean System from Bio Rad and the same reagents used for the preparative electrophoresis. Gels of 7% acrylamide concentration, 0.75 mm thick, were used. After electrophoresis, gels were stained with 0.1% (w/v) Coomassie Brilliant Blue in 12% trichloroacetic acid.

Safety Concerns. Acrylamide monomer is a neurotoxin; consequently, extreme care must be taken when handling the solid drug and when pouring gels.

Capillary Electrophoresis. Electrophoresis was performed as described recently (Rumbo and Giorgieri, 1998). A P/ACE 2100 system (Beckman, Fullerton, CA) and 25 μm i.d. (Polymicro Technologies, Phoenix, AZ), 18 cm effective-length fused silica capillary column (28 cm total length) were employed. Separations were performed using 0.1 M phosphate pH 2.50 as running buffer, containing 20% acetonitrile and 0.03% hydroxypropylmethyl cellulose (HPMC) (Sigma Chemical, Co., H-7509, viscosity of 2% solution 4000 cp). The column temperature was maintained at 40 °C. Separations were performed at constant voltage (20 kV). Samples were filtered through 0.22 μm nitrocellulose membranes immediately before injection and then injected for 20 s at low pressure. On-line detection was carried out with a diode array system (PDA) between 190 and 300 nm. Data were acquired using Beckman software accumulated at a sampling rate of 1 Hz and processed using SigmaPlot software (Jandel Scientific, San Rafael, CA). All patterns were normalized for comparison. Capillary conditioning and cleanup protocols described by Lookhart and Bean (1996) were followed.

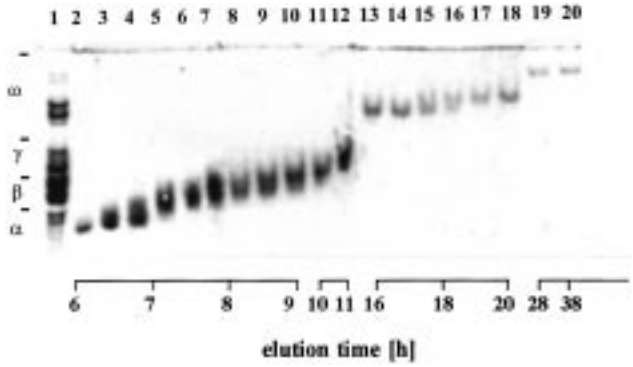

Figure 1. Analytical A-PAGE of fractions obtained from the 5 cm in length gel preparative separation. A 15 μL sample was loaded in each lane. Lane 1: ethanol extract used for the preparative separation. Lanes 2−19: analysis of the different fractions. Elution time of each fraction is indicated.

RESULTS AND DISCUSSION

Optimization of A-PAGE Preparative Fractionation of Gliadins. According to preliminary observations and in agreement with the literature (Curioni et al., 1995), the preparative system resolution was highly dependent on the separation gel length. It was found that using gels 5 cm in length, a separation of the whole gliadin fraction could be achieved. The performance of the preparative A-PAGE fractionation was evaluated studying the different collected fractions by means of analytical A-PAGE (Figure 1). Fractions were collected every 20 min up to 20 h of running; from that point to the end of the run, samples were collected every hour. As observed, different mixtures of gliadins were eluted along the preparative separation. Three different fractions containing α-gliadin were obtained between 6 and 6.6 h (lanes 2−4). Several types of β-gliadins were eluted from 7.3 to 9 h (lanes 4−9). Mixtures of β- and γ-gliadins were isolated within 8 and 9 h (lanes 8−10). γ-Gliadins were separated 8−12 h of running (lanes 11 and 12). The ω-fraction was resolved in several fractions of diverse mobility (lanes 13−18). Fast-mobility ω-gliadins were eluted within 16−22 h of running. The first fast-mobility ω-fraction was separated within 16−17 h of running (lanes 13 and 14). Between 18 and 19 h, a mixture of the two main fractions was eluted (lanes 15 and 16). The second fast-mobility ω-fraction was isolated 20−22 h of running (lanes 17 and 18). Slow ω-gliadins were eluted between 28 and 39 h (lanes 19 and 20). In five different experiments, elution profiles were similar. Comparing the elution time of the first fast-mobility ω-gliadin in the five experiments, a CV of approximately 10% was observed.

To evaluate the composition of fractions from the preparative separation, they were analyzed by CE, a methodology that has proved to be a powerful tool in the field of gliadin analysis (Lookhart and Bean, 1995a, 1996). In Figure 2, the CE fraction analysis results from a 5 cm in length preparative gel are shown. All the fractions in Figure 2 correspond to samples appearing in the A-PAGE analytical gel (Figure 1). Fractions showing minor differences were not included to simplify

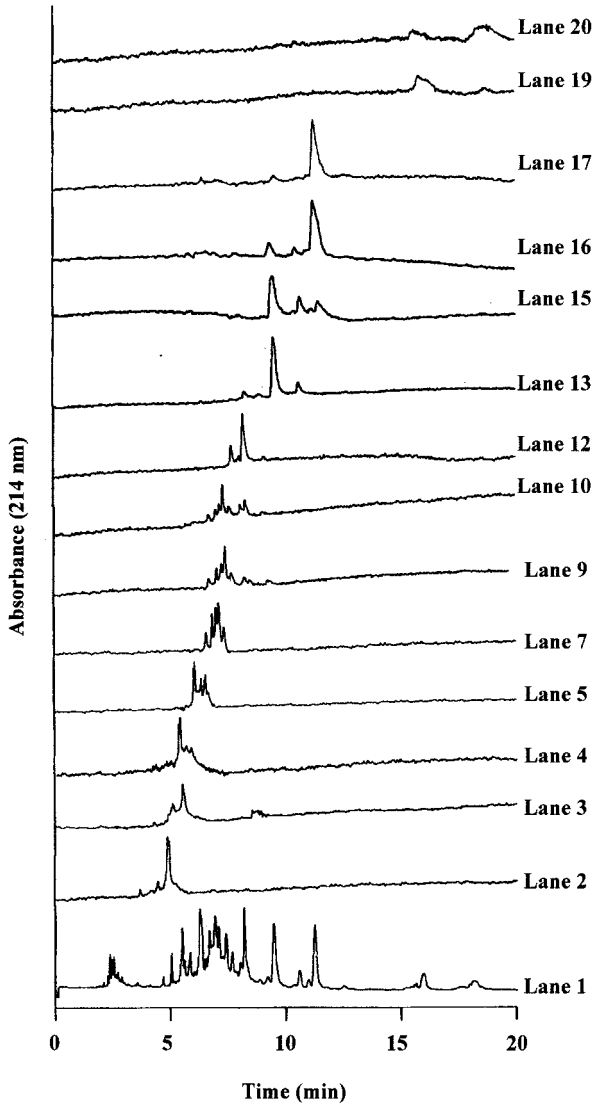

Figure 2. CE analysis of different fractions, performed at a constant 20 kV in a 0.1 M phosphate buffer. Ethanol extract was diluted 10-fold in aqueous ethanol (70%) in order to use the same loading conditions for all samples. Results were normalized for comparative purposes. Each electropherogram belongs to different fractions obtained from the 5 cm in length preparative gel. The correspondence with samples analyzed in Figure 1 is indicated.

the plot. As previously described (Lookhart and Bean, 1995b; Rumbo and Giorgieri, 1998), there is considerable concordance among gliadin mobility in both systems, capillary electrophoresis in phosphate buffer and A-PAGE. Fractions displaying higher mobility in the A-PAGE system also show low migration times in CE. Many of the fractions revealed as a wide band in A-PAGE analysis appear as several peaks in the CE analysis. As observed, most fractions are heterogeneous when analyzed by CE (see lanes 5−10) whereas others appear as a major single peak (lanes 13 or 17). Fractions of α/β- (lanes 2−8) and γ-gliadins (lanes 9−12) are formed by more than one component, while isolated ω-gliadins can be obtained (lanes 13, 17, 18). This may be due to the fact that even if differences in mobility among ω-gliadins seem small when measured as rf in an analytical gel, they are magnified in the preparative system, where they have to completely migrate through the gel to be collected. However, even in the case of heterogeneous fractions, all components showed close

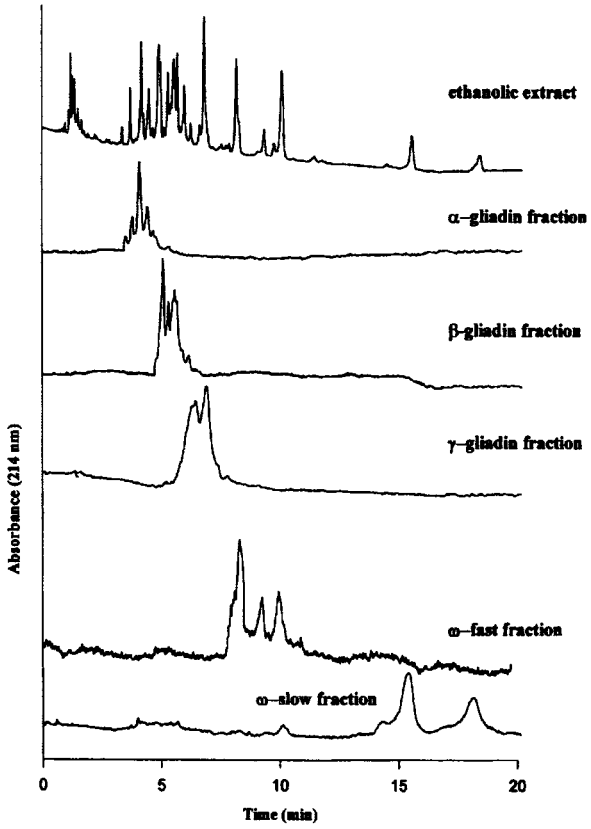

Figure 3. CE analysis of pooled fractions. The same conditions as described in Figure 2 were employed.

mobility. No cross-contamination with components of other gliadin groups could be found. This fact allowed us to pool the gliadins in groups of α-, β-, γ-, ω-fast, and ω-slow gliadins. To this purpose, only tubes containing gliadins from one subclass were mixed. The CE analysis of these pools is shown in Figure 3. Note that no cross-contamination among the different fractions is evident. In a single preparative electrophoresis of approximately 10 mg of protein loaded in the gel, the amount of each pooled fraction obtained (measured by Lowry's method on the pooled fraction) is approximately 0.2 mg of α-gliadin, 0.8 mg of β-gliadin, 0.6 mg of γ-gliadin, 0.5 mg of fast-mobility ω-gliadin, and 0.2 mg of slow-mobility ω-gliadin. These figures depend on the type of preparative gel employed and the amount of protein loaded. Fractions obtained by preparative electrophoresis can be used as starting material in other preparative systems to get isolated single gliadin components.

The acrylamide polymerization reaction at low pH is highly dependent on the proportions in which reagents are used (Friis and Shäfer-Nielsen, 1985; Huebner and Bietz, 1984). To avoid polymerization problems that affect protein separation, the same reagents and protocol to perform the analytical gel were used in the preparative system. No problems in polymerization, such as incomplete reactions during gel formation, were observed when the preparative gels were formed. During polymerization in analytical gels, the heat produced is quickly dissipated, while in the preparative system, due to its geometry, the heat produced by the reaction itself increases the temperature and consequently the polymerization rate. This factor leads to a complete polymerization in approximately 2 h.

Increasing amounts of gliadin were tested in different experiments. Results shown here were obtained loading

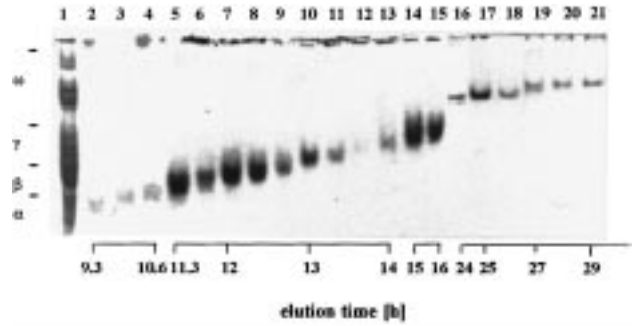

Figure 4. Analytical A-PAGE of fractions obtained from the 10 cm in length gel preparative separation. Lane 1: ethanol extract used for the preparative separation. Lanes 2−21: analysis of the different fractions. Elution time of each fraction is indicated.

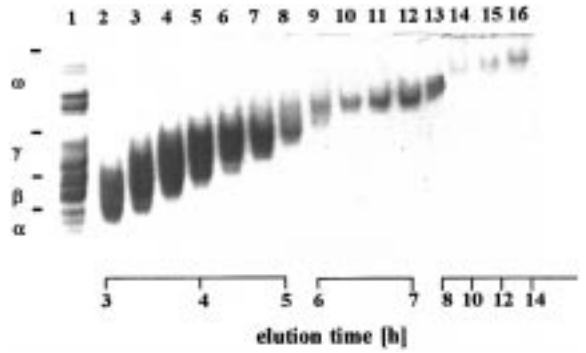

Figure 5. Analytical A-PAGE of fractions obtained from the 2 cm in length gel preparative separation. Lane 1: ethanol extract used for the preparative separation. Lanes 2−16: analysis of the different fractions. Elution time of each fraction is indicated.

10 mg of whole gliadin onto the preparative gel. Similar results were seen when 20 and 30 mg of gliadin were employed. Conversely, when 40 mg of gliadin was loaded, precipitation of proteins within the gel was observed, producing impaired resolution, independent of the gel length. This may be caused by a sharp increase in local gliadin concentration inside the gel, resulting in local gliadin aggregation and precipitation.

Two buffers were employed alternatively to collect the eluted fractions: 10 mM acetic acid and lactic acid/aluminum lactate buffer (the same used as the running buffer). The resolution achieved was similar in both cases. However, the amount of recovered proteins after the separation was very different. Protein recovery was estimated measuring the protein content of all the different fractions obtained after a preparative electrophoresis (pooled purified fractions and tubes containing fractions with subclasses overlapping). Approximately 50% of the original material was recovered using lactate buffer as a collecting buffer, and no material precipitation at the bottom of the gel was observed. It must be considered that in the extract loaded in the preparative gel, albumins and globulins were present also (Wieser et al., 1994). These fractions have an electrophoretic mobility higher than α-gliadins in A-PAGE system (Bietz and Simpson, 1992) and consequently should elute earlier from the preparative gel and were not included in the recovered protein. Apart from that, aggregated proteins that cannot enter the gel are usually observed in the top of the preparative gel. This may partially explain the deviation from the theoretically optimum recovery. Nevertheless, the recovery obtained when lactate buffer is used as the collecting buffer is at least 10−20-fold higher than that seen using acetic acid as the collecting solution. When acetic acid is used, large amounts of aggregated proteins were present in the dialysis membrane at the bottom of the gel. Gliadin precipitation apparently occurs when gliadin bands outflow from the bottom of the gel and transit from lactic/lactate solution within the gel to collecting acetic solution. This phenomenon may also depend on the local gliadin concentration. Consequently, for subsequent experiments, lactate buffer was employed as the collecting buffer.

Analysis of Separation Performance Using Different-Length Gels. To improve the preparative separation performance, longer preparative gels were employed. Results of gliadin fractionation in a gel 10 cm in length are shown in Figure 4. It can be seen that α-/β- and γ-gliadins are separated in different fractions (lanes 2−15). Three different fractions containing α-gliadins were obtained between 9.3 and 10.6 h (lanes 2−4). β-gliadins were eluted 11.3−14 h (lanes 5−13). γ-Gliadins were eluted 13.3−18 h (lanes 10−15). Fast-mobility ω-gliadins were separated in at least four different fractions, which were isolated within 24−32 h. The first ω-fraction was eluted at 24 h. According to A-PAGE analysis, it consists of two components (lane 16). A fast-moving ω-fraction was isolated between 25 and 27 h (lanes 17 and 18). At 28 h, a fraction composed by a mixture of the two main fast-mobility ω-gliadins was eluted (lane 19), and then another fast-mobility fraction was also eluted 29−32 h (lanes 20 and 21). After 42 h of running, slow-mobility ω-gliadins were not eluted. Using the same collection protocol employed in the gel 5 cm in length resulted in a higher number of different fractions, as appreciated. An increase in the elution times of all the fractions was found. A more efficient separation than that achieved in gels 5 cm in length was obtained. However, partial overlapping of fractions is still seen in A-PAGE analysis.

Table 1. Retention Time of Each Gliadin Type According to the Length of the Separation Gel

	migration time (h)		
gliadin type	10-cm gel	5-cm gel	2-cm gel
α-gliadins	9.3−10.6	6−6.6	3−3.3
β-gliadins	11.3−14	7−9	3−5
γ-gliadins	13.3−18	8−12	3.3−5
ω-fast	24−32	16−22	6−8
ω-slow	>42	28−39	10−14

Due to their low mobility, the isolation of ω-gliadins was extremely time-consuming with either 5 or 10 cm in length preparative gels, as described above. To get a faster separation of ω-gliadins, gels 2 cm in length were tested (Figure 5). At 3 h of running, a mixture of α/β-gliadins was eluted (lane 2). Between 3.3 and 5 h, different mixtures of α/β- and γ-gliadins were separated (lanes 3−8). Even in such short gels, the two main groups of ω-gliadins were well-resolved. A fast-mobility fraction was eluted within 6 and 8 h (lanes 9−13), and a slow-mobility one was isolated between 10 and 14 h (lanes 14−16).

The elution times for the different gliadin fractions in each gel tested are summarized in Table 1. Changes in the length of the preparative gel strongly influence the separation resolution and the operation times. A good resolution was achieved using 5 or 10 cm in length gels. However, as shown in Table 1, elution times of the different fractions become longer as the gel length

increases, producing very long-lasting runs. Consequently, a balance must be reached between separation efficiency and duration of the process. On the other hand, the 2 cm in length gel gives a poor separation of α/β- and γ-gliadins. However, it is suitable for ω-gliadin separation. Two groups of ω-gliadins, the fast-mobility and slow-mobility ones, were collected between 6 and 8 h and 10−14 h of running, respectively (Table 1). Short gels are useful to isolate purified ω-gliadins, which would be extremely time-consuming if high-resolution longer gels were used.

In conclusion, a preparative electrophoretic procedure to isolate native purified gliadin fractions was developed. This system is useful to produce a one-step gliadin fractionation from a crude ethanol extract. The preparative gel length is one of the critical parameters to optimize in order to achieve the expected resolution and time of separation. Using preparative electrophoresis, fractions containing α-, β-, and γ-gliadins and fast-mobility and slow-mobility ω-gliadins were obtained. Preparative A-PAGE may be a useful alternative procedure in cereal biochemistry to obtain purified gliadin fractions.

ABBREVIATIONS USED

A-PAGE, polyacrylamide gel electrophoresis at pH 3.1; RP-HPLC, reversed-phase high-performance liquid chromatography; CV, coefficient of variation; HPMC, hydroxypropylmethylcellulose; CE, capillary electrophoresis.

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