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Fractionation of secalins and hordeins by preparative electrophoresis at acid pH

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Abstract In this report, the optimization of a preparative electrophoretic method to fractionate secalins and hordeins is described. Separation was performed in preparative 7% polyacrylamide gels of 4 cm length at pH 3.1. The separation performance was tested using analytical electrophoresis at pH 3.1 and capillary electrophoresis (CE). Fractions of B- and C-hordeins were isolated in a single run from barley ethanol extract. γ- and ω-secalin fractions were isolated from rye ethanol extract. Resolution of preparative separation was maintained at a protein load of up to 30 mg in each run. Each secalin and hordein fraction showed several components of close mobility when analyzed by CE. Fractions from the preparative separation were pooled in such a way that no components from one pool were present in the others. These pooled fractions could be used as starting material for single polypeptide purification. Preparative electrophoresis at low pH allowed a simple separation of γ- and ω-secalins and B- and C-hordeins from crude material under non-denaturing conditions.

Keywords Secalin · Hordein · Preparative electrophoresis · Polyacrylamide gel electrophoresis at pH 3.1

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Introduction

The major storage proteins of the grain of barley and rye are alcohol-soluble prolamins called hordeins and secalins respectively. The polypeptides that compose both of these fractions show different properties among the different genetic varieties, so-called cultivars. Most studies performed on these proteins have used fractions purified by chromatographic methods, (reviewed in [1]).

Electrophoretic techniques have been widely employed to analyze cereal storage proteins (reviewed in [2]. Although these techniques are mainly used as analytical tools, preparative electrophoretic methods have been employed for the separation of high molecular weight (HMW) glutenins under denaturing conditions [3] and for the separation of HMW and low molecular weight glutenins using preparative electrophoresis at acid pH [4]. Recently we described the use of acidic buffers for the preparative fractionation of gliadins under non-denaturing conditions [5].

Since the use of preparative electrophoretic techniques to isolate secalins and hordeins has not yet been explored, the aim of the present work was to evaluate the use of a preparative electrophoretic system for their fractionation under non-denaturing conditions.

Materials and methods

Samples and reagents. All reagents were from Sigma (St. Louis, Mo., USA) unless otherwise stated. Flour from *Secale cereale* L. Insave 631031 and *Hordeum vulgaris* L. B1614 were kindly provided by Dr. Chidichimo from the Cátedra de Cerealicultura, Facultad de Ciencias Agrarias, Universidad Nacional de La Plata. Flour was extracted using 70% aqueous ethanol (10 ml/g) under gentle shaking for 1 h at room temperature. The prolamin fraction was obtained as a clear supernatant after centrifugation in a Sorvall centrifuge for 15 min at 10,000×*g* and 8 °C.

Protein quantification. The protein contents of ethanol extracts and fractions were determined by the method described by Lowry et al [6]. As standard, a gliadin ethanol solution quantified by Kjeldahl's method was used as previously described [7].

Preparative electrophoretic procedure. Electrophoresis was performed using a PrepCell Model 491 [equipped with the 37 mm internal diameter (ID) gel tube] from Bio Rad (Hercules, Calif., USA) as previously described [5]. Gels of 7% acrylamide concentration and a lactate buffer system were used. Briefly, two different lactate buffers were employed. Buffer A was employed as an electrode buffer, consisting of 0.028 M lactic acid/aluminum lactate, pH 3.1 (prepared by dissolving aluminum lactate and adjusting 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 lactic acid and adjusting pH with KOH (again, prepared as stock buffer 50-fold concentrated and diluted immediately before use). Buffer B was used for preparation of the gel and pre-run. All buffers and solutions employed were filtered by 0.22 μm pore diameter nylon membranes (MSI Separations, Westboro, Mass., USA) and degassed immediately before use, employing a vacuum pump. 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 were 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 unpolymeryzed 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 pre-run 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 (1 h/cm of gel). After that, the buffer system was replaced and prolamin fractionation was performed using buffer A. In each experiment, a 4 ml sample (3 ml ethanolic extract+1 ml saturated sucrose in running buffer), containing approximately 10 mg of protein was loaded. Separation was carried out at 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 the use of a peristaltic pump, the Miniplus 3 from Gilson (Villiers le Bel, France). Samples were collected at 1 ml/h flow using running buffer as the collecting buffer, in aliquots of 1 ml, employing a fraction collector Microcol TDC 80 from Gilson.

Analytical electrophoresis. Fractions collected from the preparative system were analyzed by analytical polyacrylamide gel electrophoresis at pH 3.1 (A-PAGE). Electrophoresis was performed as described in literature [8] 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.

Capillary electrophoresis. Capillary electrophoresis (CE)was performed as described recently [9]. A HP ^{3D}Capillary Electrophoresis system (Hewlett-Packard, Waldbronn, Germany) and a 50 μm ID, 22 cm effective-length fused silica capillary column (30.5 cm total length) from Polymicro (Phoenix, Ariz., USA) were employed. Separations were performed using 0.1 M imminodiacetic acid (pH 2.30) as running buffer, containing 20% acetonitrile and 0.05% hydroxypropylmethyl cellulose {Sigma, H-7509, viscosity of 2% solution, 4000 centipoise [4 kg/(s m)]} The column temperature was maintained at 40 °C. Separations were performed at constant voltage (28 kV). Samples were filtered through 0.22 μm nitrocellulose membranes immediately before injection, and then injected for 5 s at 50 psi. On-line detection was carried out with a photodiode array system between 190 nm and 300 nm. Data were acquired using HP software and processed using SigmaPlot software (Jandel, San Rafael, Calif., USA). All patterns were normalized for comparison.

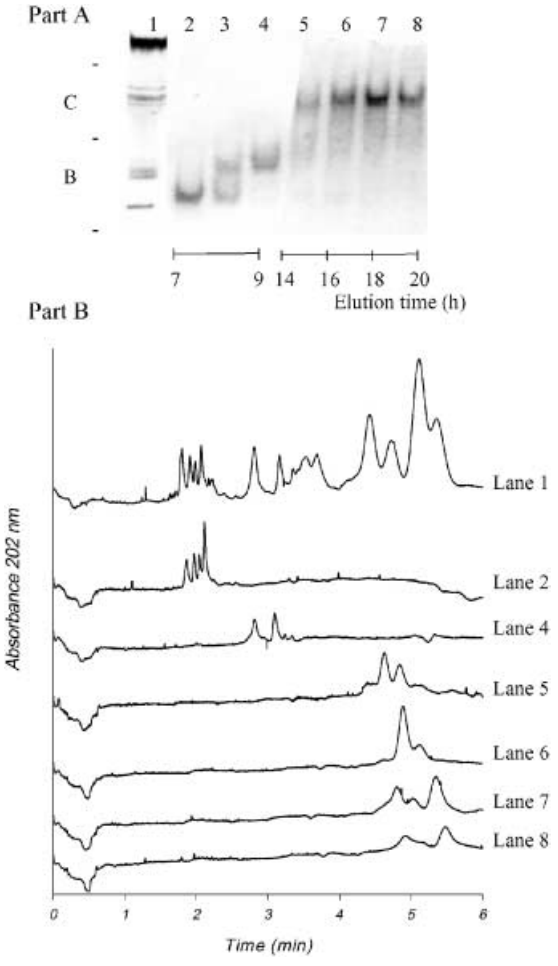

Fig. 1 **a** Analytical polyacrylamide gel electrophoresis at pH 3.1 (A-PAGE) of hordein fractions obtained from the preparative separation. Fifteen microliters of sample were loaded in each lane. *Lane 1* Ethanol extract used for the preparative separation, *lanes 2–8,* analysis of the different fractions. The elution time of each fraction is indicated. **b** Capillary electrophoresis (CE) analysis of different hordein fractions, performed at 28 kV constant in a imminodiacetic buffer 0.1 M. Ethanol extract was diluted tenfold 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 a different fraction obtained from the preparative gel. The correspondence with samples analyzed by A-PAGE is indicated

Results and discussion

Optimization of A-PAGE preparative fractionation of hordeins

As was previously described, the preparative system resolution was highly dependent on the separation gel length [5]. However, since prolamin patterns from rye and barley show a moderate number of bands, it was found that using 4 cm-length gels, a good separation of fractions could be achieved. A-PAGE analysis of hordein fractionation showed that at least three different fractions can be obtained (Fig. 1). The ethanol extract used as starting material for the preparative electrophoresis is

shown in lane 1, where the separation of the main components of ethanol extract, B and C-hordeins can be appreciated. A big amount of aggregated material that does not enter the gel is also observed. Two types of fast moving hordeins were obtained separately after 7 and 9 h of running (lanes 2 and 4), whereas a mix of both fractions was eluted at 8 h of running (lane 3). Between 14 h and 22 h of running, C-hordeins were eluted (lanes 5 to 8).

In order to evaluate the composition of fractions from the preparative separation, they were analyzed by CE using a zwitterionic buffer. The use of this buffer, that presents low conductivity, allows the use of higher ID capillaries and high voltages, lowering the analysis time and improving the loading capacity of the system, thus allowing the analysis of samples of lower concentration than other CE methods [9]. The CE analysis results and the correspondence to samples appearing in the A-PAGE analytical gel are shown in Fig. 1. Results of CE separation of hordeins are in concordance with previous reports [9, 10]. B- and C-hordeins showed different electrophoretic mobilities, having considerable concordance among prolamin mobility in both systems, CE in imminodiacetic buffer and A-PAGE. Fractions displaying higher mobility in the A-PAGE system also showed low migration times in CE. Fractions that appeared as a wide band in A-PAGE analysis were resolved in several peaks in the CE analysis. As can be observed, most fractions were heterogeneous when analyzed by CE but composed of proteins from closer mobility. No cross-contamination with components of different hordein groups could be found. This fact allowed us to pool the hordeins in groups that may be used for subsequent purification steps or for immunochemical characterization [11]. From 10 mg of proteins used in a single run, approximately 40% was recovered in the selected fractions. Unrecovered material includes precipitated protein in the top of the gel, albumin and globulin fractions that are eluted in the first tubes and tubes containing components from more than one fraction. Resolution of the preparative run was kept when increasing the protein load, until seeding 30 mg of total protein in the ethanol extract.

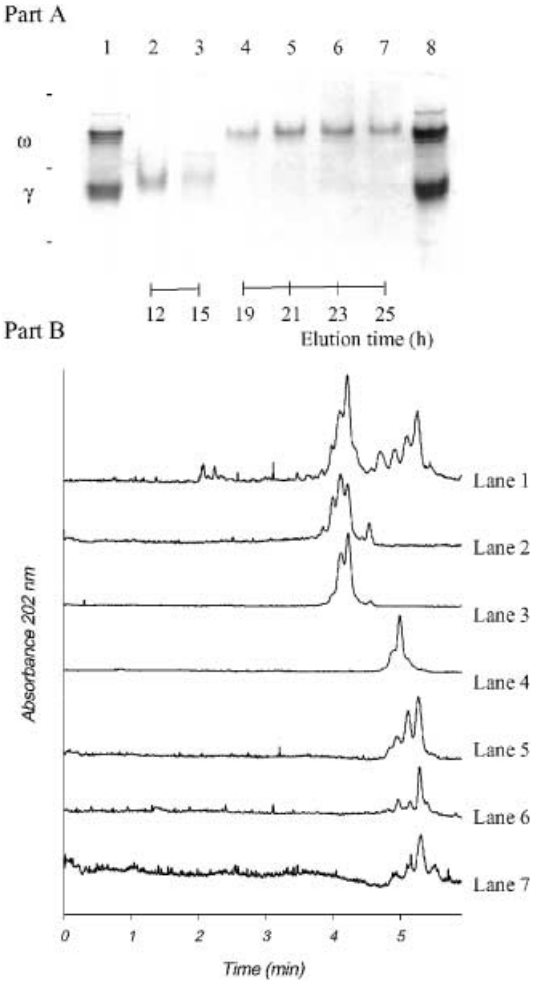

Fig. 2 **a** Analytical A-PAGE of secalin fractions from the preparative separation. *Lanes 1* and *8* Ethanol extract used for the preparative separation, *lanes 2–7* analysis of the different fractions. The elution time of each fraction is indicated. **b** CE analysis of different secalin fractions, performed in the same conditions reported in Fig. 1. Ethanol extract was diluted tenfold in 70% aqueous ethanol in order to use the same loading conditions for all samples. Results were normalized for comparative purposes. Each electropherogram belongs to a different fraction obtained from the preparative gel. The correspondence with samples analyzed by A-PAGE is indicated

Isolation of secalin fractions

Rye ethanol extract was fractionated using 4 cm-length preparative gels. A-PAGE and CE analysis of the fractions obtained is shown in Fig. 2. γ-Secalins were eluted between 12 and 15 h of separation, whereas ω-secalins were eluted between 19 and 26 h of running. The ethanol extract used as starting material for the preparative separation was also analyzed (lanes 1 and 8). Few non-resolved bands were present in each region of ω- and γ-secalins (lanes 2–7). CE analysis of these fractions showed that there are several components in each one, revealing minor compositional differences in fractions that appeared similar by the A-PAGE analysis. The composition of extracts of γ-secalins from lanes 2 and 3, that were similar by A-PAGE showed a different balance of components by CE. The same was observed for the ω-secalin fractions. This analysis allowed us to fractionate the secalins in two main groups with no cross-contamination of fractions from each other, subsequently named γ- and ω-secalins. Mobilities of components inside each fraction were very similar and no further fractionation could be achieved by increasing the length of the preparative gel (not shown). Preparative separations showed similar resolution when increasing the protein load up to 40 mg of total protein in the starting material.

It has been reported that the secalin fraction contains a group of polypeptides that have no homologous counterparts in other cereals [1]. This fraction is assigned to the γ-group due to its N-terminal primary structure, but it presents a high MW since it has undergone internal du-

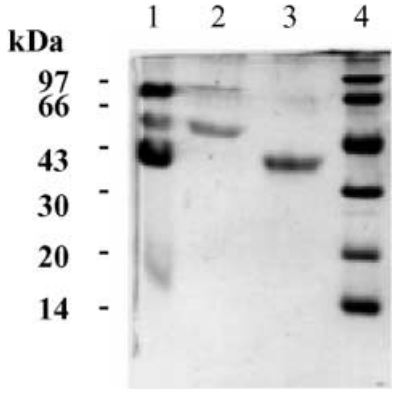

Fig. 3 SDS-PAGE analysis of purified secalin fractions. *Lane 1* Rye ethanol extract, *lane 2* A-PAGE-slow moving secalin fraction (ω+γ75-secalin, A-PAGE analysis shown in Fig. 2, lanes 4–5), *lane 3* A-PAGE-fast moving secalin fraction (γ40-secalin fraction, A-PAGE analysis shown in Fig. 2, lanes 2–3)

plication events during evolution [12]. Due to its MW of approximately 75 kDa, it is named as γ75-secalin. We performed an SDS-PAGE analysis of the purified secalin fractions in order to detect the presence of the γ75-secalin fraction (Fig. 3). Lane 1 shows the analysis of the ethanol extract used as starting material for preparative electrophoresis. Three main components were present in the rye ethanol extract, showing molecular weights of 40, 60 and 75 kDa approximately (lane 1). According to the literature, these polypeptides were respectively a γ-secalin fraction of 40 kDa, a ω-secalin fraction of about 60 kDa and a γ75-secalin fraction [13]. The fraction named γ-secalin, isolated by preparative A-PAGE was mainly composed of polypeptides of 40 kDa of MW by means of SDS PAGE (lane 3) and will be named subsequently as γ40-secalin, whereas the purified fraction named previously the ω-secalin fraction (lane 2) is composed of a mixture of ω-secalins of 60 kDa of MW and γ75-secalin. These two fractions showed similar mobility in A-PAGE and CE separations (Fig. 2, lanes 4–7).

In conclusion, it was shown that preparative electrophoresis at pH 3 is useful in producing a one-step fractionation of crude ethanol extracts from rye and barley. Fractions containing γ40-secalins, ω- and γ75-secalins and C-hordeins were obtained. Preparative A-PAGE may be a useful alternative procedure in cereal biochemistry to obtain partially purified prolamin fractions under non-denaturing conditions. Preparative electrophoresis can be combined with other methodologies for the preparation of purified prolamin components.

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