Fractionation of Wheat, Barley, and Rye Prolamins by Cation Exchange FPLC

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Wheat, rye, and barley prolamins were separated using optimized elution conditions by cation exchange FPLC on an analytical Mono-S column. Wheat gliadins were resolved into a larger number of peaks than has been obtained hitherto. Most fractions contained a mixture of components as determined by SDS–PAGE and A–PAGE. At least one fraction was obtained for each group of gliadins that contains only one major component with minimal contamination by other components. Only ω-gliadins occurred in five of the fractions. A preparative S-Sepharose Fast Flow column, run under similar chromatographic conditions, was used to isolate ω-gliadins. This procedure could be used as the first step for the purification of individual gliadin components. Chromatography on the Mono-S column was highly reproducible and showed promise as a means of differentiating wheat cultivars. Furthermore, FPLC was applied to fractionate prolamins from barley and rye.

Keywords: *FPLC; gliadin fractionation; prolamin fractionation*

INTRODUCTION

The major wheat storage protein groups are gliadin and glutenin, which are differentiated on the basis of their extractability. Up to 112 components can be found using high-resolution 2-D electrophoresis separation (Tkachuk and Mellish, 1987). The gliadin fraction is classified into α-, β-, γ-, and ω-gliadins, according to their relative mobilities by electrophoresis at pH 3 (Bushuk and Zillman, 1978). These proteins have a characteristic amino acid composition, notable features being the presence of large proportions of glutamine and proline residues. Their sequences contain extensive regions of tandem repeats of five to nine amino acid consensus sequences (Kasarda et al., 1983, 1984; Shewry et al., 1986). Although there are some differences, these repetitive sequences are found in all groups of gliadins, hordeins, and secalins (the last two being the prolamin fractions of barley and rye, respectively) (Kreis et al., 1985; Shewry and Tatham, 1990).

These highly polymorphic proteins contain many structurally related components with some similar biochemical properties. They have unusual extractability properties and have a marked tendency to aggregate. Such properties present many difficulties for the fractionation and characterization of the polypeptides. The structural homology among prolamins is also responsible for their known high degree of immunological cross-reactivity, thus making their purification difficult, even by immunochemical methods (Skerritt and Underwood, 1986; Festenstein et al., 1987).

Gliadin polypeptide have been separated traditionally by conventional chromatographic methods, such as gel filtration followed by ionic exchange on sulfoethylcellulose (Huebner and Rothfus, 1968), (carboxymethyl)-cellulose (Patey and Evans, 1973) or sulfopropyl-Sephadex (Charbonnier and Mossé, 1980). The best analytical separation has been obtained by employing reversed-phase high-performance liquid chromatography (RP-HPLC) (Bietz, 1985), but only small amounts of proteins could be recovered. Higher resolution and shorter analysis times than those of low-pressure chromatography have been reported recently using fast protein liquid chromatography (FPLC) on anion exchanger (Mono-Q) (Batey, 1984) or cation exchanger (Mono-S) (Larre et al., 1991).

In the study presented here we have developed improved conditions for the analytical and preparative cation exchange FPLC of gliadin proteins and have explored the usefulness of the procedure for the differentiations of wheat cultivars. Furthermore, this is the first study in which cation exchange FPLC was applied to fractionate hordein and secalin proteins.

MATERIALS AND METHODS

Cereal Cultivars. Wheat cultivars included *Triticum aestivum* L. (hard red spring wheat) INTA La Paz, Staparka, Buck Bagual, Buck Pucara, INTA Marcos Juarez, Buck Fogon, Buck Catriel, ProINTA Pigue, and ProINTA Oasis. The barley cultivar used was *Hordeum vulgare* L. Linea 371; the rye cultivar used was *Secale cereale* L. Insave FH.

Prolamin Preparation. The gliadin fraction was extracted from ground grain using 70% (v/v) aqueous ethanol extraction (10 mL/g, 2 h at room temperature) after prior extraction of the albumin–globulin fraction using 0.15 M NaCl (10 mL/g, 4 × 1 h at room temperature). After each extraction step, a clear supernatant was obtained by centrifugation at 10000*g* for 15 min at 8 °C. The whole gliadin fraction was lyophilized, dissolved in and dialyzed against the FPLC starting buffer, and finally filtered through a 0.22 μm Nucleopore membrane (Millipore). The protein concentration was determined according to the Lowry method (Lowry et al., 1951) using a gliadin solution as standard.

Hordein and secalin fractions were obtained by the same procedure.

Cation Exchange FPLC. FPLC separations were carried out on a Pharmacia FPLC system. Two columns were used:

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0021-8561/94/1442-2460$04.50/0

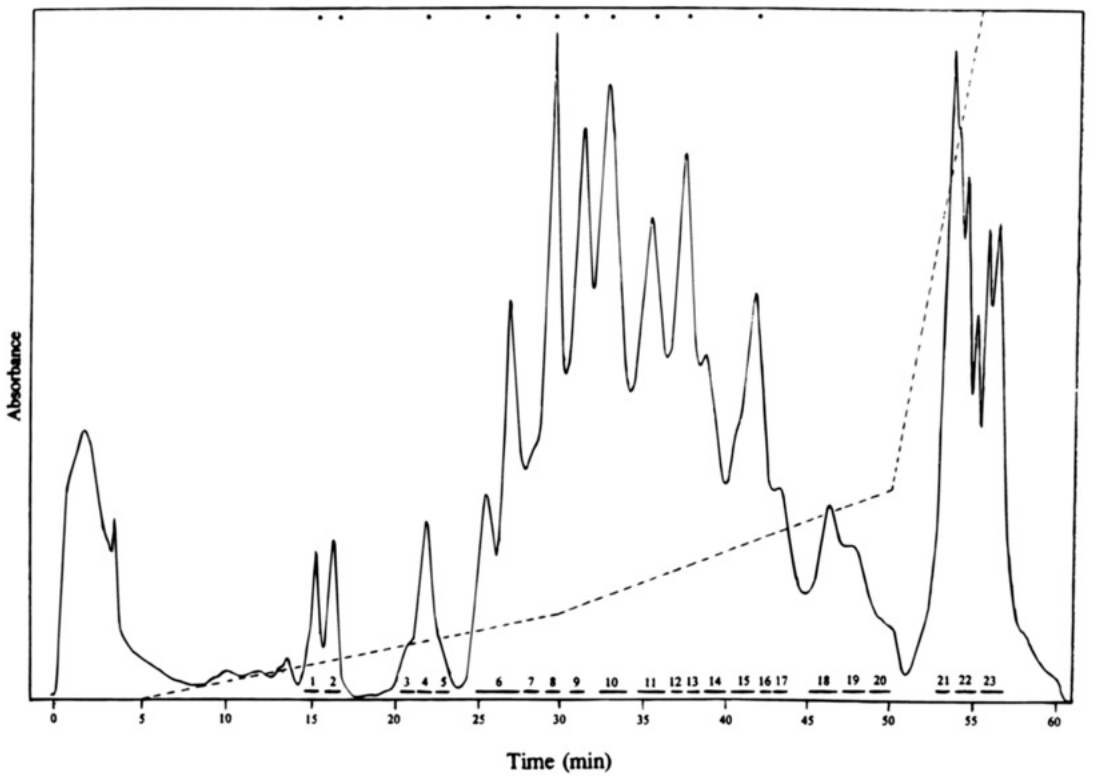

Figure 1. Elution profile of gliadin proteins from cv. INTA La Paz wheat fractionated by cation exchange FPLC. Four milligrams of gliadin extract in 2 mL was applied to a Mono-S column. Elution was at 1 mL/min, fraction volume 1 mL. Dashed line indicates the gradient of NaCl concentration: 0%, 0–5 min; 0–12%, 5–30 min; 12–30%, 30–50 min; 30–100%, 50–55 min; and 100%, 55–60 min. Column effluent was monitored at 280 nm with 0.2 AUFS. (●) Peaks used to calculate the coefficient of variation of retention time.

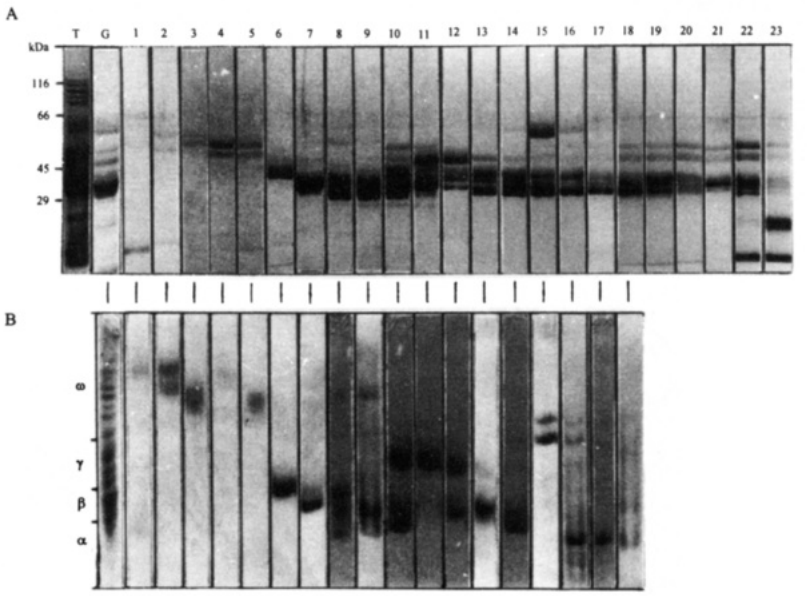

Figure 2. SDS–PAGE (A) and A–PAGE (B) patterns of gliadin fractions separated by cation exchange FPLC. T, total wheat proteins; G, gliadin extract. Fraction numbers correspond to those in Figure 1.

a Mono-S HR 5/5 column with a flow rate of 1 mL/min, back pressure of 1.5–2 MPa, and fraction volume of 1 mL and a Pharmacia XK 16/40 column packed with S-Sepharose gel (about 60 mL), flow rate of 4 mL/min, back pressure of 0.5 MPa, fraction volume of 4 mL. The columns were equilibrated with starting buffer (buffer A: 2 M urea, 0.05 M citric acid adjusted to pH 3 with 0.1 M NaOH).

All elutions were performed at room temperature with linear NaCl gradient concentration steps (buffer B: 0.8 M NaCl, 2 M urea, 0.05 M citric acid, adjusted to pH 3 with 0.1 M NaOH). Protein elution was detected by measuring absorbance at 280 nm. The columns were washed after two or three runs with buffer B and 2 M NaOH until no material was detected at 280 nm; the columns were then equilibrated with buffer A.

Collected fractions were lyophilized. The fractions were dissolved and dialyzed against 0.01 M acetic acid before using.

Electrophoresis. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed under reducing conditions according to the discontinuous buffer system of Laemmli (1970) with an acrylamide concentration gradient (10–15%).

Polyacrylamide gel electrophoresis at acid pH (A–PAGE) was carried out according to the method of Lafiandra and Kasarda (1985) with a 7% acrylamide gel concentration.

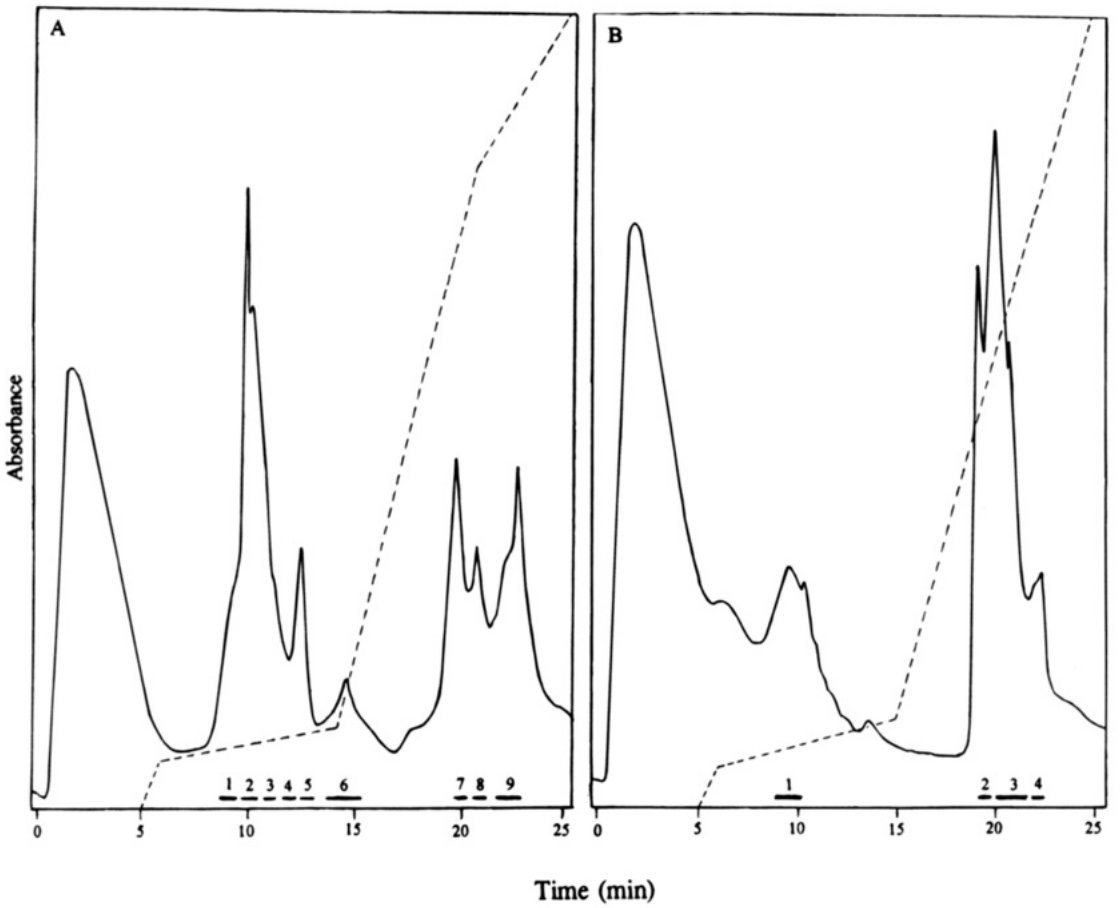

Figure 3. Elution patterns of hordein (A) and secalin (B) proteins fractionated by cation exchange FPLC on the Mono-S column. Volume injected was 2 mL. Flow rate was 1 mL/min. Fraction volume was 1 mL. Column effluent was monitored at 280 nm with 0.2 AUFS. (A) Protein injected, 0.8 mg. Gradient of NaCl concentration: 0%, 0–5 min; 0–6%, 5–6 min; 6–10%, 6–15 min; 10–80%, 15–22 min; 80–100%, 22–25 min; 100%, 25–30 min. (B) Protein injected, 1.6 mg. Gradient of NaCl concentration: 0%, 0–5 min; 0–6%, 5–6 min; 6–11%, 6–15 min; 11–100%, 15–25 min; 100%, 25–30 min.

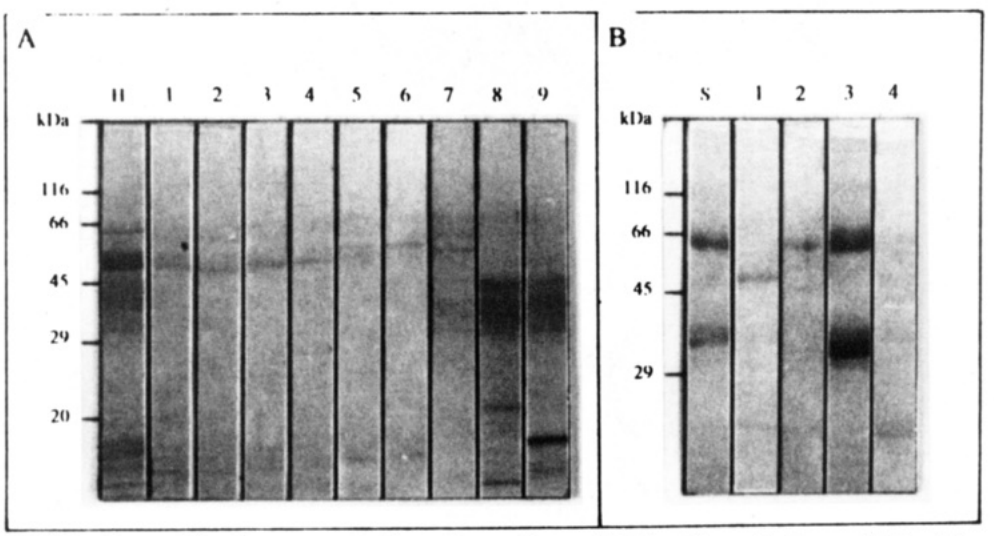

Figure 4. SDS–PAGE patterns of fractions separated by cation exchange FPLC on Mono-S column: (A) hordeins and (B) secalins. H and S, hordein and secalin extracts, respectively. Fraction numbers correspond to those in Figure 3.

After both electrophoretic separations, proteins were stained with 0.1% Coomassie Brilliant Blue R-250 in acetic acid/methanol/water (10:25:65).

RESULTS

Gliadin Fractionation. Wheat prolamins were fractionated by cation exchange FPLC, and all of the fractions obtained were analyzed by SDS–PAGE and A–PAGE. Figure 1 shows the elution profiles from FPLC separation of gliadin proteins from INTA La Paz wheat using a Mono-S column.

Four milligrams of gliadin extract was injected onto the column for FPLC fractionation. After chromatography, the gliadin components were observed to be distributed among many fractions. To prepare enough protein for the electrophoretic analysis, successive runs of gliadin extracts were performed and the corresponding fractions were pooled. The reproducibility of the

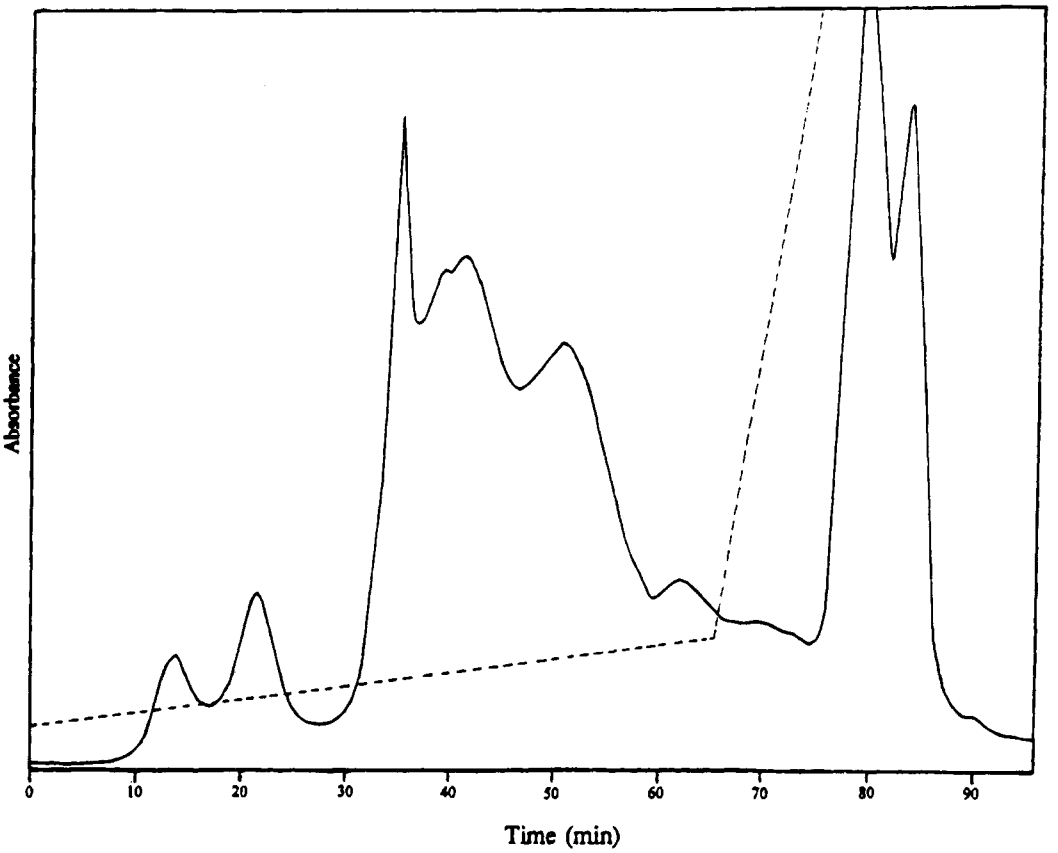

Figure 5. Elution pattern of gliadin proteins from cv. INTA La Paz wheat fractionated by a cation exchange FPLC on S-Sepharose preparative column. Thirty-five milligrams in 10 mL was injected. Flow rate was 4 mL/min. Fraction volume was 4 mL. Column effluent was monitored at 280 nm with 0.2 AUFS. Gradient of NaCl concentration: 6–17%, 0–65 min; 17–100%, 65–75 min; 100%, 75–90 min.

protein separation was assessed after seven runs of the same sample under the same conditions. The elution profile was found to be highly reproducible; only minor differences in intensity could be observed in some peaks. The mean coefficient of variation (CV) of the retention times calculated from the 11 best defined peaks indicated in Figure 1, for seven replicates, was 0.52%.

The elution conditions were optimized so as to obtain good resolution for all groups of gliadins. The elution pattern in Figure 1 shows about 25 peaks. Some of the lanes shown in Figure 2 correspond to pooled fractions. Pooling was carried out only in those cases in which contiguous fractions appeared to have identical composition after SDS–PAGE and A–PAGE analysis. By SDS–PAGE and A–PAGE, 23 fractions were obtained that differed in polypeptide composition. The elution order was generally related to the electrophoretic mobility observed in A–PAGE. In accordance with Larre et al. (1991), the first fractions eluted (components weakly retained by the column) contained non-gliadin components. The ω-gliadins were eluted by 23 min. As shown by Popineau et al. (1986), a small increase in ionic strength (0.07 M NaCl) was sufficient for the complete elution of ω-gliadins. Five of the fractions (1–5) contained ω-gliadins; SDS–PAGE and A–PAGE showed (Figure 2) that these fractions, which are different from one another, are mainly composed of ω-gliadins with only minor contaminants.

As described by other authors (Larre et al., 1991), when the ionic strength was increased, the separation was not so efficient, and in one fraction components of different groups of gliadin can be detected (e.g., fraction 8 contains α-, β-, and ω-gliadin, and fraction 10 contains α-, β-, and γ-gliadin). Nevertheless, at least one fraction consisting of a major component of each group of gliadins, with small amounts of contaminants, was obtained (e.g., γ-gliadins, lane 6; β-gliadins, lane 7; and α-gliadins, lane 17).

Fractions 18–20 have components of all groups of gliadins. The three fractions eluting last, fractions 21–23 (material strongly retained by the column), correspond to a mixture of glutenin and gliadin components, as judged by SDS–PAGE under reducing conditions; a similar result was reported by other authors (Larre et al., 1991; Popineau et al., 1986).

Hordein and Secalin Fractionation. Prolamins of barley and rye were fractionated by FPLC cation exchange. The best results were obtained using a procedure similar to that applied to gliadins. Both separations were carried out in just 25 min (Figure 3). A greater number of peaks was not obtained with a longer separation time (up to 60 min). The first peak eluted contained neither hordein nor secalin proteins.

Hordein fractions 1–6 (Figure 4A) comprised different B- and C-hordeins as major components, together with small amounts of other components. Fractions 7–9 (components eluted at high ionic strength) are a mixture of B-, C-, and D-hordeins.

A low number of different fractions was obtained in secalin fractionation (Figure 4B). According to their M_r, fractions 1 and 2 are mainly formed by ω- and γ-secalins. Fractions 3 and 4 are composed of a mixture of several secalin components.

Preparative FPLC. The amount of protein injected onto the analytical column was approximately 4 mg. Even though the maximum recommended loading for the Mono-S column is 25 mg, an increase in the amount injected led to a lower degree of resolution. On the other

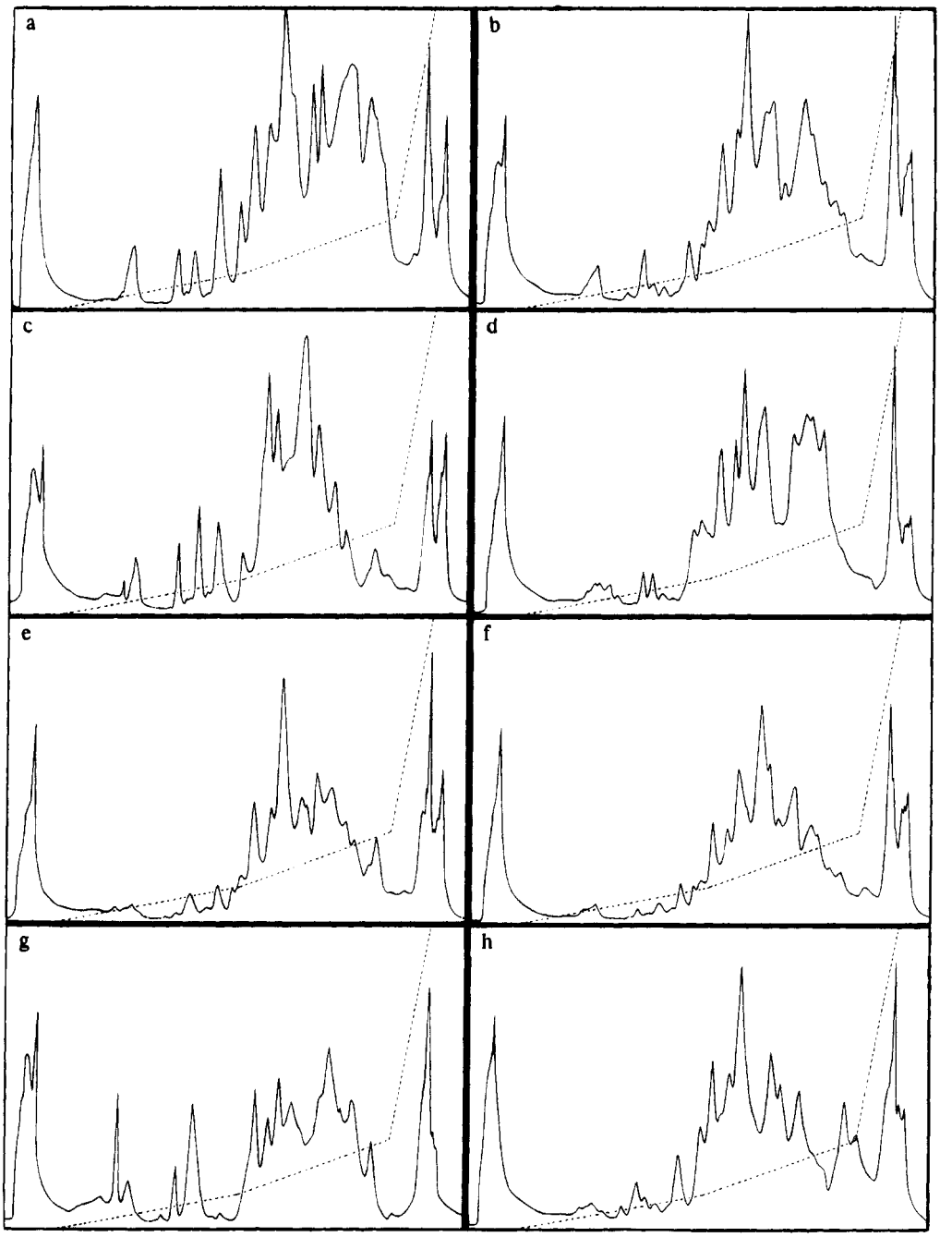

Figure 6. Comparison of cation exchange FPLC profiles of gliadin extracts from different hard red spring wheat cultivars: (a) Staparka, (b) Buck Bagual, (c) Buck Pucara, (d) INTA Marcos Juarez, (e) Buck Fogon, (f) Buck Catriel, (g) ProINTA Pigue, (h) ProINTA Oasis. Elution conditions were as Figure 1. In all cases approximately 4 mg of gliadin extracts was injected.

hand, the low solubility of gliadins does not make it possible to work at higher concentrations under optimal separation conditions. Consequently, the use of columns of higher capacity was necessary to recover higher amounts of the isolated prolamins.

To obtain larger amounts of purified gliadins, an S-Sepharose XK 16/40 column injected with approximately 35 mg of protein was employed. The resolution of the preparative column was lower than that of the Mono-S column (Figure 5). Preparative chromatography was performed in a shorter time (90 min) than other preparative methods.

After SDS–PAGE analysis of all the collected fractions, it could be observed that fractions 1 and 2 consisted of ω-gliadins only, while the other fractions (3–8) included different mixtures of α-, β-, and γ-gliadins (not shown).

FPLC Gliadin Patterns from Different Wheat Cultivars. Eight wheat cultivars were analyzed by FPLC on the Mono-S column under the same elution conditions as used for gliadin separation. The elution profiles are depicted in Figure 6. Important qualitative and quantitative differences among the patterns corresponding to the different wheat cultivars were observed. For example, cultivars giving profiles a, c, and g had higher contents of components with elution times up to 30 min than other cultivars, although differences among those were also apparent. On the other hand, cultivars giving profiles c, e, and f had lower proportions of components in the region eluted between 35 and 45 min than cultivars giving profiles a, b, and d.

Analysis of gliadins from some varieties of hard red spring wheat by means of the chromatographic separa-

tion described should make possible the differentiation between wheat cultivars.

DISCUSSION

Several methods, such as low-pressure chromatography (Huebner and Rothfus, 1968; Patey and Evans, 1973; Charbonnier and Mossé, 1980), RP-HPLC (Bietz, 1985), and recently ion exchange FPLC (Batey, 1984; Larre et al., 1991), have been employed for the fractionation of crude gliadin. Separation of cereal proteins is difficult because of their biochemical characteristics, inextractability, homology, etc. This difficulty led to the use of different methods, most of which yield fractions containing several components or only small amounts of individual proteins. The problems of obtaining large enough amounts of pure proteins or proteins of adequate purity pose drawbacks to the study of their physicochemical and functional properties, immunochemical characterization, amino acid sequence, etc. A procedure to separate wheat, barley, and rye prolamins by cation exchange FPLC, which makes possible a rapid and efficient separation of several of those proteins, is described here.

Recently, Batey (1984) was able to differentiate wheat cultivars using anion exchange FPLC, but the elution profiles showed relatively few peaks. Larre et al. (1991), using cation exchange FPLC on a Mono-S column, obtained a greater number of peaks. The procedure described here, when applied to wheat, gave profiles containing more peaks and in shorter time than those described above (Larre et al., 1991). Twenty-three different fractions were obtained by chromatography. Some of these fractions, as judged by SDS–PAGE and A–PAGE analysis, comprised isolated components of only one group of gliadins with small amounts of contaminants.

The degree of resolution obtained with the analytical column was higher than that of the preparative one. Some fractions comprising mainly ω-gliadins could be obtained with the preparative column. The other fractions, although containing several components of α-, β-, and γ-gliadins, nevertheless provide good starting material for subsequent purification by other techniques.

Significant differences were observed in the profiles of the different wheat cultivars analyzed. This technique has a lower level of resolution than RP-HPLC (Bietz, 1985). Nevertheless, the good resolution obtained, with a high number of peaks and high reproducibility of the separations, means that the technique may be useful as a preliminary means of characterizing cultivars.

On the other hand, the system employed here made possible the separation of several components of prolamins of barley and rye. It is worth noting that no previous reports of hordein and secalin separation by FPLC have been found in the literature.

This paper reports an improved separation of wheat gliadins by cation exchange FPLC. Other workers have previously reported similar separations, but the conditions given in the present work offer a far superior resolution. In addition, it extends the separation to prolamins from barley and rye.

ACKNOWLEDGMENT

This work was financed with grants from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET). C.A.F. is also member of the Instituto de Estudios de la Inmunidad Humoral (IDEHU), where part of the experiments were performed.

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Received for review April 18, 1994. Accepted August 23, 1994.[⊗]

[⊗] Abstract published in *Advance ACS Abstracts,* October 15, 1994.