Separation of Wheat Gliadins by Preparative Reversed-Phase High-Performance Liquid Chromatography

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ABSTRACT

A preparative method is described for separating wheat gliadins in significant quantities by using reversed-phase high-performance liquid chromatography. The method has advantages over previous methods in simplicity and speed. It separates proteins by hydrophobicity rather than by size or ionic strength. This, in conjunction with separations by molecular size and by ion-exchange chromatography, enabled further separation of four of those fractions into at least 15 subfractions. The separated fractions were also analyzed by aluminum lactate gel electrophoresis and by isoelectric focusing, which showed that each of the above fractions still contained more than one component.

Gliadin proteins extracted with 70% ethanol from wheat endosperm are highly heterogeneous (Huebner and Wall 1966, Wrigley 1970, Jones et al 1982). Their molecular weights (mol wt) range from 27,000 to 125,000 with gliadins of higher molecular weight consisting of smaller subunits linked by disulfide bonds (Huebner et al 1967, Bietz et al 1977, Bietz and Wall 1980). However, subunits that form gliadin proteins of higher molecular weight differ significantly from gliadins of lower molecular weight, consisting of single polypeptide chains having molecular weight of 27,000–36,000; a method for isolating milligram quantities of these lower molecular weight gliadins is described in this paper.

Various gliadin components have been separated by ion-exchange and gel-filtration column chromatography (Huebner et al 1967; Charbonnier 1974; Ewart 1977a, 1977b, 1981; Terce-Laforgue et al 1980). Although most resulting fractions appeared pure by one-dimensional electrophoresis, two-dimensional gel electrophoresis (isoelectric focusing [IEF] followed by pH 3.2 aluminum lactate electrophoresis or sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE]) showed that most consist of more than one component (Wrigley 1970, Brown and Flavel 1981). Isolating individual gliadins is difficult because of their great heterogeneity resulting from gene duplication, mutation, and polyploidy (Bietz 1982).

Recently, a new method of separating cereal proteins was developed through the use of reversed-phase (RP) high-performance liquid chromatography (HPLC) (Bietz 1983). This procedure has advantages over traditional column chromatography or electrophoresis in terms of rapidity, simplicity, high resolving power, and high sensitivity. RP-HPLC resolves proteins by differences in surface hydrophobicity, and therefore complements other separation methods that differentiate proteins on the basis of molecular size or ionic charge. Initial tests of large-pore RP-HPLC columns have demonstrated that preparative separations of gliadin proteins can be achieved with resolutions comparable to analytical columns, and that resulting fractions, though still heterogeneous, are considerably purified (Burnouf and Bietz 1984). The availability of preparative RP-HPLC columns has now also facilitated separation of large quantities of gliadins from ion-exchange chromatography fractions in a short period of time. This paper describes such a use of preparative RP-HPLC to isolate gliadin proteins, and the characterization of the isolated proteins by electrophoresis.

[1]Mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

MATERIALS AND METHODS

Chemicals and Reagents

Acetonitrile (ACN) was obtained from Mallinckrodt (Chrom AR grade) or Fisher (HPLC grade). Trifluoroacetic acid (TFA) (Sequanal grade) was from Pierce. Aluminum lactate (lot 39141-A) was from ICN Pharmaceuticals, Inc. Acrylamide (electrofocusing grade) and ampholines were obtained from LKB. All other materials were of reagent grade or better. Water used for HPLC was obtained from a Barnstead NANO pure system, and had a specific conductance of, at most, 15 megohm-cm. All HPLC solvents were filtered (0.22 μm) and deaerated before use.

Gliadin Preparation

The hard red winter wheat variety Centurk was grown in 1979 near Peoria, IL. It was milled on a Buhler laboratory flour mill to 65% flour extraction, defatted twice with dry *n*-butanol (7:1 ratio) for 20 min, washed for 10 min with petroleum ether, air-dried, and then extracted with 0.1 *N* NaCl (at 4–5° C) to remove most albumins and globulins. Gliadins were then extracted with 70% ethanol (at room temperature) and fractionated according to molecular weight on Sephadex G-100 (Beckwith et al 1966). Resulting low-molecular-weight gliadins were next fractionated on sulfoethyl (SE) cellulose (2.5 × 26 cm column) using a salt gradient in a solvent consisting of 2*M* DMF, 0.03*N* acetic acid, and 0.005*N* HCl (pH 2.45 ± 0.05) (Huebner and Wall 1966). The salt gradient was generated using seven chambers of a Varigrad device (Buchler Instruments), each of which contained 200 ml of solvent containing 0.045, 0.06, 0.06, 0.07, 0.08, 0.10, and 0.16*N* NaCl, respectively. Fractions from the SE cellulose column were then further fractionated by preparative RP-HPLC.

HPLC

The apparatus used for preparative RP-HPLC included a Spectra-Physics SP8700 solvent-delivery system, WISP 710A automatic sample injector, Beckman model 165 dual-channel variable-wavelength detector usually set at 276 and 225 nm, and a Pharmacia FRAC-100 fraction collector. The support used was a preparative (250 × 10 mm i.d.) SynChropak RP-P reversed-phase (C_{18}) column having 300Å pores (SynChrom, Linden, IN). The column was operated at room temperature (22–25° C) at 3 ml/min, and samples were eluted using linear gradients generated from solvent A (15% aqueous ACN + 0.05% TFA) and solvent B (80% aqueous ACN + 0.05% TFA). These conditions were similar to those used by Bietz (1983), except that the TFA concentration was reduced from 0.1% in an attempt to avoid protein deamidation. Samples of 35–50 mg of lyophilized protein were dissolved in 0.3–0.4 ml of 20% ACN + 0.05% TFA, found to be a good solvent for gliadins. All samples were centrifuged (5 min, in a small desk-type clinical centrifuge) before RP-HPLC analyses; generally 270 μl was applied for each run. Up to 50–60 mg of protein could be applied to the preparative RP-HPLC column without serious

overloading, but closely eluting components were not as well resolved using this sample size. Data were recorded on a Kipp and Zonen BD41 recorder. Samples were usually removed from the fraction collector within 20 min after elution and stored in a refrigerator for no more than a few hours before being frozen and lyophilized. Recoveries were estimated gravimetrically or on the basis of relative peak areas; Bietz (1983) previously observed near-quantitative recovery of gliadins from SynChropak RP-P.

Electrophoresis

An LKB 2001 vertical slab-gel electrophoresis apparatus was used to analyze separated proteins, using pH 3.2 aluminum lactate buffer according to a modification of the method of Tkachuk and Mellish (1980). Because we experienced difficulty polymerizing gels at pH 3.1, we raised the pH to 3.2, which is often used by other workers. Also, the use of H_2O_2 (Tkachuk and Mellish 1980) requires cooling solutions to near 0°C, and even then polymerization is very fast; sometimes nonuniform polymerization results that is not discernible until the gel is removed from the glass plates. By changing the concentrations of ferrous sulfate and ascorbic acid to those used by Maier and Wagner (1980) for acetic acid-glycine buffers (1.7 mg ferrous sulfate and 53 mg ascorbic acid per 100 ml), and by using 13 mg of ammonium persulfate per 100 ml of gel in place of H_2O_2, polymerization could be done reliably at room temperature within 2–4 min.

Isoelectric focusing (IEF) was performed on 5% gels using 6*M* urea and equal parts of pH 6–8 and pH 3–10 ampholines (LKB) (total 2%) on an LKB Multiphor apparatus (Wall et al 1984).

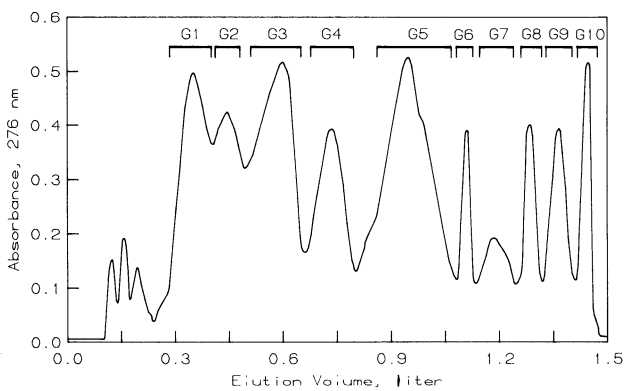

Fig. 1. Column chromatography of low molecular weight gliadins on sulfoethyl cellulose (SE cellulose). The column was eluted at room temperature with 2*M* DMF, 0.03*N* acetic acid, 0.005*N* HCl, pH 2.45 ± 0.05 containing 0.045*M* to 0.16*M* NaCl. Dark lines above the peaks indicate volumes pooled for each fraction.

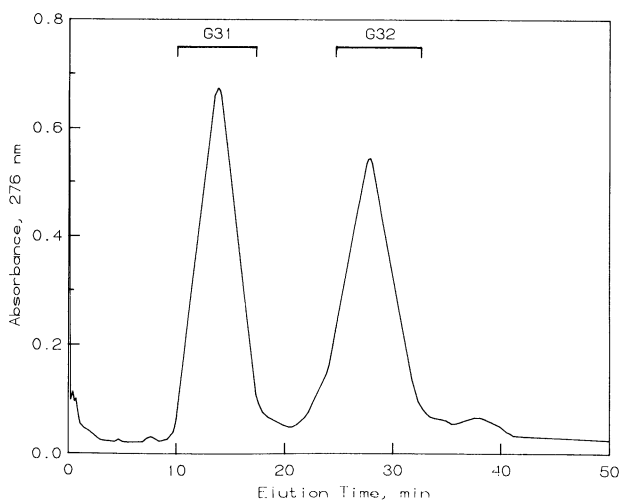

Fig. 2. RP-HPLC of gliadin fraction G3 from sulfoethyl cellulose (Fig. 1). Flow rate, 3 ml/min. Samples of 30–50 mg were eluted at room temperature during 50 min with a linear gradient from 28 to 50% acetonitrile (containing 0.05% trifluoroacetic acid). Samples were pooled as indicated by bars above the peaks.

RESULTS

RP-HPLC of Gliadin Fractions

Gliadin fractions for subsequent separation by preparative RP-HPLC were obtained by ion-exchange column chromatography on SE cellulose (Fig. 1). Peaks numbered G1–G7 correspond to γ- through α-gliadins (Huebner and Wall 1966). A small amount of undefined protein eluted before peak G1, and peaks G8–10 correspond to water-soluble proteins coextracted with gliadins by 70% ethanol (Huebner and Wall 1966).

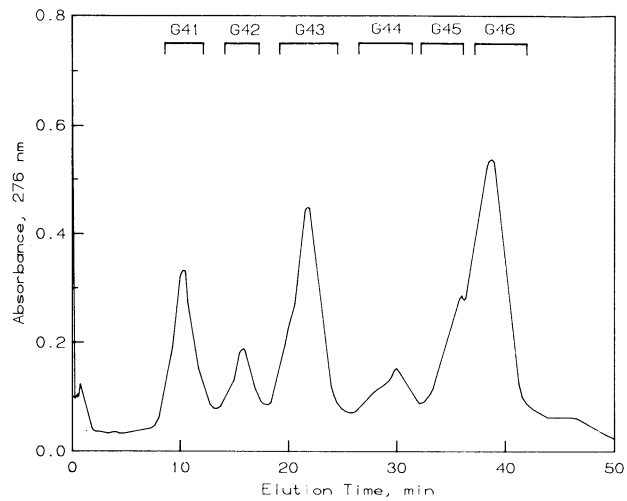

Fig. 3. RP-HPLC of gliadin fraction G4 from sulfoethyl cellulose (Fig. 1). Flow rate, 3 ml/min. Samples of 30–50 mg were eluted at room temperature during 50 min with a linear gradient from 28 to 50% acetonitrile (containing 0.05% trifluoroacetic acid). Samples were pooled as indicated by bars above the peaks.

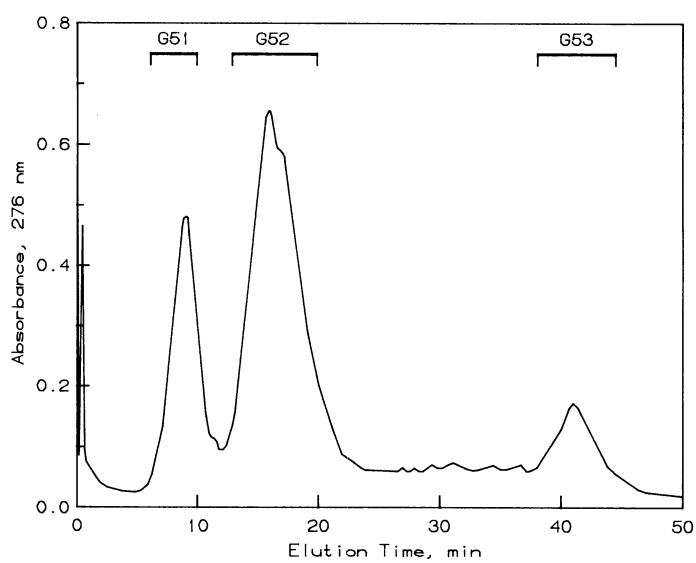

Fig. 4. RP-HPLC of gliadin fraction G5 from sulfoethyl cellulose (Fig. 1). Flow rate, 3 ml/min. Samples of 30–50 mg were eluted at room temperature during 50 min with a linear gradient from 28 to 50% acetonitrile (containing 0.05% trifluoroacetic acid). Samples were pooled as indicated by bars above the peaks.

Figure 2 shows the preparative RP-HPLC separation of gliadin fractions G3 (Fig. 1). The fraction was separated on the basis of differences in surface hydrophobicity into two main peaks, plus a small amount of unretarded material and several small peaks (approximately 16% of the material recovered). Dried samples were weighed to determine the percentage of each fraction. Although the area of peak G31, as determined by absorbance at 276 nm, was larger than that of peak G32, the actual amount of recovered protein was 32% for peak G31 and 52% for peak G32, apparently reflecting different proportions of tryptophan and tyrosine. Previously, a γ-gliadin was found with apparently no tryptophan (Huebner et al 1967). Although wavelengths in the range 200–230 nm accurately measure protein on the basis of peptide bonds, the most commonly used wavelength, 210 nm, cannot usually be used with preparative RP-HPLC because the resulting absorbance exceeds the range of the detector. The less sensitive wavelength, 225 nm, can be used, however, to detect all proteins, including those having little absorbance at 276 nm.

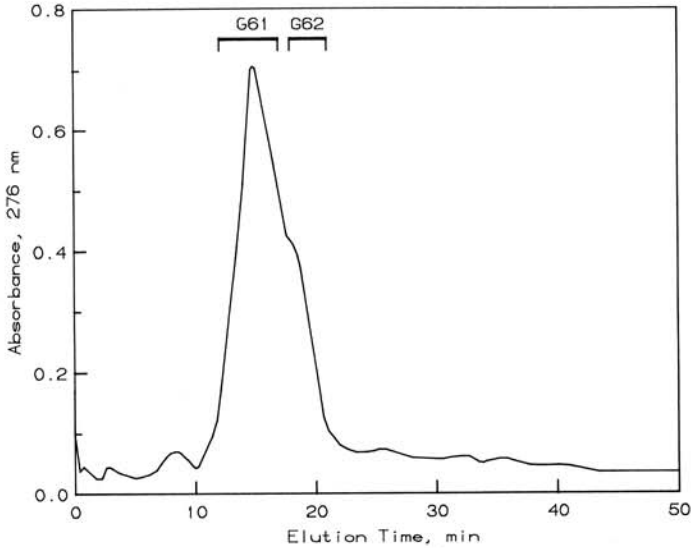

Fig. 5. RP-HPLC of gliadin fraction G6 from sulfoethyl cellulose (Fig. 1). Flow rate, 3 ml/min. Samples of 30–50 mg were eluted at room temperature during 50 min with a linear gradient from 28 to 50% acetonitrile (containing 0.05% trifluoroacetic acid). Samples were pooled as indicated by bars above the peaks.

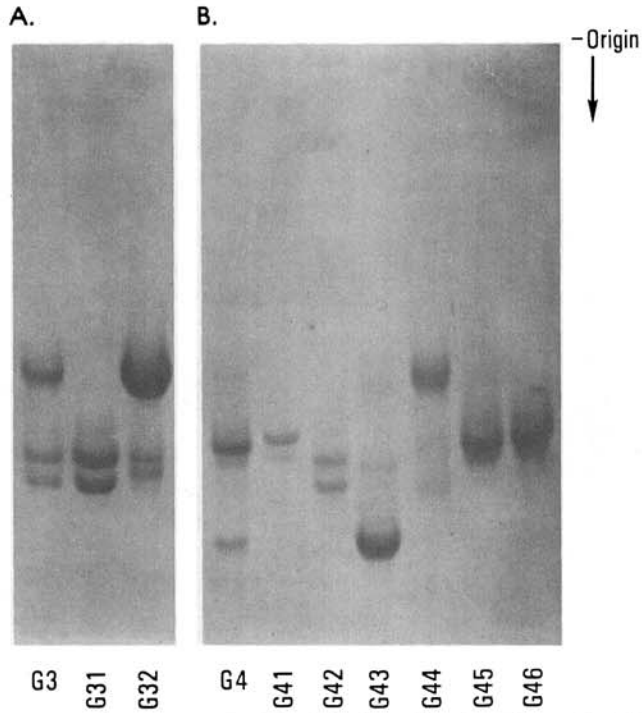

Fig. 6. Aluminum lactate PAGE of gliadin subfractions from sulfoethyl cellulose and RP-HPLC. **A,** Gliadin sulfoethyl cellulose fraction G3 (Fig. 1) and HPLC subfractions G31 and G32 (Fig. 2). **B,** Gliadin sulfoethyl cellulose fraction G4 (from Fig. 1) and HPLC subfractions G41-46 (from Fig. 3).

RP-HPLC separated gliadin SE cellulose fraction G4 (Fig. 1) into six subfractions (Fig. 3); obviously, SE cellulose fraction G4 is more complex than fraction G3; furthermore, G43 and G44 also appear to contain unresolved shoulders (Fig. 3).

Gliadin SE cellulose fraction 5 (Fig. 1) separated into two main peaks, one of which (peak G52) was itself heterogeneous, plus several minor components (Fig. 4). Rechromatography of peak G52 on the RP-HPLC column using a shallower gradient (32–38% ACN) also failed to completely separate these components; possibly other chromatographic conditions, such as different solvents, organic modifiers, or bonded phases, could enhance this separation.

The preparative RP-HPLC separation of gliadin SE cellulose fraction 6 (Fig. 1) resulted in a single major peak containing two unresolved components, plus at least six minor peaks (Fig. 5).

Electrophoresis

Figure 6 shows aluminum lactate PAGE patterns of SE cellulose fractions G3 and G4 (Fig. 1), and resulting subfractions obtained

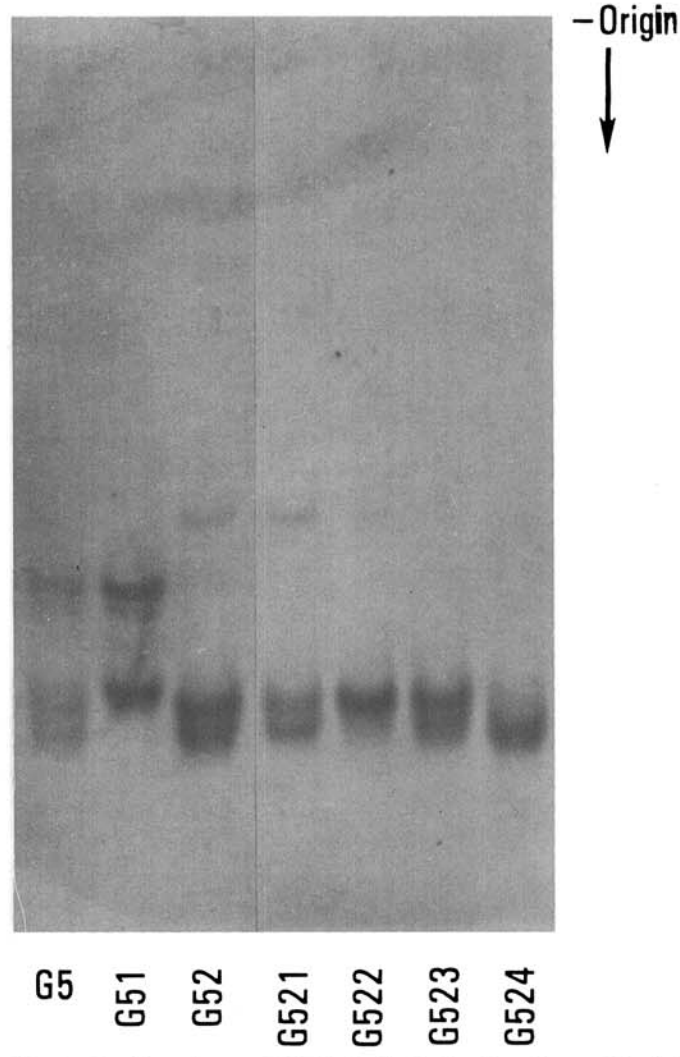

Fig. 7. Aluminum lactate PAGE of gliadin fractions from sulfoethyl cellulose and RP-HPLC. Gliadin sulfoethyl cellulose fraction G5 (from Fig. 1) and HPLC subfractions G51 and G52 (from Fig. 4). Patterns labeled G521-G524 were obtained by rechromatography of fraction G52 using a 32–38% ACN gradient.

by preparative RP-HPLC (Figs. 2 and 3). The results demonstrate that a significant purification of each SE cellulose fraction was achieved through preparative RP-HPLC. Most subfractions now exhibit only one or two major components upon PAGE, although some that had contained unresolved shoulders are obviously still heterogeneous. In addition, each polypeptide generally is found in only a single HPLC peak, with the possible exception of the two most rapidly migrating components of fraction G3, which may associate to a minor degree with the slower migrating component.

Figure 7 illustrates aluminum lactate PAGE patterns of SE cellulose fraction G5 (Fig. 1), and of subfractions of SE cellulose fraction G5 obtained via preparative RP-HPLC (Fig. 4). Subfractions G51 and G52 of SE cellulose fraction G5 again have very different mobilities in this type of electrophoresis. When fraction G52 was rechromatographed by RP-HPLC, using a shallow (32–38% ACN) gradient (results not shown), it partially resolved into its two major components, as demonstrated by PAGE (Fig. 7, G521-G524). These results demonstrate that it is sometimes possible to achieve improved resolution of gliadins by RP-HPLC through the use of shallow gradients, even with the same column. Subfractions of gliadin SE cellulose fraction G6 (Fig. 1) obtained through preparative RP-HPLC (Fig. 5) had similar mobilities

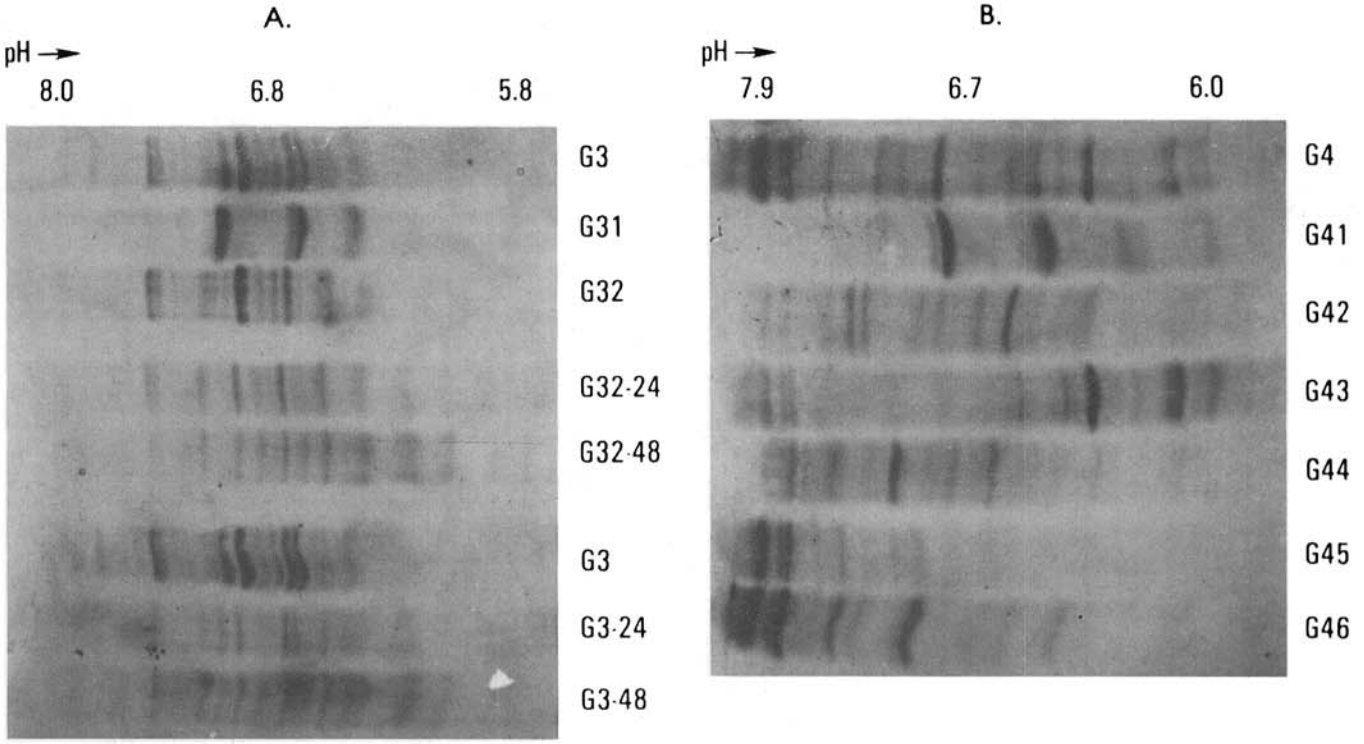

Fig. 8. Isoelectric focusing of gliadin fractions from sulfoethyl cellulose (SE cellulose) and RP-HPLC. **A,** Gliadin sulfoethyl cellulose fraction G3 (from Fig. 1), and RP-HPLC subfractions G31, G32 (Fig. 2). Patterns labeled G32-24, G32-48, G3-24, and G3-48 represent samples G32 and G3 held for 24 and 48 hr at 32.5°C in 45% acetonitrile + 0.1% trifluoroacetic acid. **B,** Gliadin SE cellulose fraction G4 (Fig. 1) and RP-HPLC subfractions G41-46 (from Fig. 3).

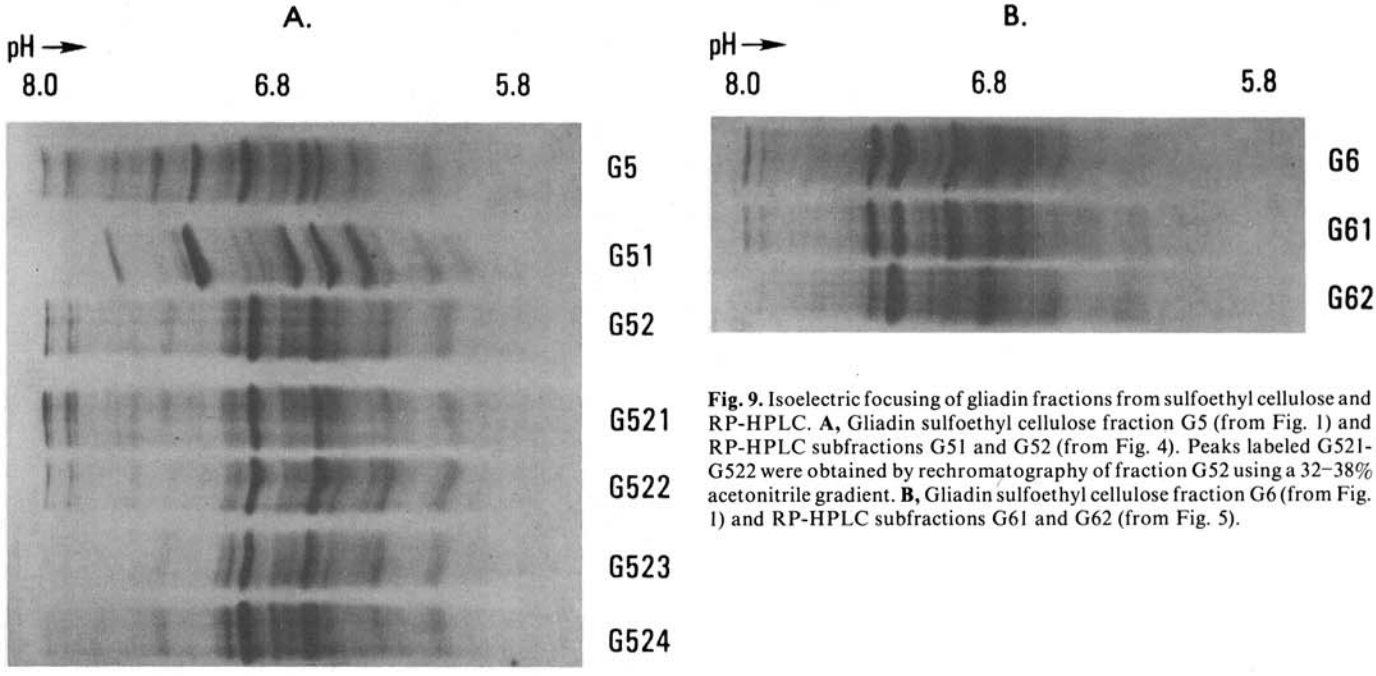

Fig. 9. Isoelectric focusing of gliadin fractions from sulfoethyl cellulose and RP-HPLC. **A,** Gliadin sulfoethyl cellulose fraction G5 (from Fig. 1) and RP-HPLC subfractions G51 and G52 (from Fig. 4). Peaks labeled G521-G522 were obtained by rechromatography of fraction G52 using a 32–38% acetonitrile gradient. **B,** Gliadin sulfoethyl cellulose fraction G6 (from Fig. 1) and RP-HPLC subfractions G61 and G62 (from Fig. 5).

upon aluminum lactate PAGE, suggesting that little resolution had been achieved by HPLC.

IEF

Isoelectric focusing patterns for gliadin SE cellulose fractions G3 and G4 and their subfractions, resulting from preparative RP-HPLC, are shown in Fig. 8. Subfractions G31 and G32 of fraction G3 (Fig. 8A) consist of completely different components, and together contain all components in the starting material, G3. It is apparent that IEF resolves many more components from these subfractions than were resolved by aluminum lactate PAGE (Fig. 6A).

It also seemed possible that the low pH of the SE cellulose chromatographic solvent or of the ACN-TFA RP-HPLC solvent could cause partial deamidation of glutamine and asparagine residues, thereby producing multiple species having additional carboxyl groups and different isoelectric points that could separate upon IEF. To test this, two protein samples (gliadin SE cellulose fraction G3 and its subfraction G32 from RP-HPLC) were held in 45% ACN + 0.1% TFA (observed pH 1.9 + 0.05) at 32.5° C for 24 and 48 hr. The IEF patterns of these samples are also shown in Fig. 8A. In both samples, after 24 and 48 hr the main bands decreased in relative intensity, and new, more acidic, bands with increased intensity formed with time. These bands are consistent with more acidic groups on the proteins, formed by deamidation. For this reason, we decreased the TFA concentration to 0.05% (pH 2.0) and kept samples cold before lyophilization.

IEF patterns of RP-HPLC subfractions of gliadin SE cellulose fraction G4 (Fig. 3) appear less complex than their parent fraction (Fig. 8B), but they still contain a number of minor components. Subfractions G42, G43, and G44 all contain numerous components; in contrast, aluminum lactate PAGE (Fig. 6B) showed two major bands in subfraction G42 and one major band in subfractions G43 and G44.

IEF analysis of the RP-HPLC subfractions of gliadin SE cellulose fraction G5 is shown in Fig. 9A. Approximately 15 unique bands are visible in each subfraction, compared to only two major bands visible upon aluminum lactate PAGE in each subfraction (Fig. 7A). When subfraction G52 was rechromatographed on HPLC, as described earlier, most major bands appeared in all four fractions, and only some basic bands were concentrated in subfractions G51 and G52.

Gliadin SE cellulose fraction G6 (Fig. 1) did not separate well upon RP-HPLC (Fig. 5), and subfractions were nearly identical upon aluminum lactate PAGE (results not shown). IEF (Fig. 9B) reveals that even the slight separation achieved by RP-HPLC resulted in very different compositions for the two resulting heterogeneous subfractions.

DISCUSSION

Although the heterogeneity of gliadins still prevents RP-HPLC from isolating individual components in large quantities, preparative RP-HPLC does separate gliadins into much simpler fractions, in reasonable quantities, and in a much shorter time than was previously possible. Preparative gel electrophoresis has yielded fractions of up to a few milligrams that appear homogeneous upon one-dimensional electrophoresis (Caldwell 1984), but these fractions may still exhibit heterogeneity when examined by other techniques. But the time and work involved in purifying gliadins by gel electrophoresis is considerable, whereas with RP-HPLC, separated proteins can be obtained within 24 hr, in dry form, with little effort. Also, this report is the first to investigate in detail the ability of preparative RP-HPLC to fractionate cereal proteins, and only one other study (Burnouf and Bietz 1984) has tested the method with gliadin proteins. Thus, it is possible that other solvents, perhaps using dissociating agents in conjunction with hydrophobic solvents, could give considerably improved separations. New column types are also now becoming available that could further facilitate gliadin separations by RP-HPLC.

Initial studies using analytical RP-HPLC (Bietz 1983) separated gliadins into more than 40 peaks, some only slightly resolved; PAGE revealed additional charge heterogeneity in many fractions, showing that gliadins having similar hydrophobicities may have different ionic characteristics. Similarly, the present study shows that gliadins having identical ionic characteristics (eluting in single peaks from SE cellulose) exhibit heterogeneity with respect to hydrophobic character when analyzed by RP-HPLC. Thus, the combined use of independent complementary methods, such as used in this study, may be necessary to produce the additional fractionation of gliadins on a preparative scale.

Two complications may exist when aluminum lactate PAGE is used to examine gliadins. First, components found in small quantity may not be visible until concentrated (as in Fig. 6B, G42 and G44). Second, many gliadins migrate at the same rate under acidic conditions because of similar molecular weight and similar contents of basic amino acids. With IEF, however, separations occur independently of protein molecular weight, based upon number of all ionizable amino acids, as represented by differing isoelectric points. Therefore, total resolution and sensitivity in IEF exceeds that which can be achieved in aluminum lactate PAGE. It is possible that some IEF bands may be artifacts, however (Catsimpoolas 1973, Righetti et al 1977), and acid-catalyzed deamidation may also increase apparent heterogeneity of relatively homogeneous fractions.

Gliadins contain large concentrations of glutamine, which can easily undergo deamidation under acidic conditions. Thus, considerable care must be exercised when fractionating gliadins at low pH, as in SE cellulose chromatography or preparative RP-HPLC, as noted previously (Charbonnier 1974). We have therefore taken precautions such as decreasing the TFA concentration in the RP-HPLC eluent to 0.05%, refrigerating fractions after elution, and minimizing time of exposure to acidic conditions to attempt to prevent deamidation. It remains necessary, however, to determine whether deamidation occurs during SE cellulose chromatography of gliadins, to determine actual amide contents of whole gliadin and of gliadin subjected to acidic conditions for various times, to determine to what extent deamidation occurs at low temperatures or on the presence of various concentrations of TFA.

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