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SEPARATION OF CEREAL PROTEINS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

J. A. BIETZ
Northern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, 1815 North University Street, Peoria, IL 61604 (U.S.A.)

SUMMARY

Cereal proteins have been extremely difficult to purify and characterize owing to their heterogeneity, poor solubility and tendency to polymerize. High-performance liquid chromatography (HPLC) on a 300 Å reversed-phase (RP) (C_{18}) support (Syn-Chropak RP-P), using acetonitrile as organic modifier in the presence of trifluoroacetic acid, has been found to be capable of high-resolution separations of these proteins; the resolution is often better than that obtained by any other chromatographic or electrophoretic method. Examples are presented showing separations of low-molecular-weight gliadins, omega-gliadins and ethanol-soluble reduced glutenin subunits from wheat and of zein from corn. In addition, proteins may be directly extracted from ground single kernels and subsequently analyzed by RP-HPLC; applications in genetic studies, in breeding programs and in varietal identification are proposed.

In addition to its high resolution, RP-HPLC is superior to most other methods in speed, sensitivity, reproducibility and suitability for quantitation. Polypeptide chains of molecular weight up to 133,000 are recovered in high yields and the column capacity is high, demonstrating that RP-HPLC is suitable for both preparative and analytical separations of proteins. RP-HPLC resolves proteins primarily on the basis of differences in surface hydrophobicity, so it therefore complements, rather than duplicates, other techniques that separate proteins on the basis of size or charge. RP-HPLC promises to become an invaluable technique for the fractionation and characterization of proteins from cereals and other sources.

INTRODUCTION

The endosperm storage proteins of cereal grains are mainly of two types, differentiated by their solubility (prolamins) or insolubility (glutelins) in aqueous alcoholic solutions following extraction with water and/or salt solutions. Prolamins are heterogeneous mixtures of monomers of molecular weight (MW) 20,000–70,000, but oligomeric prolamins also exist. Glutelins are also very heterogeneous and generally consist of polypeptides uniquely different from prolamins, having MW from 10,000 to 130,000, which are joined covalently and non-covalently into polymers with

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MW ranging to $20 \cdot 10^6$ or more. Most prolamins and glutelins are enriched in glutamine, proline and hydrophobic amino acids; they are insoluble in water or buffered salt solutions but soluble in solutions containing alcohols, acids or alkali, or following treatment with detergents, denaturants or disulfide-cleaving reagents. The prolamins and glutelins from wheat[1–4], termed specifically gliadins and glutenins, are representative of these complex protein fractions of all cereals. Gliadin, for example, has more than 40 components, and glutenin, after disruption of disulfide and hydrophobic bonds, contains at least 50 subunits.

Methods used to isolate, characterize and compare cereal endosperm proteins include selective extraction, gel permeation chromatography, ion-exchange chromatography, various electrophoresis methods and, most recently, hydrophobic interaction chromatography (HIC)[5,6]. High-performance molecular exclusion chromatography is now beginning to be applied to cereal endosperm proteins[7], but reversed-phase high-performance liquid chromatography (RP-HPLC) of these proteins has not been reported.

RP-HPLC is now frequently used for the isolation, characterization and comparison of low-MW peptides[8–13]. Columns are usually packed with 5–10 μm silica particles having average pore sizes of 80–100 Å[14,15]. Bonded to these particles is an alkylsilane phase, typically octadecyl (C_{18}). Most separations employ gradients of increasing amounts of the organic phases acetonitrile, methanol or 1- or 2-propanol, so bound peptides are eluted in order of increasing hydrophobicity. The ionic strength of the mobile phase is frequently elevated to 0.1–0.2 *M* to suppress residual silanol group interactions. The pH is usually between 2.1 and 3.2 to protonate free amino groups and thus reduce elution times of peptides; high-resolution separations at pH 6 may also be achieved, however[10]. Ion-pairing reagents are also frequently added to modify peptides, the support or both, and thereby alter their interaction; examples include acids such as phosphoric and trifluoroacetic acids and buffered salts such as triethylammonium phosphate, ammonium acetate and pyridine formate.

The RP-HPLC of high-MW peptides and proteins has, in general, been less successful than for low-MW peptides. Proteins may bind so tenaciously that they are recovered only in low yield, or may be unable to penetrate the 80–100 Å pores of standard supports, and may bind to a very limited extent. Nevertheless, standard pore size supports have been used for the RP-HPLC of some proteins[12,15–17]. The limitations of these supports have been recognized, however[14,18,19], and RP supports having pore sizes of 300–500 Å have been developed. These more porous supports, with appropriate ion-pairing reagents, are now beginning to be successfully applied to the RP-HPLC of high-MW peptides and proteins[14,19–21].

During studies using a 300 Å pore size C_{18} column to characterize large peptides, attempts to separate intact cereal proteins were also made. The resolution achieved for these samples equalled and frequently surpassed that resulting from any previously used chromatographic or electrophoretic technique. This paper reports initial experiences with the RP-HPLC of cereal proteins, describes the optimization of separation conditions and gives examples of the application of the method to several heterogeneous cereal protein fractions.

MATERIALS AND METHODS*

Chemicals and reagents

Unless noted otherwise, all chemicals were of reagent grade. Acetonitrile was obtained from Mallinckrodt (ChromAR grade) or Fisher (HPLC grade), methanol came from MCB (OmniSolv grade) and 2-propanol (certified ACS spectranalyzed grade) was a product of Fisher Scientific. Trifluoroacetic acid (TFA) and heptafluorobutyric acid (HFBA) (Sequanal grade) were obtained from Pierce. Water having a specific resistance of at least 15 MΩ cm was obtained using a Barnstead NANO pure system. All solvents were filtered (0.45 μm) and deaerated by sonication prior to use.

Protein samples

Low-MW gliadin and omega-gliadin were separated from whole Ponca wheat gliadin on Sephadex G-100 and subsequently were reduced and pyridylethylated (PE)[22]; these samples correspond to fractions f and c, respectively, in Fig. 1 of ref. 22. Ethanol-soluble reduced PE-glutenin from Ponca wheat[23] was also characterized previously[22]. Zein was isolated from normal corn hybrid P-A-G SX52 and converted to PE derivatives[24].

Single kernels of the wheat variety Chinese Spring, obtained from Dr. E. R. Sears, were ground with a mortar and pestle and subsequently extracted for 30 min, with periodic agitation, with 70% ethanol or with 50% solvent A (15% acetonitrile + 0.1% TFA) + 50% glacial acetic acid (0.04 ml/mg). Following centrifugation (10 min at 27,000 *g*), samples were analyzed immediately or stored frozen. Alternatively, single ground kernels were first extracted with 0.1 *M* sodium chloride solution (0.01 ml/mg) under these conditions, and the residues were washed with water and then further extracted with 70% ethanol (0.04 ml/mg).

Apparatus

All data were obtained using Waters Assoc. (Milford, MA, U.S.A.) Models M6000A and M45 solvent-delivery systems controlled by a Model 660 solvent programmer, a WISP 710A automatic sample injector and a Model 450 variable-wavelength detector. A capillary restrictor (Alltech) eliminated outgassing in the detector flow cell. SynChropak RP-P (250 × 4.1 mm I.D.), a reversed-phase (C_{18}) support having 300 Å pores[19], and SynChropak RSC (dry packed into a 25 × 3.9 mm I.D. guard column) were products of SynChrom (Linden, IN, U.S.A.). Zorbax ODS (250 × 4.6 mm I.D.) was obtained from DuPont. Columns were maintained at 30–31°C in an aluminum block connected to a constant-temperature water-bath.

Data were recorded on a Houston Instruments Omniscribe recorder (10 mV full-scale) and were quantitated using a ModComp computer system. Auxiliary programs permitted further analysis of chromatographic data, as well as replotting raw data so that the largest peak in any interval represents full-scale deflection. This program was used to plot the chromatographic data in this paper; because the solvent peak, eluting at the breakthrough volume, typically has the highest absorbance, this

* The mention of names of firms or trade products does not imply that they are endorsed by the U.S. Department of Agriculture over other firms or similar products not mentioned.

region frequently was not plotted so that the presentation of subsequent protein-containing peaks was optimized.

Chromatographic conditions

Most cereal proteins were eluted in the presence of 0.1% TFA[10,25–27] by solvents containing 15–80% of acetonitrile. Combining water with 100% acetonitrile causes occasional outgassing at the detector, so solvents A and B were chosen to be 15% and 80% aqueous acetonitrile, respectively, both containing 0.1% of TFA. For some studies, acetonitrile was replaced by methanol or 2-propanol, or 0.1% TFA was replaced by 0.13% HFBA[20,27,28]. Before use, the columns were regenerated at 1.0 ml/min with either solvent B or 100% acetonitrile and were equilibrated at initial solvent conditions. Samples were dissolved in any appropriate solvent, such as solvent A, solvent B, 50% solvent A + 50% glacial acetic acid, 70% ethanol, 0.1 *M* sodium chloride or 0.1 *M* sodium phosphate, pH 7.0, containing 0.1% sodium dodecyl sulfate (SDS). Proteins were generally dissolved at concentrations of 0.1–1.0 mg/ml, depending on their heterogeneity. When the proteins were not completely soluble or were extracted from ground single kernels, samples were filtered (0.45 μm) using a centrifugal filtration apparatus (Bioanalytical Systems) or were centrifuged (10 min at 27,000 *g*). Volumes of 20–100 μl were generally injected, although large (*e.g.*, 1.0 ml) samples could be applied when the initial solvent conditions were so polar that no proteins were eluted. Proteins were eluted from the column, usually at 1.0 ml/min (0.5–1.5 ml/min), using a linear gradient of increasing organic modifier (0.25–1.0% B/min) over 30–120 min. The final gradient conditions were then maintained for 10 min, and the column was re-equilibrated for 10 min at the initial solvent conditions prior to application of the next sample. The column effluent was typically monitored at 210 nm using 0.2 (0.02–2.0) absorbance units full-scale (a.u.f.s.). For semi-preparative runs (up to 5 mg of protein) or to monitor aromatic amino acids, the effluents were monitored at 280 or 254 nm.

Electrophoresis

Polyacrylamide gel electrophoresis at pH 3.2 was performed in a Bio-Rad Protean vertical electrophoresis cell by the method of Maier and Wagner[29]. Isoelectric focusing was performed on an LKB Multiphor apparatus, utilizing a mixture of equal volumes of pH 6–8 and pH 7–9 Ampholines. Isoelectric points of focused proteins were measured using an Ingold surface electrode, and focused proteins were then revealed as precipitation bands following immersion in 12.5% trichloroacetic acid.

RESULTS

Optimization of conditions

With 15% and 80% acetonitrile containing 0.1% of TFA as solvents A and B, respectively, unknown samples are generally resolved initially using a 50-min linear gradient from 0 to 100% B at 1.0 ml/min. Subsequently, the separation conditions are optimized, using gradients of 0.25–1.0% B/min, on the basis of the elution positions of the sample constituents. Typical optimized limits were found to be 20–50% B for wheat gliadins (28–47.5% acetonitrile), 0–55% B for albumins and globulins (15–

50.75% acetonitrile) and 50–70% B for corn zeins (47.5–60.5% acetonitrile). A gradient slope of 0.25–0.5% B/min gave optimal resolution for most samples; these conditions compromised the effects of band spreading due to diffusion in long runs and of decreased resolution in short runs.

A slow flow-rate (0.5 ml/min) did not significantly improve the resolution over that obtained at 1.0 ml/min, and a flow-rate of 1.5 ml/min only slightly decreased the resolution; consequently, 1.0 ml/min (*ca.* 1000 p.s.i.) was used routinely. However, higher flow-rates, combined with a steeper gradient slope, can be used to minimize analysis times. For heterogeneous samples, 0.1 mg in 100 µl is generally applied, and the effluent is monitored at 210 nm using a detector sensitivity of 0.2 a.u.f.s. For purified proteins, the amounts applied were 1–10%, by weight, of that applied for mixtures. The amounts of protein applied to the column can be increased or decreased by 1–2 orders of magnitude, with appropriate adjustment of detector sensitivity, without significantly affecting the resolution. For high sample loads (1–5 mg of protein), the column effluent was monitored at 254 or 280 nm, as the absorbance was too great at 210 nm. Monitoring at more than one wavelength is also useful for differentiating proteins on the basis of their tryptophan and tyrosine contents.

Prior to a series of analyses, and periodically thereafter, it is desirable to regenerate the column for 5–10 min with acetonitrile or with solvent B to remove any hydrophobic proteins not eluted by the chosen gradient; a 10-min re-equilibration (*ca.* 5 column volumes) at the initial solvent conditions is then sufficient to restore a stable baseline. It also is desirable periodically to analyze a "blank", consisting of the solvent in which proteins are dissolved; this will show any baseline shift during the gradient, will reveal any peaks due to contaminants in the various solvents, and can be used to ascertain the need for column regeneration. It also is desirable to analyze a heterogeneous reference sample periodically (*e.g.*, daily) to indicate any instrumental

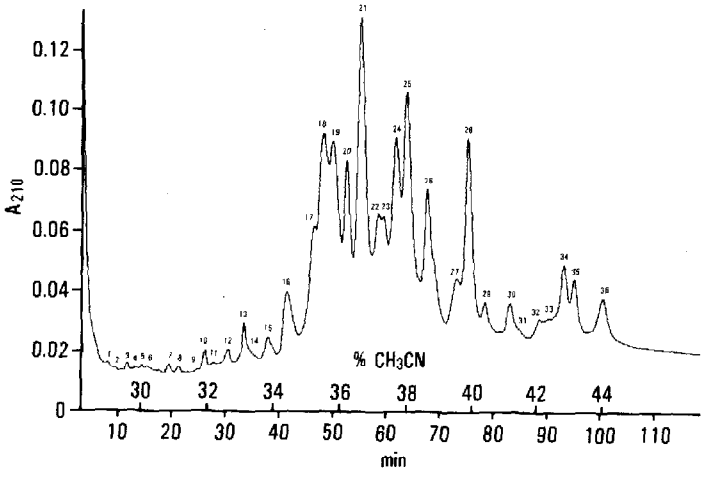

Fig. 1. RP-HPLC separation of PE-low-MW gliadin proteins from Ponca wheat. A sample of 0.1 mg in 100 µl was applied to a SynChropak RP-P column (250 × 4.1 mm), and eluted with a linear gradient from 20% to 50% B during 120 min. The column effluent was monitored at A_{210} = 0.1 a.u.f.s./10 mV. Solvent A, 15% acetonitrile + 0.1% TFA; solvent B, 80% acetonitrile + 0.1% TFA. Peaks are numbered in order of increasing elution volume.

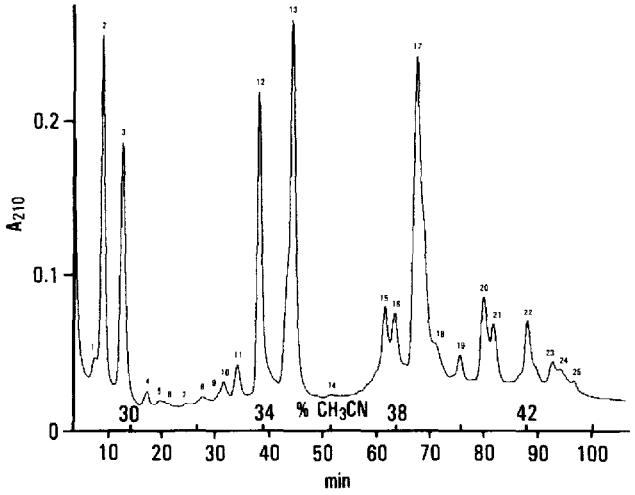

Fig. 2. RP-HPLC separation of PE-omega-gliadin from Ponca wheat. A sample of 0.1 mg was applied in a volume of 100 μl, and eluted with a linear gradient from 20% to 50% B during 120 min. The column effluent was monitored at A_{210} = 0.2 a.u.f.s.; other conditions as in Fig. 1.

malfunction or any differences in solvent concentrations. Solvents typically are filtered and deaerated immediately prior to use and are changed daily.

Analysis of isolated protein fractions

Typical RP-HPLC separations of three heterogeneous wheat protein fractions are illustrated in Figs. 1–3. The abscissa of each chromatogram indicates both the run time and acetonitrile concentration, displaced 2.0 min to the right (corresponding to the column's void volume, determined to be 2.0 ml) to reflect the effluent composition at the detector.

Figs. 1–3 illustrate the high resolving power of RP-HPLC for cereal proteins, and the ability or RP-HPLC to differentiate wheat protein fractions and classes. Low-MW gliadin (Fig. 1) was resolved into 36 peaks (including minor peaks and unresolved shoulders) eluting between 34 and 45% acetonitrile. For this and for all other figures, additional small peaks and unresolved shoulders could be revealed by computer-assisted scale expansion (as demonstrated in Fig. 7).

In comparison, omega-gliadin (Fig. 2), which is enriched in 65,000–80,000 MW proteins[30], is resolved into at least 25 components, including four major ones (peaks 2, 3, 12 and 13 in Fig. 2) that elute prior to most major low-MW gliadins (Fig. 1). The unique nature of these four major early eluting omega-gliadin components was further emphasized by column monitoring at 254 nm (data not shown): their absorbance at 254 nm relative to that at 210 nm is only about 15% of that for later eluting peaks, suggesting a very low content of tryptophan and tyrosine in these omega-gliadins compared with low-MW gliadins; this is supported by comparative amino acid analyses of isolated gliadin proteins[1,31]. The omega-gliadin fraction (Fig. 2) also contains numerous components having apparent surface hydrophobicities similar to low-MW gliadins, such as the major component (peak 17) eluting at *ca.* 39% acetonitrile (Fig. 2).

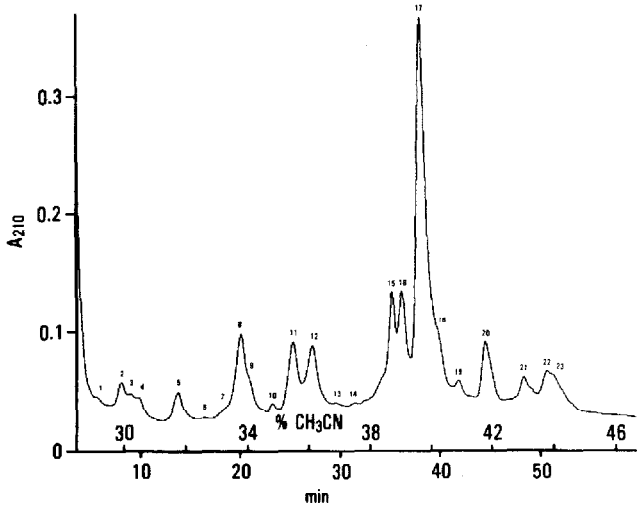

Fig. 3. RP-HPLC separation of PE ethanol-soluble reduced glutenin (0.1 mg) from Ponca wheat. The sample was applied in a volume of 100 μl, and eluted with a linear gradient from 20% to 50% B during 60 min. The column effluent was monitored at A_{210} = 0.2 a.u.f.s.; other conditions as in Fig. 1.

Ethanol-soluble reduced glutenin (Fig. 3) exists in the kernel as subunits of high-MW glutenin rather than as monomers (as in Figs. 1 and 2). RP-HPLC resolves ethanol-soluble reduced glutenin into approximately 23 components (Fig. 3), eluting between 30 and 44% acetonitrile, and dominated by one peak (peak 17) at 39.9% acetonitrile; most of these components differ uniquely from polypeptides in other wheat gluten classes (Figs. 1 and 2).

The same HPLC methods used for wheat proteins, with gradient modifications to compensate for differences in protein hydrophobicity, are applicable to all cereal protein fractions examined to date. Another example, the corn prolamin fraction

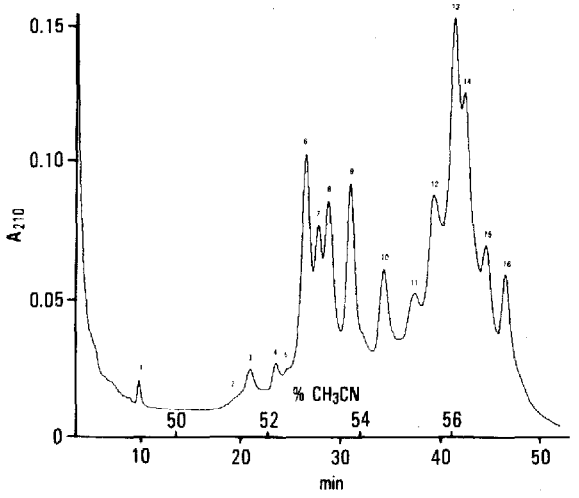

Fig. 4. RP-HPLC separation of PE-zein (0.1 mg applied in 100 μl). The chromatogram was developed with a linear gradient from 50% to 70% B during 60 min, and monitored at A_{210} = 0.2 a.u.f.s.; other conditions as in Fig. 1.

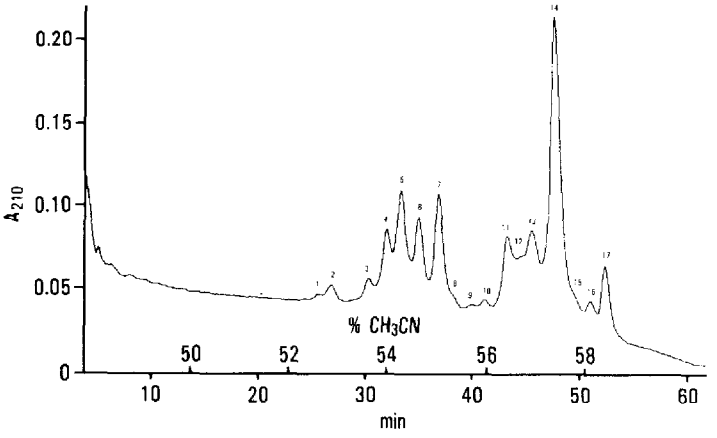

Fig. 5. RP-HPLC separation of PE-zein (0.1 mg applied in 100 µl). The chromatogram was developed with a linear gradient from 50% to 70% B during 60 min, and monitored at A_{210} = 0.2 a.u.f.s. For this chromatogram only, solutions A and B contained 0.13% HFBA in place of 0.1% TFA. Other conditions as in Fig. 1.

zein, is shown in Fig. 4. Sixteen peaks are resolved, most of which elute between 51.7 and 57.2% acetonitrile. These proteins are significantly more hydrophobic than most wheat proteins (Figs. 1–3), agreeing with the abundance of hydrophobic amino acids in zein and with zein's limited solubility except in the presence of denaturants, detergents or high concentrations of organic solvents.

Fig. 5 shows an example of how selectivity can be modified by changing the ion-pairing reagent. Using 0.13% HFBA rather than 0.1% TFA, zein resolves into about 17 components, most eluting between 53.0 and 58.5% acetonitrile. The overall separations of major zein species and of other cereal protein fractions using HFBA seem slightly inferior to those obtained using TFA (compare, for example, Figs. 4 and 5); nevertheless, such modification of column selectivity may be useful in difficult separations of cereal proteins, as suggested for peptides by Bennett *et al.*[32]. As noted for peptides[32], cereal proteins also exhibit longer retention times in the presence of HFBA than with TFA.

Analysis of single-kernel extracts

Fig. 6 shows four typical chromatograms obtained by analyzing extracts of ground single kernels of the wheat variety Chinese Spring. Fig. 6a and b show the results for sequential extraction of a kernel with 0.1 *M* sodium chloride solution to extract albumins and globulins, followed by 70% ethanol to extract gliadins. Forty-one peaks were resolved in the sodium chloride extract (Fig. 6a), and 24 in the subsequent ethanol extract (Fig. 6b). The chromatograms differ greatly; most major constituents of the sodium chloride extract elute below 40% acetonitrile, whereas most in the ethanol extract elute above 40% acetonitrile. Some peaks in the two chromatograms have similar elution characteristics; *e.g.*, peaks 17, 19, 23, 24 and 25 in Fig. 6a are similar to peaks 7, 8, 10, 11 and 12, respectively, in Fig. 6b. This suggests that some proteins are soluble in both extractants, and that the initial sodium chloride extraction was not exhaustive. Fig. 6c shows the results of a direct extraction of a ground kernel with 70% ethanol; approximately 30 peaks are resolved. The chroma-

togram is qualitatively very similar to that in Fig. 6b, but significant quantitative differences occur, particularly for peaks 9, 15 and 16, which appear to correspond to proteins soluble in 0.1 *M* sodium chloride solution (peaks 17, 24 and 25, Fig. 6a). Fig. 6d shows the results for a single wheat kernel extracted with equal volumes of solvent A and glacial acetic acid, a useful solvent for most proteins examined; approximately 34 peaks result. Although peaks characteristic of albumins, globulins and gliadin are present, the pattern is dominated by those which are characteristic of gliadin, owing to its greater relative abundance in the kernel. In addition, however, some uniquely different major peaks are present in the chromatogram of this extract, such as peaks 4 and 5 (Fig. 6d). The proteins in these peaks must have limited solubility in 0.1 *M* sodium chloride solution or 70% ethanol, and probably represent components of glutenin. The chromatograms in Fig. 6a–d demonstrate the value of selective extraction procedures for wheat kernels, and show the value of RP-HPLC for monitoring such fractionations and for differentiating the resulting fractions.

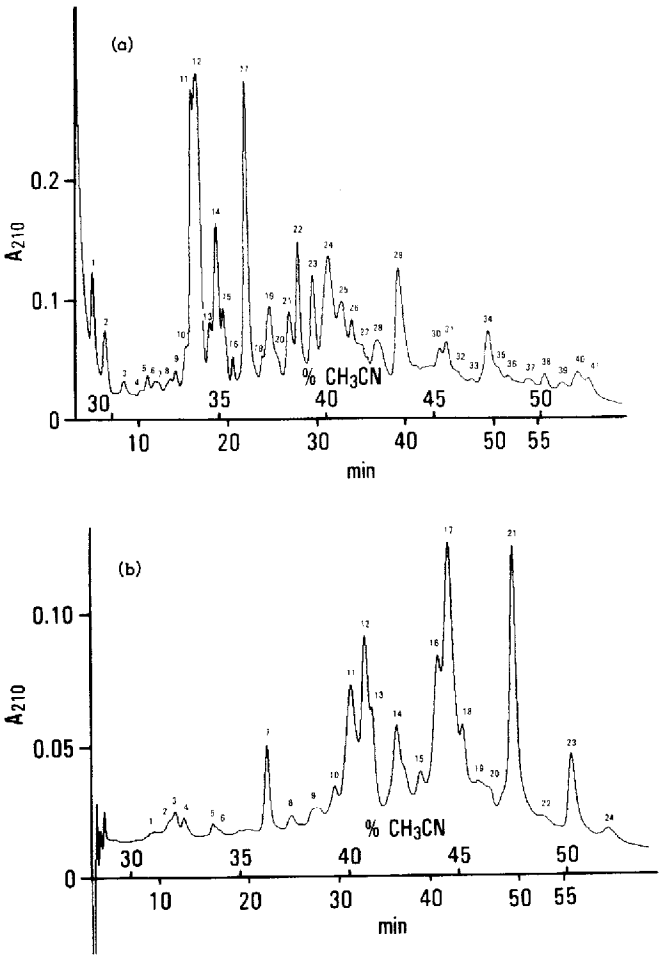

Fig. 6.

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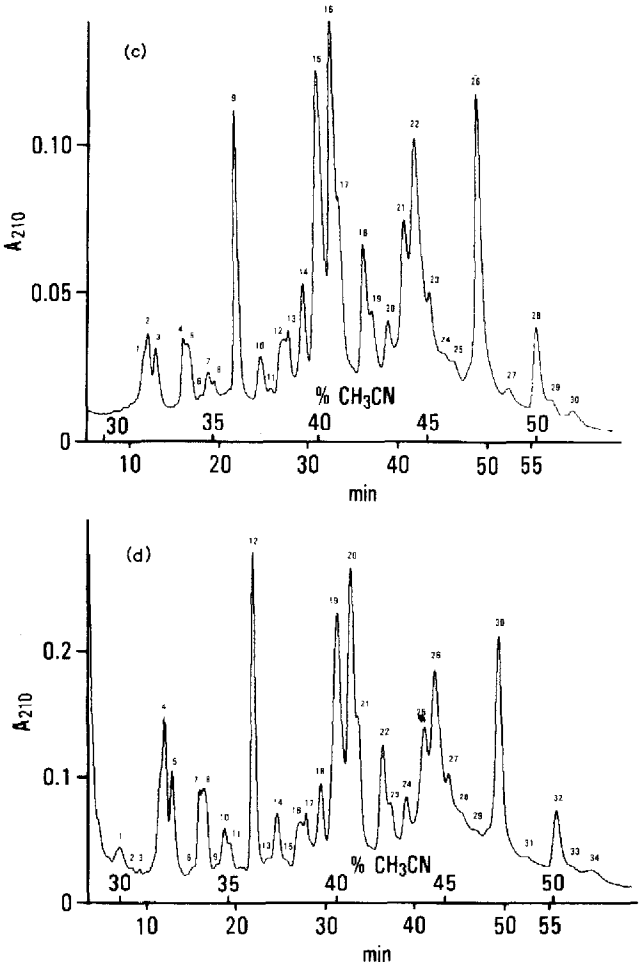

Fig. 6. RP-HPLC separations of proteins extracted from single kernels of the wheat variety Chinese Spring by (a) 0.1 *M* NaCl; (b) 70% ethanol subsequent to extraction with 0.1 *M* NaCl; (c) 70% ethanol (without prior extraction); and (d) 50% solvent A + 50% glacial acetic acid. Sample volumes of 100, 150, 50 and 100 μl, respectively, were applied to the column; extraction conditions and concentrations are described in Materials and methods. The column was eluted with a linear gradient from 20% to 55% B during 55 min, and the column effluent was monitored at A_{210} = 0.2 a.u.f.s.; other conditions as in Fig. 1.

The gradient used for HPLC of the 0.1 *M* sodium chloride extract (Fig. 6a) was, to facilitate comparisons, the same as for the other single-kernel extracts. Still better resolution of this fraction, particularly for early eluting components (prior to 30–35% acetonitrile), can be obtained by using a 0–55% gradient (Fig. 7a). This improved resolution is more clearly seen in a computer-expanded portion of the chromatogram (Fig. 7b). Whereas Fig. 7a indicates approximately 46 components in the sample, the expanded-scale chromatograms reveal at least 86 major and minor peaks in the total chromatogram. As illustrated in Fig. 7b, for example, approximately 14 additional minor peaks are visible in the first part of the chromatogram. These results (Fig. 7a and b) illustrate that proteins differing only slightly in hydrophobicity are readily separated, and also demonstrate the value of computer-assisted scale expansion in detecting trace components.

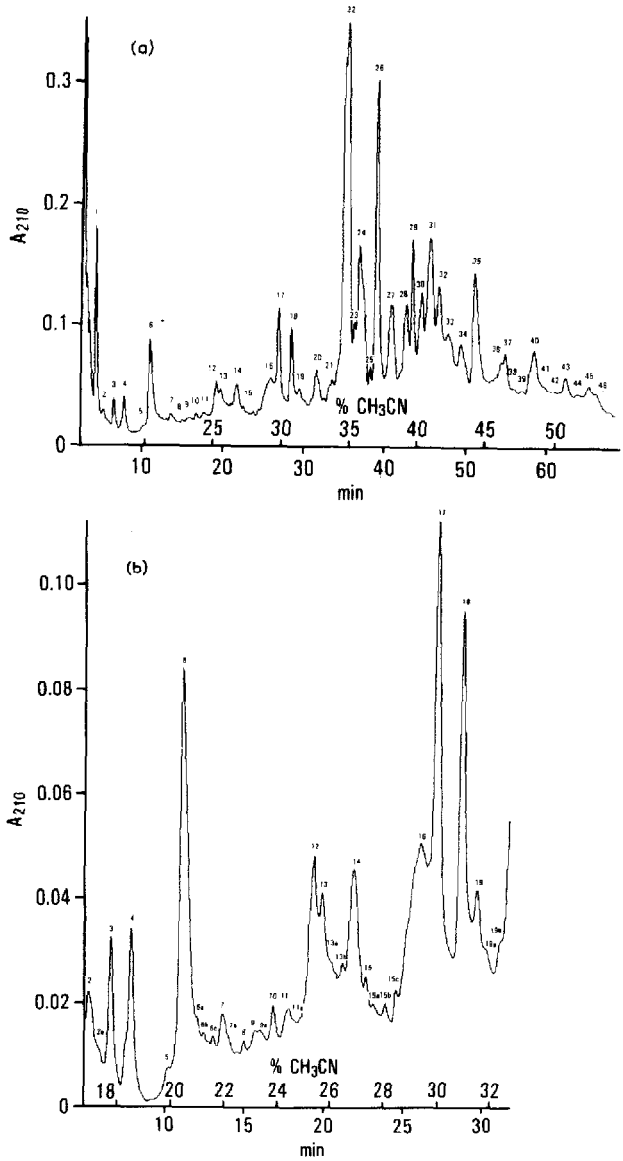

Fig. 7. RP-HPLC separations of proteins extracted from a single kernel of the wheat variety Chinese Spring with 0.1 *M* NaCl. A sample of 100 μl was applied to the column, and eluted with a linear gradient from 0% to 55% B during 60 min; the column effluent was monitored at A_{210} = 0.2 a.u.f.s.; other conditions as in Fig. 1. (a) Entire chromatogram; (b) expanded portion of (a).

Potential of RP-HPLC as a preparative method

The size of sample applied to an RP-HPLC column can be increased markedly without significantly decreasing the resolution, demonstrating the method's potential for preparative applications. As shown in Fig. 8a, for example, 39 peaks were resolved from a 5.0-mg sample of PE-low-MW gliadin. This is very similar to the

number of peaks obtained for a 0.1-mg sample (Fig. 1), but the resolution of major peaks seems slightly inferior. It should be emphasized, however, that this separation was performed on an analytical-scale column, so that considerably improved resolution, together with increased sample size, should be possible on a larger column. It is also interesting that the relative peak heights in Fig. 8a differ from those in Fig. 1, owing to the column effluent being monitored at 254 nm (necessary because of the increased sample size); such spectral differences can be valuable in characterizing and differentiating proteins having different hydrophobicities.

Similarly, a 5.0-mg sample of PE-zein was separated on the analytical RP-P column; detection at 254 nm revealed at least 24 major and minor components (Fig. 9a). The resolution and number of peaks were roughly comparable to that for a 0.1-mg sample (Fig. 4).

Electrophoretic characterization of peaks separated by RP-HPLC

To assess more accurately the separations of cereal proteins obtained by RP-HPLC, fractions were collected from the two semi-preparative separations shown in Figs. 8a and 9a and aliquots were analyzed by electrophoresis.

Fig. 8b shows analyses of representative peaks from the chromatogram of PE-low-MW gliadin (Fig. 8a) by electrophoresis at pH 3.2. It is apparent that, compared with the parent fraction, the peaks contain significantly purified components. Owing to the extreme heterogeneity of the sample, few, if any, peaks contain homogeneous proteins; nevertheless, it is most significant to note that most peaks contain only one or two major components, and that relatively little overlap occurs, even between adjacent peaks. Further, it is apparent that the RP-HPLC separation differs markedly from separations based primarily on charge, demonstrating the complementary nature of RP-HPLC to other methods. These results are only meant to be representative of the separations obtainable by RP-HPLC; more detailed characterization and comparison of separated protein fractions will be reported later. Nevertheless, these results suggest that excellent preparative RP-HPLC separations of cereal proteins

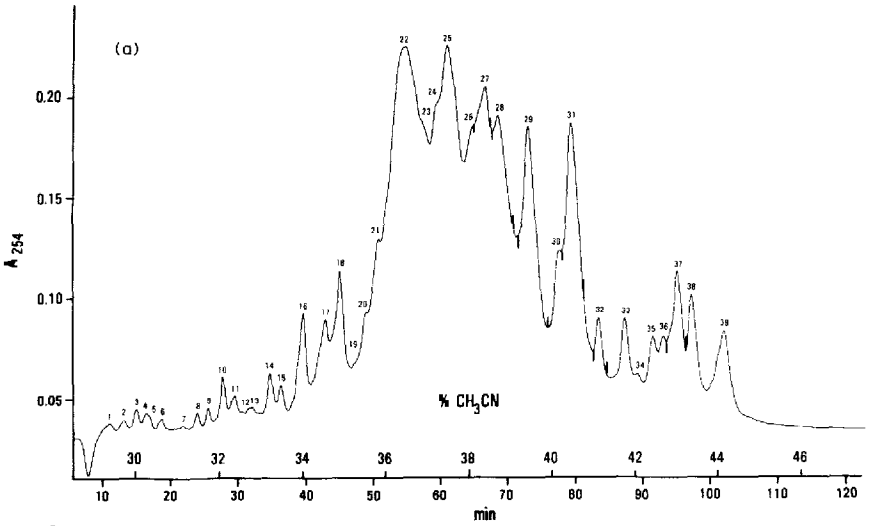

Fig. 8.

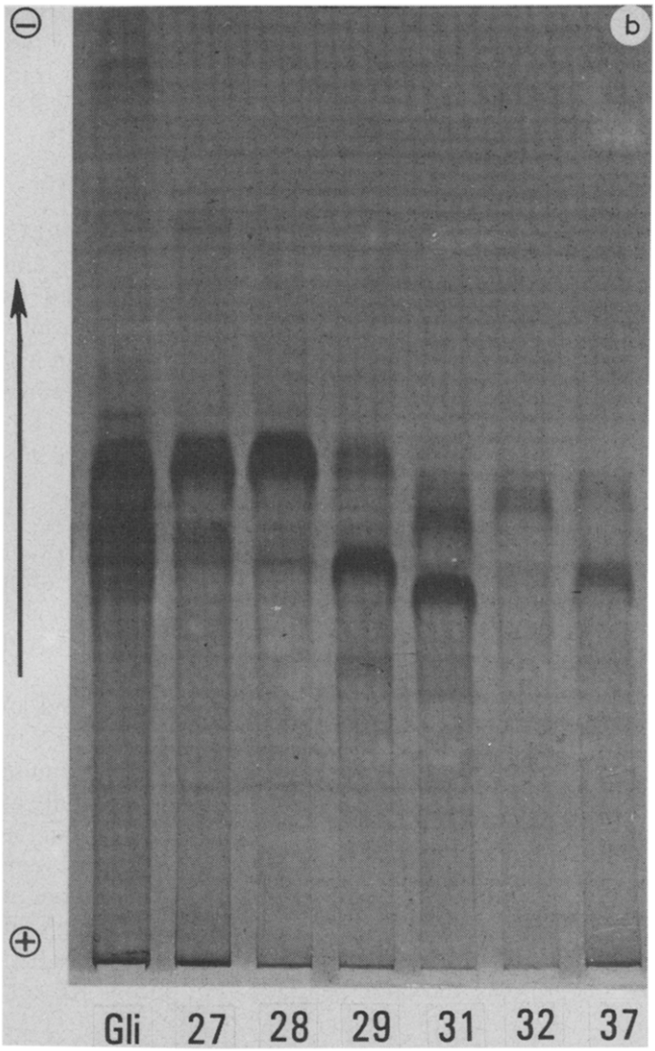

Fig. 8. (a) RP-HPLC separation of 5.0 mg of Ponca PE-low-MW gliadin on SynChropak RP-P (250 × 4.1 mm). Sample [1.0 ml of a 5 mg/ml solution in 50% solvent A (15% acetonitrile + 0.1% TFA) + 50% glacial acetic acid] was applied to the column equilibrated with solvent A, and then eluted with a linear gradient from 20% to 50% solvent B (80% acetonitrile + 0.1% TFA) during 120 min. The column effluent was monitored at A_{254} = 0.4 a.u.f.s./10 mV. Individual peaks (as numbered) were collected for subsequent analyses; those fractions examined in Fig. 8b are indicated by descending and ascending marks superimposed on the chromatogram, which indicate, respectively, the beginning and end of fraction collection. (b) Polyacrylamide gel electrophoresis at pH 3.2 of Ponca PE-low-MW gliadin (Gli) and fractions obtained by RP-HPLC. Numbers designating fractions correspond to numbered peaks in Fig. 8a; *ca.* 10% of each collected fraction was analyzed.

may be obtained. These separations complement other separations based on charge or size, and should greatly facilitate the isolation of cereal proteins.

Fig. 9b presents isoelectric focusing results for representative fractions from the RP-HPLC separation of PE-zein (Fig. 9a). Most fractions are still heterogeneous,

in accord with zein's complex nature, but all represent a considerable purification compared with the parent fraction; in addition, it is apparent that only one or two species predominate in each fraction, and that overlap, even between adjacent fractions, is minimal. Thus, these results also demonstrate the validity and value of RP-HPLC separations of cereal proteins.

General considerations

Reproducibility and stability. To test the reproducibility of gradient RP-HPLC, a sample of ethanol-insoluble reduced glutelin subunits[23] was injected repeatedly, and the elution times of corresponding peaks were compared. The relative standard deviation, averaged for eleven peaks from four separate analyses, was 0.3295; typically, this means that even late in a chromatogram, the elution times for any protein will differ by no more than 0.10–0.15 min between runs. Column performance and reproducibility also have been demonstrated by collection of individual eluted peaks, followed by concentration, re-injection and analysis; identical elution times are obtained.

The RP column used in these studies has been remarkable stable. No change in performance is apparent after more than 1000 analyses. The only precautions that have been taken include careful filtration of all solutions, centrifugation or filtration of all samples, occasional regeneration of the column with 80–100% acetonitrile, and repacking of the guard column when any increase in operating pressure is noted (typically after 200–300 analyses).

Recovery. A standard solution of PE-low-MW gliadin was prepared and an aliquot was injected on to the RP-P column and eluted with a gradient from 20% to 90% B during 10 min, followed by a 13-min hold at 90% B. The entire column effluent was collected, and its absorbance was determined spectrophotometrically at 220 and 254 nm. Appropriate corrections were made for buffer and for solvents in the sample solution, and the corrected absorbance values for the recovered effluent were compared with the absorbance of an aliquot of the same initial sample. Recoveries of 98.2% and 96.2% were indicated at 220 and 254 nm, respectively, suggesting that nearly quantitative recovery is routinely obtained. This agrees with other studies that report recoveries of 80–100% for proteins and large peptides from porous RP columns[14,20,21].

Optimization of gradient, flow-rate and run time. Using the described acetonitrile–TFA solvents A and B with SynChropak RP-P, it is possible to optimize or alter the resolution by adjusting the gradient slope, run time or both. Generally, a gradient slope of *ca.* 0.5% B/min (*i.e.*, 0.325% acetonitrile/min) is most suitable. The resolution can be improved slightly by using 0.25% B/min (0.1625% acetonitrile/min) (*e.g.*, 20–50% B/120 min, as in Figs. 1 and 2), but the improved resolution is partially countered by increased band spreading due to diffusion. Alternatively, gradient slopes of 1.0 or even 2.0% B/min (0.65–1.30% acetonitrile/min) are satisfactory for screening unknown samples and for less demanding separations. Total run times of 60–120 min therefore seem optimal for most separations, although runs of *ca.* 30 min may be used. For more rapid runs, it may be desirable to increase the flow-rate to *ca.* 1.5 ml/min to reduce the possibility of precipitation of proteins by concentrated organic modifier in the mobile phase before the proteins elute from the column. As the elution of proteins in RP-HPLC is governed primarily by the polarity of the

mobile phase, the flow-rate generally has little effect on separations; for example, resolution at 0.5 ml/min is not noticeably better than at 1.0 ml/min. Under very different chromatographic conditions, proteins can elute at slightly different (*e.g.*, about 1 %) apparent acetonitrile concentrations; consequently, describing chromatographic peaks in terms of acetonitrile concentrations at which they elute, although useful to differentiate proteins, must refer to a specific set of chromatographic conditions.

Comparison of organic modifiers. Substitution of methanol or 2-propanol for acetonitrile in solvents A and B led to increases or decreases, respectively, compared with acetonitrile, in the percentage of organic modifier required to elute wheat gliadins, in accordance with known solvent strengths[26]. Although the resolution in all instances seemed best with acetonitrile, some differences in column selectivity were apparent using alternative organic modifiers, which may be used to advantage for difficult separations[33].

Effect of TFA concentration. In the absence of TFA or when only 0.01 % of TFA is present in solvents A and B, extreme peak broadening and poor resolution occur. Increasing the TFA concentration to 0.2 or 0.3 % leads to slight increases (3.7 % and 6.3 %, respectively) in retention times. Some slight differences in selectivity occur for different peaks, however, suggesting that the resolution can be enhanced through modification of TFA concentration.

Use of alternative ion-pairing reagents. HFBA can replace TFA in the RP-HPLC system (Fig. 5), giving a possibly advantageous change in selectivity. Sodium dodecyl sulfate (SDS) also can be used successfully in the system. Initially, RP-HPLC was used to analyze glutenin proteins that had been reduced, fully complexed to SDS and separated by high-performance gel filtration chromatography on TSK-3000SW[7]. The resolution was only slightly inferior to that observed with no SDS present; the retention times increased slightly. Excellent chromatographic separations can also be obtained when SDS (0.1 %) is included in solvents A and B. For example, glutenin extracted from a single kernel of Ponca wheat with a solvent containing SDS and β-

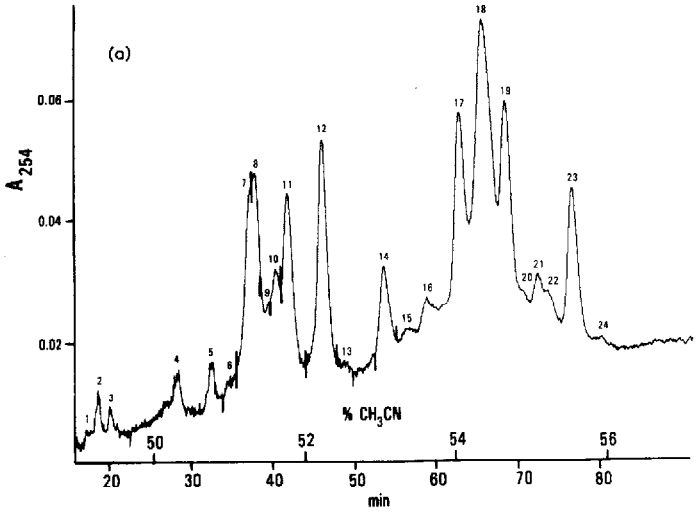

Fig. 9.

(Continued on p. 234)

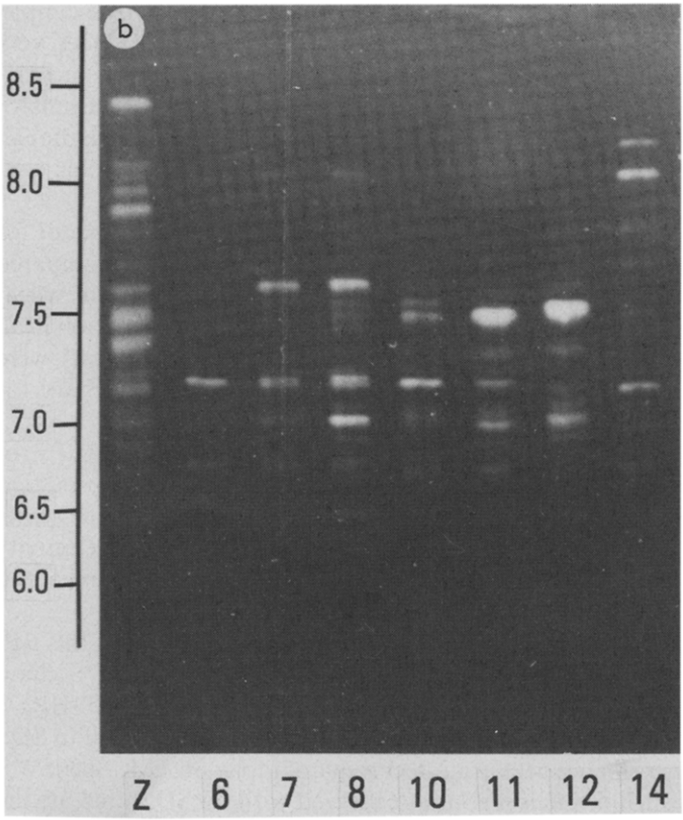

Fig. 9. (a) RP-HPLC separation of 5.0 mg of PE-zein on SynChropak RP-P (250 × 4.1 mm). Sample [1.0 ml of a 5 mg/ml solution in 50% solvent A (15% acetonitrile + 0.1% TFA) + 50% glacial acetic acid] was applied to the column equilibrated with 20% solvent B (80% acetonitrile + 0.1% TFA) + 80% solvent A, and then eluted with a linear gradient from 50% to 70% B during 120 min. The column effluent was monitored at A_{254} = 0.4 a.u.f.s./10 mV. Individual fractions subsequently analyzed in Fig. 9b are indicated as in Fig. 8. (b) Isoelectric focusing of PE-zein (Z) and fractions obtained by RP-HPLC. Numbers designating fractions correspond to numbered peaks in Fig. 9a; *ca.* 25% of each collected fraction was analyzed. Determined isoelectric points are indicated at the left-hand margin.

mercaptoethanol[34] was resolved by RP-HPLC into 37 peaks, most eluting between 41.0 and 73.5% acetonitrile. It is obvious that the apparent hydrophobicity of most proteins increases in the presence of SDS.

The role of SDS and the nature of SDS–protein complexes in RP-HPLC is uncertain. SDS has been used as an ion-pairing reagent to separate peptides[35,36], and it dramatically increases retention times. However, the mechanism of the ion-pairing phenomenon is uncertain. These studies have demonstrated that SDS does not mask differences in surface hydrophobicity between proteins and suggest either that SDS–protein complexes dissociate during RP-HPLC or that SDS does not react with those surface areas which determine selectivity. The potential of SDS in RP-HPLC is considerable, both because it can act as an ion-pairing reagent and because it has such strong solvating properties.

Use of other chromatographic supports. RP-HPLC of proteins has been found to be most successful on supports having a large pore size (*e.g.*, 300–500 Å)[14,37]. To test this, PE-low-MW gliadin (as in Fig. 1) was chromatographed, using the same acetonitrile–TFA solvent system, on DuPont Zorbax ODS, which is typical of supports having pore sizes of about 100 Å[14]. Poor initial retention was observed, apparently as proteins could not penetrate the pores of the support; strong adsorption also occurred, so that a high acetonitrile concentration was necessary to elute all proteins. Pearson *et al.*[19] also noted poor recovery and resolution for large peptides on Zorbax ODS.

Potential for varietal identification and genetic studies. Cereal prolamins are accurate genotypic indicators owing to their heterogeneity and invariant expression by the genome; consequently, they are frequently analyzed by electrophoresis to identify cereal varieties[38] and may also serve as biochemical markers. As shown in Figs. 6 and 7, RP-HPLC resolves numerous polypeptides from various single kernel extracts; further, it can differentiate most wheats, permitting varietal identification. RP-HPLC has the potential of complementing electrophoresis methods and, in some instances, of serving as an alternative to them. These studies will be described in a subsequent publication. Similarly, it is possible to differentiate and study relationships of corn inbreds through analysis of extracted zein proteins, similar to the separation shown in Fig. 4, and to determine the chromosomal control of proteins through analysis of cereal aneuploids.

Applicability to other proteins. During these studies, several non-cereal protein fractions have also been examined by RP-HPLC. In all instances, excellent separations of components were achieved. For example, reduced soy glycinin polypeptides were resolved into approximately 13 major and several minor components eluting between 35.5 and 65.0% acetonitrile; the two major peaks eluted at 45.8 and 46.3% acetonitrile. These results agree well with studies showing six acidic and six basic proteins within the glycinin protein complex[39]. These and other results indicate that RP-HPLC using porous supports may become an extremely valuable method for most, if not all, protein separations.

DISCUSSION

RP-HPLC is the newest, and may become the best, method for analyzing most proteins, including those from cereal grains. RP-HPLC separates proteins primarily by hydrophobic interaction or adsorption chromatography; therefore, one of its major advantages is that it complements most other chromatographic and electrophoretic methods, which separate proteins on the basis of differences in size or charged amino acid residues. The success of RP-HPLC is due primarily to three factors, namely pore size, particle uniformity and ion-pairing reagents. A support having 300 Å or larger pores potentially permits unhindered penetration by virtually any single polypeptide chain and by proteins with molecular weights up to several hundred thousand[14]. The uniform coating, end capping of residual silanol groups and uniform small particle size of supports result in highly efficient RP-HPLC columns, in contrast to HIC on modified agarose supports. In addition, ion-pairing reagents, such as TFA, modify protein and/or support surfaces so that proteins are retained under relatively polar solvent conditions; they are then selectively eluted by increasing the

organic modifier concentration. Seemingly minor variations in surface hydrophobicity lead to high-resolution separations of proteins. In addition, initial polar solvent conditions, combined with the relatively high capacity of the support, effectively concentrate applied samples into narrow zones on the column.

RP-HPLC separates most of the heterogeneous cereal protein fractions that have been examined into as many or more components than does any other chromatographic or electrophoretic method used to characterize these samples. In most instances, the number of peaks observed in RP-HPLC agrees well with estimates of heterogeneity from these other methods. In addition, electrophoretic analysis of fractions isolated by RP-HPLC (Figs. 8b and 9b) has indicated that each HPLC peak is enriched in one or only a few polypeptides, which differ from those in adjacent peaks. Apparently minor differences in charged residues or in size, which may not be readily detected by other analytical methods, lead to significant differences in surface hydrophobicity that are easily recognized by RP-HPLC.

Using the acetonitrile-TFA RP-HPLC system, low-MW gliadin (Fig. 1) and omega-gliadin (Fig. 2) were separated into 36 and 25 components, respectively; most compounds were unique to one or the other fraction. Together, low-MW gliadin and omega-gliadin constitute the major gliadin polypeptides often analyzed by electrophoresis in genetic studies and for varietal identification. Two-dimensional electrophoresis of whole gliadin, employing a combination of isoelectric focusing and electrophoresis at pH 3.1, resolves a maximum of approximately 46 components[40]. Similarly, ion-exchange chromatography and starch gel electrophoresis reveal omega-gliadin to have eight major components and several minor ones[31], agreeing well with the results in Fig. 2. Thus, the number of peaks resolved by RP-HPLC generally agrees well with estimates of heterogeneity from other methods.

It is also interesting to compare RP-HPLC results with those from HIC and with estimates of hydrophobicity of various gliadins. Both Caldwell[6] and Popineau and Godon[5] noted that major omega-gliadins elute first upon HIC on octyl- or phenyl-Sepharose CL 4B, but other omega-gliadins have longer retention times. For other gliadins, no clear relationship was noted between electrophoretic mobilities and surface hydrophobicities. Charbonnier *et al.*[30] also demonstrated that omega-gliadin hydrophobicities, calculated from amino acid compositions, do not predict HIC retention characteristics because surface rather than overall hydrophobicity determines retention. These results are supported by the present studies, which found low retention for four major omega-gliadins (Fig. 2) but noted that omega-gliadin also contains numerous components similar in hydrophobicity to low-MW gliadins (Fig. 1). We also found no clear relationship between RP-HPLC retention characteristics and electrophoretic mobilities of gliadins. It may be necessary, therefore, to develop a new nomenclature for cereal proteins, based on apparent hydrophobicities; it will certainly be necessary to correlate fully the RP-HPLC behavior of cereal proteins with their characteristics in other separation systems.

The resolution achieved by RP-HPLC for other protein samples also generally equals or exceeds that obtained by previous methods. Ethanol-soluble reduced glutenin was resolved into 23 components (Fig. 3), compared with approximately 20 separated by electrophoresis at pH 3.2 (ref. 22). PE-zein was separated into a total of 16–24 peaks (Figs. 4 and 9), similar to the number of components typically resolved by isoelectric focusing[41] or two-dimensional electrophoresis[42].

RP-HPLC has several additional advantages for analysis of cereal proteins. It is significantly faster (30–120 min per analysis) than most chromatographic or electrophoretic methods, and automatic sample application further increases the capability of RP-HPLC to analyze numerous samples. Detection at 210 nm is another significant advantage; it is possible because of the transparency of the acetonitrile–TFA solvents at this wavelength. The molar absorptivity of most cereal proteins at 210 nm is 50–100 times greater than that at 254 or 280 nm, and detector response at 210 nm is independent of amino acid composition. Hence very small protein samples, such as from single kernels, can readily be analyzed under our standard conditions. For isolated polypeptides, approximately 5 μg can be easily analyzed; by increasing detector sensitivity, the minimum detectable amount of protein per peak in RP-HPLC can be reduced to approximately 50 ng.

As noted above, a significant advantage of RP-HPLC over most electrophoretic methods is its excellent reproducibility; another major advantage is the ability to apply automated data processing to quantitate HPLC results rapidly. As baselines are very noise-free and retention times are very reproducible, excellent quantitative results are routinely obtained; in comparison, electrophoresis combined with densitometry suffers from variable background, different sample migration distances, limited detector sensitivity and possible non-linear detector response.

The chromatograms shown here demonstrate that polypeptides of MW 20,000–80,000 all elute satisfactorily from the porous RP-HPLC column. Further, as all reduced glutenin subunits are recovered quantitatively, polypeptides of MW up to at least 133,000 (ref. 43) may be analyzed; therefore, molecular size should be no deterrent to the RP-HPLC analysis of any single-chain polypeptide. However, the suitability of this procedure for higher MW multi-chain proteins remains to be determined. We have also shown that the support matrix has a relatively high capacity for proteins, as 5-mg samples were successfully separated on an analytical column; undoubtedly separations of increased sample amounts on larger RP-HPLC columns will prove invaluable for preparative purposes.

In conclusion, we have shown that RP-HPLC can be applied successfully to characterize protein samples, determine homogeneity or heterogeneity, compare samples and follow the progress of fractionation schemes. In addition, RP-HPLC analysis of single kernel extracts promises to be useful for genetic studies, for selection and early generation analysis in breeding programs, for quality control and for varietal identification and registration. Because it is so powerful, RP-HPLC could potentially replace numerous other methods of protein analysis for some applications. However, it may not completely do so for two reasons: first, it is more complex and expensive than some techniques; and second, as the mode of separation of proteins by RP-HPLC differs from that in any other chromatographic or electrophoretic method (with the possible exception of HIC), it complements the other methods. It is certain that RP-HPLC is an extremely valuable addition to the available techniques for characterizing and separating proteins.

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REFERENCES

1 D. D. Kasarda, J. E. Bernardin and C. C. Nimmo, *Advan. Cereal Sci. Technol.*, 1 (1976) 158.
2 J. A. Bietz, *Cereal Foods World*, 24 (1979) 199.
3 J. S. Wall, in D. L. Laidman and R. G. Wyn Jones (Editors), *Recent Advances in the Biochemistry of Cereals*, Academic Press, London, 1979, pp. 275–311.
4 J. A. Bietz and F. R. Huebner, *Ann. Technol. Agr.*, 29 (1980) 249.
5 Y. Popineau and B. Godon, *Cereal Chem.*, 59 (1982) 55.
6 K. A. Caldwell, *J. Sci. Food Agr.*, 30 (1979) 185.
7 J. A. Bietz and L. A. Cobb, *Cereal Foods World*, 26 (1981) 484.
8 C. S. Fullmer and R. H. Wasserman, *J. Biol. Chem.*, 254 (1979) 7208.
9 E. C. Nice and M. J. O'Hare, *J. Chromatogr.*, 162 (1979) 401.
10 C. Y. Yang, E. Pauly, H. Kratzin and N. Hilschmann, *Hoppe-Seyler's Z. Physiol. Chem.*, 362 (1981) 1131.
11 P. Böhlen and G. Kleeman, *J. Chromatogr.*, 205 (1981) 65.
12 W. S. Hancock, J. D. Capra, W. A. Bradley and J. T. Sparrow, *J. Chromatogr.*, 206 (1981) 59.
13 F. E. Regnier and K. M. Gooding, *Anal. Biochem.*, 103 (1980) 1.
14 R. V. Lewis, A. Fallon, S. Stein, K. D. Gibson and S. Udenfriend, *Anal. Biochem.*, 104 (1980) 153.
15 E. C. Nice, M. W. Capp, N. Cooke and M. J. O'Hare, *J. Chromatogr.*, 218 (1981) 569.
16 W. Mönch and W. Dehnen, *J. Chromatogr.*, 147 (1978) 415.
17 M. J. O'Hare and E. C. Nice, *J. Chromatogr.*, 171 (1979) 209.
18 B. N. Jones, R. V. Lewis, S. Paabo, K. Kojima, S. Kimura and S. Stein, *J. Liquid Chromatogr.*, 3 (1980) 1373.
19 J. D. Pearson, W. C. Mahoney, M. A. Hermodson and F. E. Regnier, *J. Chromatogr.*, 207 (1981) 325.
20 M. van der Rest, H. P. J. Bennett, S. Solomon and F. H. Glorieux, *Biochem. J.*, 191 (1980) 253.
21 A. Fallon, R. V. Lewis and K. D. Gibson, *Anal. Biochem.*, 110 (1981) 318.
22 J. A. Bietz and J. S. Wall, *Cereal Chem.*, 57 (1980) 415.
23 J. A. Bietz and J. S. Wall, *Cereal Chem.*, 50 (1973) 537.
24 J. A. Bietz, J. W. Paulis and J. S. Wall, *Cereal Chem.*, 56 (1979) 327.
25 C. E. Dunlap, III, S. Gentleman and L. I. Lowney, *J. Chromatogr.*, 160 (1978) 191.
26 W. C. Mahoney and M. A. Hermodson, *J. Biol. Chem.*, 255 (1980) 11199.
27 H. P. J. Bennett, C. A. Browne and S. Solomon, *Biochemistry*, 20 (1981) 4530.
28 H. P. J. Bennett, S. Solomon and D. Goltzman, *Biochem. J.*, 197 (1981) 391.
29 G. Maier and K. Wagner, *Z. Lebensm.-Unters.-Forsch.*, 170 (1980) 343.
30 L. Charbonnier, T. Tercé-Laforgue and J. Mossé, *Ann. Technol. Agr.*, 29 (1980) 175.
31 L. Charbonnier, *Biochim. Biophys. Acta*, 359 (1974) 142.
32 H. P. J. Bennett, C. A. Browne and S. Solomon, *J. Liquid Chromatogr.*, 3 (1980) 1353.
33 M. T. W. Hearn and B. Grego, *J. Chromatogr.*, 218 (1981) 497.
34 J. A. Bietz, K. W. Shepherd and J. S. Wall, *Cereal Chem.*, 52 (1975) 513.
35 W. S. Hancock, C. A. Bishop, L. J. Meyer, D. R. K. Harding and M. T. W. Hearn, *J. Chromatogr.*, 161 (1978) 291.
36 W. S. Hancock, C. A. Bishop, J. E. Battersby, D. R. K. Harding and M. T. W. Hearn, *J. Chromatogr.*, 168 (1979) 377.
37 W. S. Hancock, H. J. Pownall, A. M. Gotto and J. T. Sparrow, *J. Chromatogr.*, 216 (1981) 285.
38 C. W. Wrigley, J. C. Autran and W. Bushuk, *Advan. Cereal Sci. Technol.*, 5 (1982) 211.
39 R. A. Badley, D. Atkinson, H. Hauser, D. Oldani, J. P. Green and J. M. Stubbs, *Biochim. Biophys. Acta*, 412 (1975) 214.
40 C. W. Wrigley and K. W. Shepherd, *Ann. N. Y. Acad. Sci.*, 209 (1973) 154.
41 M. Motto, F. Salamini, R. Reggiani and C. Soave, *Maydica*, 24 (1979) 223.
42 G. Hagen and I. Rubenstein, *Plant Sci. Lett.*, 19 (1980) 217.
43 J. A. Bietz and J. S. Wall, *Cereal Chem.*, 49 (1972) 416.