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REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF REDUCED GLUTENIN, A DISULFIDE-BONDED PROTEIN OF WHEAT ENDOSPERM*

THIERRY BURNOUF*,** and JEROLD A. BIETZ
Northern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Peoria, IL 61604 (U.S.A.)
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SUMMARY

The subunits of glutenin, the disulfide-bonded, high-molecular weight protein fraction of wheat, have been separated by reversed-phase high-performance liquid chromatography (RP-HPLC) on large-pore silica-based columns. Glutenin was first isolated from bulk samples, and effects of dissociating, reducing and alkylating agents on subsequent RP-HPLC separations of glutenin subunits were compared. Eight RP-HPLC columns (C_{18}, C_8, diphenyl or cyanopropyl) were compared to achieve optimal resolution of glutenin subunits. Proteins were eluted by a gradient of increasing acetonitrile concentration in the presence of 0.1% trifluoroacetic acid and were detected at 210 nm. Excellent separations occurred when glutenin was dissociated in the presence of 8 *M* urea or 6 *M* guanidine hydrochloride, reduced with 5% 2-mercaptoethanol or 0.1% dithiothreitol, alkylated with 4-vinylpyridine and chromatographed on C_{18} or C_8 bonded-phase columns. Using these conditions, fifteen to twenty major glutenin subunits were resolved. Glutenin subunits were then extracted from single kernels of aneuploid lines of the variety Chinese Spring and fractionated by RP-HPLC. Four early eluting peaks were shown to contain major glutenin subunits coded by the long arms of chromosomes 1D and 1B, and thus were assumed to correspond to the high-molecular-weight subunits. Their elution volume indicated that these subunits have lower surface hydrophobicities than do most lower-molecular-weight glutenin subunits which eluted later. Significant differences in glutenin subunit composition among bread wheat varieties suggest possible relationships between specific polypeptides and breadmaking quality, and show that RP-HPLC of glutenin subunits has the potential of identifying most wheat varieties. These results demonstrate that RP-HPLC is a valuable complement to other chromatographic and electrophoretic methods for analysis of glutenin subunits.

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** Post-doctoral research associate at the Department of Agronomy, University of Nebraska, Lincoln, NE, U.S.A.; stationed at the Northern Regional Research Center.

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INTRODUCTION

Glutenin is a heterogeneous class of wheat endosperm protein that is insoluble in water, diluted salt and alcohol solutions; it represents about 40% of all endosperm proteins. Glutenin is difficult to study in its native form because of its insolubility in most commonly used solvents. It has a strong tendency to associate and forms aggregates having molecular weights (MWs) of several million daltons[1]. Glutenin consists of a large number of smaller polypeptides joined by disulfide bonds; after reduction of the disulfide bonds, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS–PAGE) separates these subunits into 15 or more components having different MWs[1,2]. The subunits of low molecular weight (LMW) (mostly 36,000 and 44,000 daltons) are identical in size, solubility characteristics, and amino acid composition[3], as well as N-terminal sequence[4] and genetic control[5] to the subunits of high-molecular-weight (HMW) gliadin, an alcohol-soluble endosperm protein. HMW glutenin subunits range in apparent size, as indicated by SDS-PAGE, from about 80,000 to 150,000 daltons or more[6–8]; they contain almost six times as much glycine as do LMW subunits and are insoluble in neutral alcohol solutions[3,9]. Each bread wheat variety contains 3–5 HMW glutenin subunits, but much compositional variation occurs among varieties[10–12]. Glutenin is known to contribute significantly to elasticity and strength of dough[1,13]; more recently, a relationship between the presence of specific HMW subunits and the physical quality of flour has been revealed[7,10,14,15]. Glutenin subunits are coded by co-dominant genes that are transmitted, through crosses, from parental varieties to offspring[16–19]; these polypeptides can thus be used as breadmaking quality markers.

To understand better the nature and genetics of glutenin, a new high-resolution technique is needed to complement other methods that separate subunits by molecular weight and/or charge[20–25]. Recently, reversed-phase high-performance liquid chromatography (RP-HPLC), which differentiates proteins by surface hydrophobicity, has been applied successfully to the separation of cereal proteins[26]. RP-HPLC is valuable for separating gliadins and LMW glutenin subunits[26–30]. However, the potential of RP-HPLC to fractionate HMW glutenin subunits has not been evaluated.

In this paper, we define experimental conditions suitable for extraction, derivatization, and RP-HPLC analysis of HMW and LMW glutenin subunits, and compare their resolution on columns having various bonded phases. In addition, we show that RP-HPLC can reveal differences among glutenin subunits from wheat aneuploid lines and from bread wheat varieties. The methodology developed for these analyses should prove useful for RP-HPLC studies of other disulfide-bonded proteins, which must be reduced or reduced and alkylated before analysis.

MATERIALS AND METHODS*

Plant material

A bulk sample of glutenin was prepared from undefatted flour (cv. Centurk)

* The 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.

by a sequential extraction procedure[6]. Lyophilized pellets containing isolated glutenin were stored at room temperature until analyses. Samples of 20 mg were used to investigate conditions for optimum processing of the protein prior to chromatography of the subunits.

Aneuploid lines of the variety Chinese Spring were generously provided by E. R. Sears[31] (University of Missouri, Columbia, MO, U.S.A.). Bread wheat varieties were obtained from V. Youngs (Spring and Durum Wheat Quality Laboratory, USDA, Fargo, ND, U.S.A.) and from P. Poullard (Momont-Hennette Breeding Center, Mons-en-Pevele, France). Glutenin was prepared from single kernels[6] for analysis of these lines.

Preparation of glutenin for RP-HPLC

Glutenin was solubilized from dried pellets (20 mg) with 750 μl of a medium containing 0.05 *M* Tris(hydroxymethyl)aminomethane plus dissociating agent [8 *M* urea, 6 *M* guanidine hydrochloride (GuHCl) or 2% SDS] adjusted to pH 7.5 with nitric acid. Glutenins were reduced with 5% 2-mercaptoethanol[7] (2-ME) or 0.1% dithiothreitol[32] (DTT) for 2 h at room temperature, with occasional shaking. In some experiments, sulfhydryl groups were subsequently alkylated[3,22,33,34] for 2 h with freshly distilled 4-vinylpyridine, iodoacetic acid or acrylonitrile using a 1:1 molar ratio of alkylating agent to total sulfhydryl groups. Alkylation was terminated by addition of 500 μl glacial acetic acid (to *ca.* pH 3). Reduced and reduced-alkylated glutenin samples were centrifuged (35,000 *g*, 15 min) and 30–50 μl of the clear supernatants were injected for RP-HPLC.

Chromatographic conditions and columns used

The HPLC apparatus and computer system used were described previously[26]. Acetonitrile (CH_3CN) was obtained from MCB (Omnisolv grade); trifluoroacetic acid (TFA) was a product of Fisher Scientific. Water was obtained using a Barnstead NANO pure system. Solvent A was 12% CH_3CN + 0.1% TFA in water and solvent B was 80% CH_3CN + 0.1% TFA in water. Both solvents were filtered (0.45 μm) and deaerated prior to use. Proteins were eluted at 1 ml/min using a gradient from 1 to 70% B over 65 min, or when optimized, from 15 to 60% B over 65 min. Columns were maintained at 30–31°C. The column effluent was monitored at 210 nm using 0.2 a.u.f.s. Columns were from Synchrom (SynChropak RP-P C_{18} and RP-P C_8, 250 × 4.1 mm I.D., particle size 6.5 μm), J. T. Baker (Bakerbond C_{18}, C_8, diphenyl and cyanopropyl, 250 × 4.6 mm I.D., 5 μm), Brownlee Labs. [Aquapore RP-300 (a C_8 bonded phase, Brownlee Labs., personal communication), 250 × 4.6 mm I.D., 10 μm], and Whatman (diphenyl, 250 × 4.6 mm I.D., 10 μm). All packing materials were based on 300 or 330 Å wide pore silicas[35]. These columns were used for 25–30 h before this study to compare their resolution power for gliadins; no loss or change in resolution was observed during these experiments.

RESULTS

Preparation of glutenin

Effect of dissociating agent. To ensure complete reduction, glutenin was first dissolved in Tris buffer containing one of three dissociating agents which, by breaking

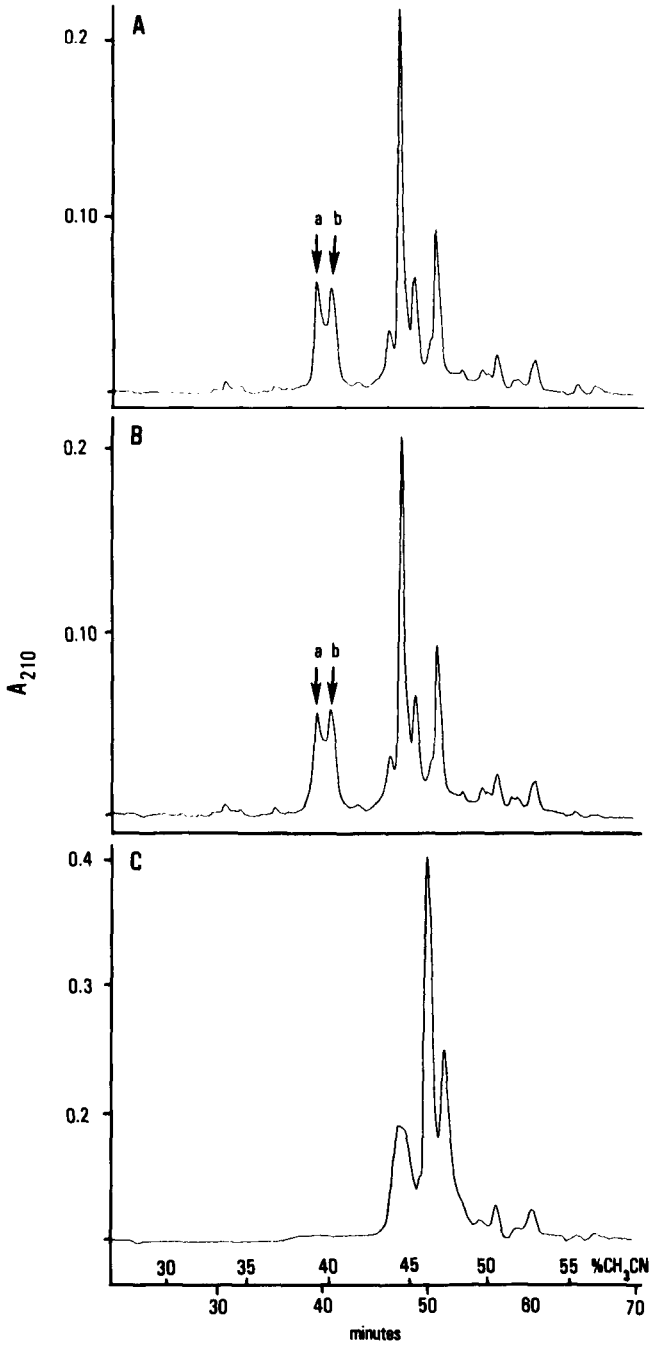

Fig. 1. RP-HPLC separation of glutenin reduced with 5% 2-mercaptoethanol, in Tris buffer containing (A) 8 *M* urea, (B) 6 *M* guanidine hydrochloride or (C) 2% SDS. Samples (50 μl) were applied onto a SynChropak RP-P C_{18} column (250 × 4.1 mm) and eluted with a linear gradient from 1 to 70% B during 65 min. Column effluent was monitored at A_{210} = 0.2 a.u.f.s./10 mV. Solvent A, 12% acetonitrile + 0.1% TFA; solvent B, 80% acetonitrile + 0.1% TFA. Arrows a and b show early protein components present when the sample was prepared in the presence of 8 *M* urea or 6 *M* GuHCl but not detected, or having a different retention time, when prepared in the presence of SDS. The solvent peak is not plotted.

hydrogen and hydrophobic bonds, permit better accessibility of disulfide bonds. Fig. 1 shows elution profiles of glutenin subunits reduced by 5% 2-ME in the presence of 8 *M* urea (A), 6 *M* GuHCl (B) or 2% SDS (C). When reduction was conducted in the presence of 8 *M* urea or 6 *M* GuHCl, six to eight major and similar peaks were resolved. Early components (a and b in Fig. 1A and 1B) were eluted with *ca.* 38–42% CH_3CN and later components with *ca.* 44–55% CH_3CN. However, when glutenin was reduced in the presence of 2% SDS (Fig. 1C), only three to five major chromatographic fractions were separated; the elution profile lacked peaks a and b, and most polypeptides eluted with *ca.* 44–52% CH_3CN. Nearly identical separations were obtained when glutenin was reduced with 0.1% DTT instead of 5% 2-ME (results not shown). Because of the poor resolution obtained in SDS, urea and GuHCl were chosen as dissociating agents for subsequent experiments.

Effect of alkylation. When glutenin was reduced by 2-ME and alkylated with 4-vinylpyridine to yield S-pyridylethyl (PE) derivatives, RP-HPLC on SynChropak C_{18} (Fig. 2) resolved fifteen to twenty major subunits, eluting between *ca.* 33 and 48% CH_3CN. This separation was significantly better than that of glutenin reduced under the same conditions but not alkylated (Fig. 1A). Thus, alkylation of liberated sulfhydryl groups improved separations of glutenin subunits, particularly early eluting ones.

Comparison of alkylating agents. S-carboxymethyl (CM) and S-cyanoethyl (CN) derivatives of reduced glutenins, prepared by reaction with iodoacetic acid and acrylonitrile, respectively, were analyzed on a Brownlee Aquapore RP-300 column and compared to PE-derivatives. CM- and CN-glutenin chromatograms (Fig. 3A and 3B, respectively) had a group of three to five early eluting peaks and a major peak in the middle of each chromatogram. PE-derivatives eluted in a pattern similar to the one found before (Fig. 2). CM-glutenins eluted slightly earlier than did PE-glutenins (*ca.* 30–48% CH_3CN as compared to *ca.* 33–48%) but CN-derivatives eluted later (*ca.* 38–50% CH_3CN). Resolution of reduced-alkylated glutenin subunits differed greatly according to alkylating agent used. About fifteen CM-glutenin peaks could be distinguished, but resolution was poorer than that of CN-glutenins. When 4-vinylpyridine was employed, resolution was far better, particularly for early eluting subunits. Consequently, 4-vinylpyridine was used for subsequent analyses.

Comparison of reducing agents. 5% 2-ME and 0.1% DTT may be used interchangeably to reduce glutenin; both permit comparable subsequent alkylation. In general, resolution obtained using 5% 2-ME was slightly better than that resulting when 1% 2-ME was used. Fig. 4 shows a separation of PE-glutenins reduced with DTT in the presence of 6 *M* GuHCl; the elution profile is extremely similar to that obtained for PE-glutenins reduced with 2-ME (Fig. 2). In addition, comparison of Figs. 2 and 4 demonstrates the excellent reproducibility of RP-HPLC separations of glutenin subunits and show that glutenin can be reduced and alkylated in either 8 *M* urea or 6 *M* GuHCl to give equivalent subunit chromatograms.

These results suggest optimal conditions for preparing glutenin for RP-HPLC. Briefly, glutenin is dissolved in 0.05 *M* Tris–HNO_3 (pH 7.5), containing either 8 *M* urea or 6 *M* GuHCl and is reduced with either 5% 2-ME or 0.1% DTT for 2 h with occasional agitation. Alkylation preferably is done with 4-vinylpyridine for 2 h, yielding S-PE derivatives; the optimal molar ratio of alkylating agent to total sulfhydryl groups is near 1:1. Alkylation is terminated by acidification with glacial acetic acid

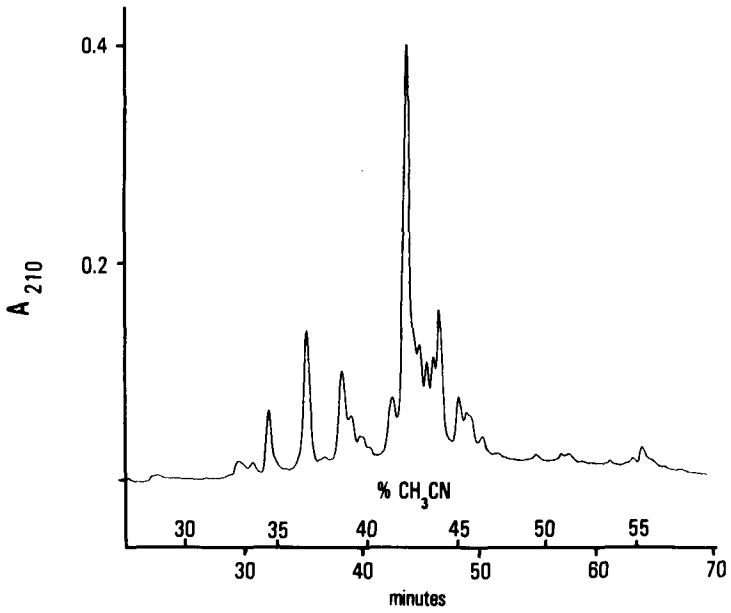

Fig. 2. RP-HPLC separation of glutenin dissolved in Tris buffer containing 8 *M* urea, reduced with 5% 2-ME and alkylated with 0.7 *M* 4-vinylpyridine. Other conditions as in Fig. 1.

to a pH of about 3. Since the TFA-containing chromatographic solvent system is acidic (pH *ca.* 2.1), acidification of the alkylation mixture can be avoided if samples are analyzed shortly after preparation. Clear supernatants obtained by centrifugation of alkylation mixtures may thus be directly analyzed by RP-HPLC. Acidified samples were stable for at least one month at room temperature, and they separated into reproducible RP-HPLC patterns.

Comparison of columns and effects of various bonded phases. PE-glutenin subunits, prepared by reducing glutenin with 2-ME in Tris buffer containing 8 *M* urea, were chromatographed using the same gradient conditions on eight different RP-HPLC columns. Representative results are shown in Fig. 5. Columns having aliphatic bonded phases (Fig. 5 (A), SynChropak RP-P C_{18}; (B) Brownlee Aquapore RP-300 C_8; (C) Bakerbond C_8 gave very similar separations; only minor differences could be observed, such as those indicated by arrows a, b and c (Fig. 5), where better separation occurred on the Brownlee and Bakerbond than on the SynChropak columns. Similar resolution was also obtained on another SynChropak C_{18} column and on a Bakerbond C_{18} column (results not shown). PE-glutenin subunits eluted earlier from a Bakerbond diphenyl column (Fig. 5D) than from aliphatic columns; however, overall separations were similar, and fifteen to twenty components were resolved. Retention times on the Whatman diphenyl column (Fig. 5E) were longer (PE-glutenins eluted with 35–53% CH_3CN) than those obtained using the Baker diphenyl column, but only about ten major peaks and shoulders could be resolved. The less hydrophobic Bakerbond cyano-bonded phase (Fig. 5F) caused glutenin subunits to elute much earlier (30–40% CH_3CN) than on the other columns, and resolution was decreased; obviously, chromatographic conditions were not optimized for this column,

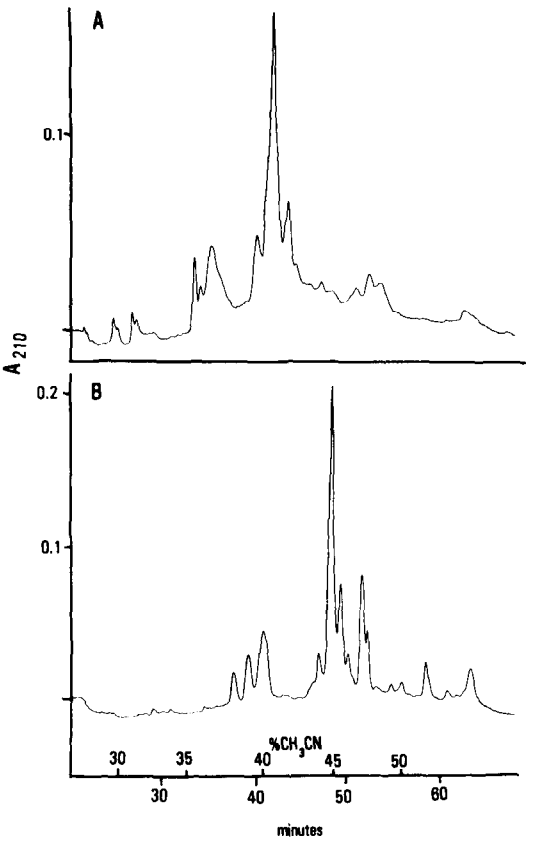

Fig. 3. RP-HPLC separation of glutenin dissolved in Tris buffer containing 8 *M* urea, reduced with 5% 2-ME and alkylated with 0.7 *M* (A) iodoacetic acid or (B) acrylonitrile. Samples were injected onto a Brownlee Aquapore RP-300 column (250 × 4.6 mm) and eluted with a linear gradient from 15 to 60% B during 65 min. Other conditions are as in Fig. 1.

and improved resolution may be obtained by modifying the gradient. Since resolution of glutenin subunits is generally best on aliphatic bonded phases, these columns were used in subsequent experiments. For many applications, the Brownlee Aquapore column was preferred because its larger particle size (10 μm) gives lower back pressure (*ca.* 400 p.s.i. at initial conditions) than most other columns, such as Bakerbond C_8 (5 μm; *ca.* 1600 p.s.i.).

Analysis of aneuploid lines. The aneuploid lines of Chinese Spring, developed by Sears[31], are invaluable for revealing the genetic control of wheat proteins. Fig. 6 shows representative elution profiles of: PE-glutenins from (A) Chinese Spring; (B), nulli-1D tetra-1A (N1DT1A); (C) nulli-1B tetra-1A (N1BT1A); and (D) ditelo $1B_S$ Chinese Spring lines. N1DT1A does not have chromosomes 1D, while N1BT1A lacks chromosomes 1B; both lines have double the normal complement of homoeologous chromosomes 1A. Consequently N1DT1A will lack glutenin subunits coded by genes on chromosomes 1D, and N1BT1A will lack those coded by genes on chromosomes 1B; both lines will have twice the normal amount of subunits coded by genes on

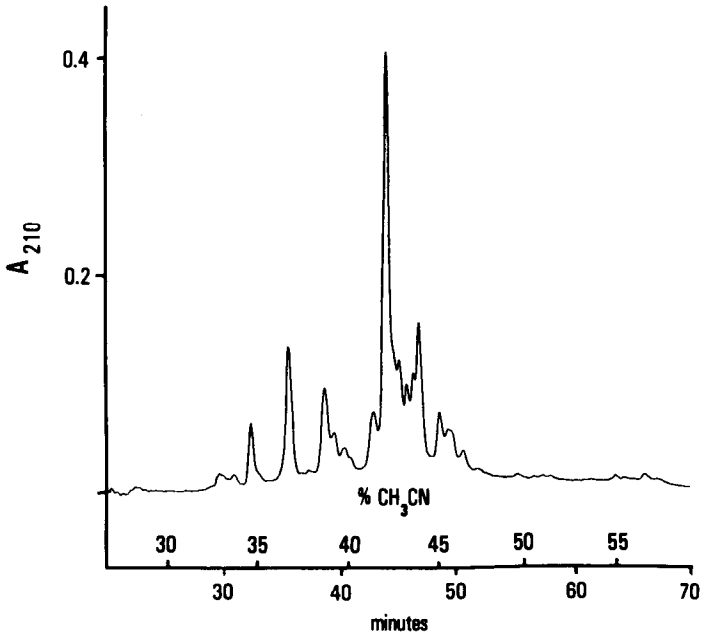

Fig. 4. RP-HPLC separation of glutenin dissolved in Tris buffer containing 6 *M* GuHCl, reduced with 0.1% DTT and alkylated with 0.014 *M* 4-vinylpyridine. Chromatographic conditions as in Fig. 1.

chromosomes 1A. By comparing PE-glutenin patterns of Chinese Spring and its aneuploid lines, several differences can be observed, which will be discussed in detail elsewhere[36]. For instance, two major peaks (a and b in Fig. 6A) in Chinese Spring are absent in N1DT1A; thus, these peaks contain glutenin subunits coded by genes on chromosomes 1D. Similarly, peaks c and d are absent in N1BT1A and, thus, contain subunits coded by genes on chromosomes 1B. Glutenin from the ditelocentric line $1B_S$ (Fig. 5D), which lacks the long arm of chromosomes 1B, also lacks peaks c and d, indicating that proteins in these peaks are coded by genes on the long arm of chromosomes 1B. Similarly, we found glutenin subunits peaks a and b to be controlled by the long arm of chromosomes 1D, whereas most later eluting polypeptides were coded by genes on the short arms of homoeologous chromosomes 1. Among gluten proteins, only glutenin's HMW subunits are coded by genes on the long arms of chromosomes 1B and 1D in Chinese Spring[6,37]; so, we concluded that peaks a–d (Fig. 6) correspond to HMW glutenin subunits. Similarly, LMW glutenin subunits and many gliadins are controlled by genes on the short arms of homoeologous group 1 chromosomes[5,37]. Thus, late-eluting chromatographic peaks (Fig. 6) likely contain LMW glutenin subunits plus gliadin contaminants, if any, entrapped in glutenin during extraction.

Analysis of hexaploid wheat varieties. Glutenins were extracted from single kernels of several hexaploid wheat varieties. Fig. 7 shows representative RP-HPLC separations of PE-glutenins from (A) Marquis, (B) Hardi and (C) Maris-Huntsman. In each variety, about 20 components could be separated. Numerous major quantitative and qualitative differences exist among these chromatograms (*e.g.*, peaks c–h) show-

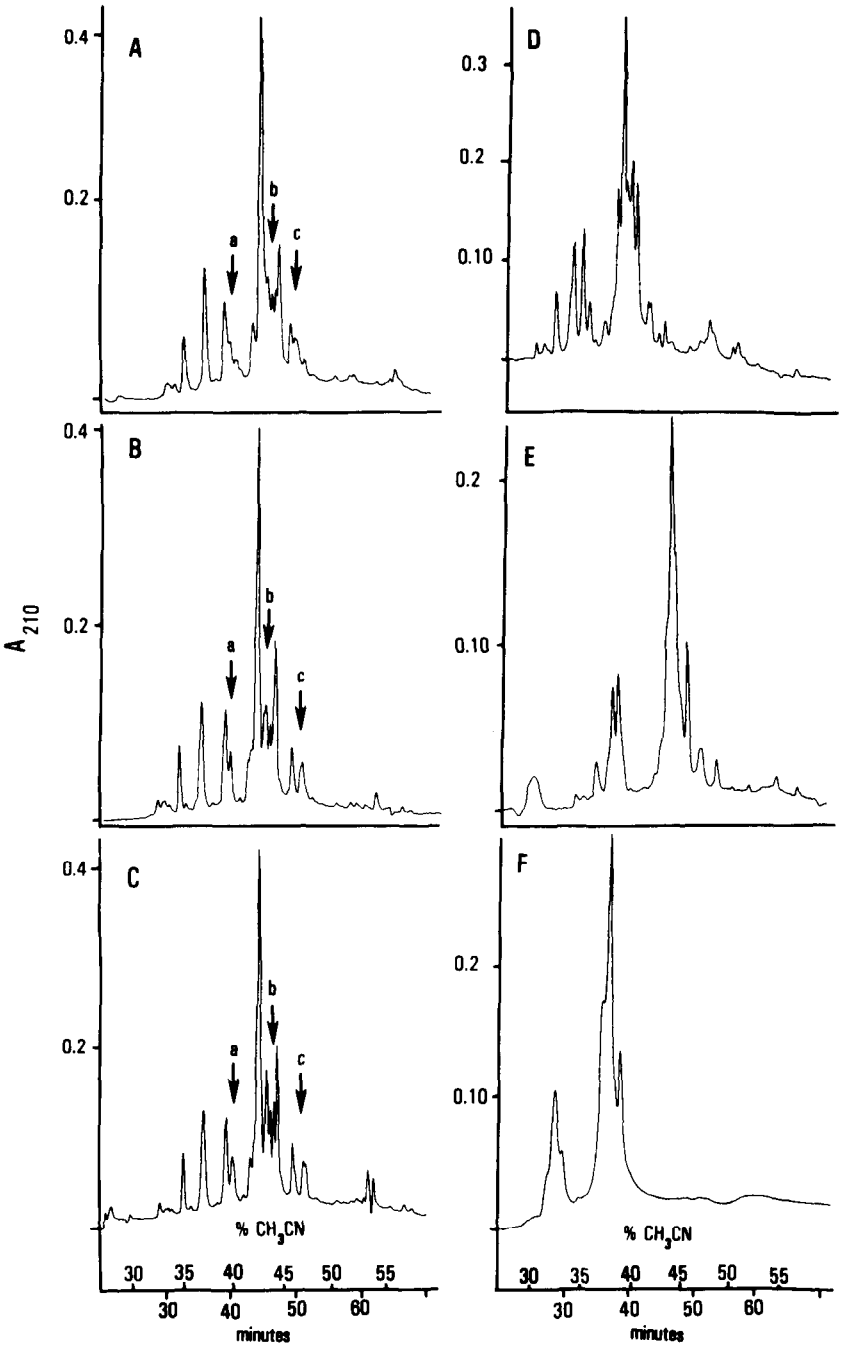

Fig. 5. Effects of various columns and bonded phases on resolution of PE-glutenins. (A) SynChropak RP-P C_{18}; (B) Brownlee Aquapore RP-300; (C) Bakerbond C_8; (D) Bakerbond diphenyl; (E) Whatman diphenyl; and (F) Bakerbond cyanopropyl. Glutenin was prepared in the presence of 8 *M* urea, reduced with 5% 2-ME and alkylated with 0.7 *M* vinylpyridine. Chromatographic conditions as in Fig. 1. Arrows a–c indicate components resolved better on the Brownlee and Bakerbond C_8 columns than on the SynChrom column.

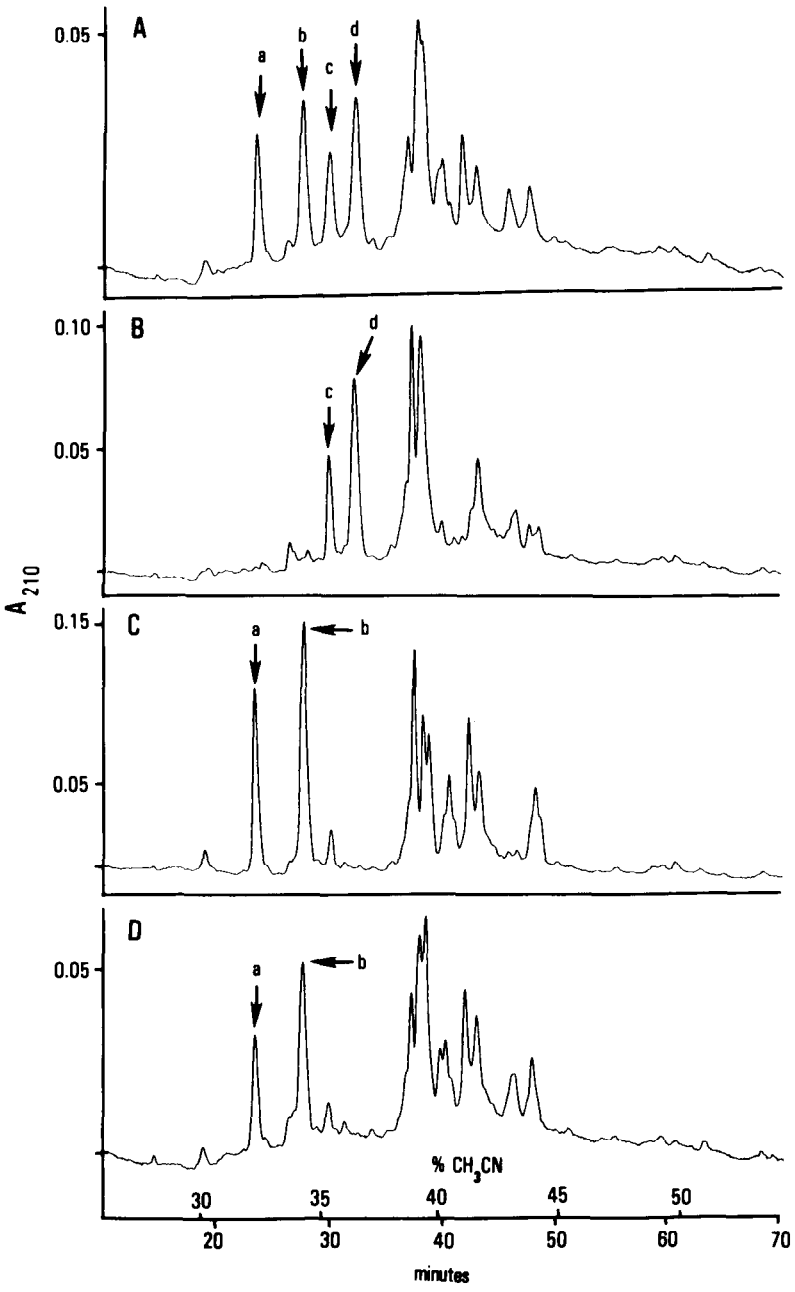

Fig. 6. RP-HPLC of PE-glutenins, reduced with 5% 2-ME in the presence of 8 *M* urea, from (A) Chinese Spring and from aneuploid lines, (B) N1DT1A, (C) N1BT1A, and (D) Ditelo $1B_S$. Samples were applied to a Brownlee Aquapore RP-300 column, and eluted with a linear gradient from 15 to 60% B during 65 min. Other conditions as in Fig. 1. Arrows indicate early peaks present in Chinese Spring but absent in aneuploid lines; peaks a and b are coded by the long arm of chromosome 1D, and peaks c and d by the long arm of chromosome 1B.

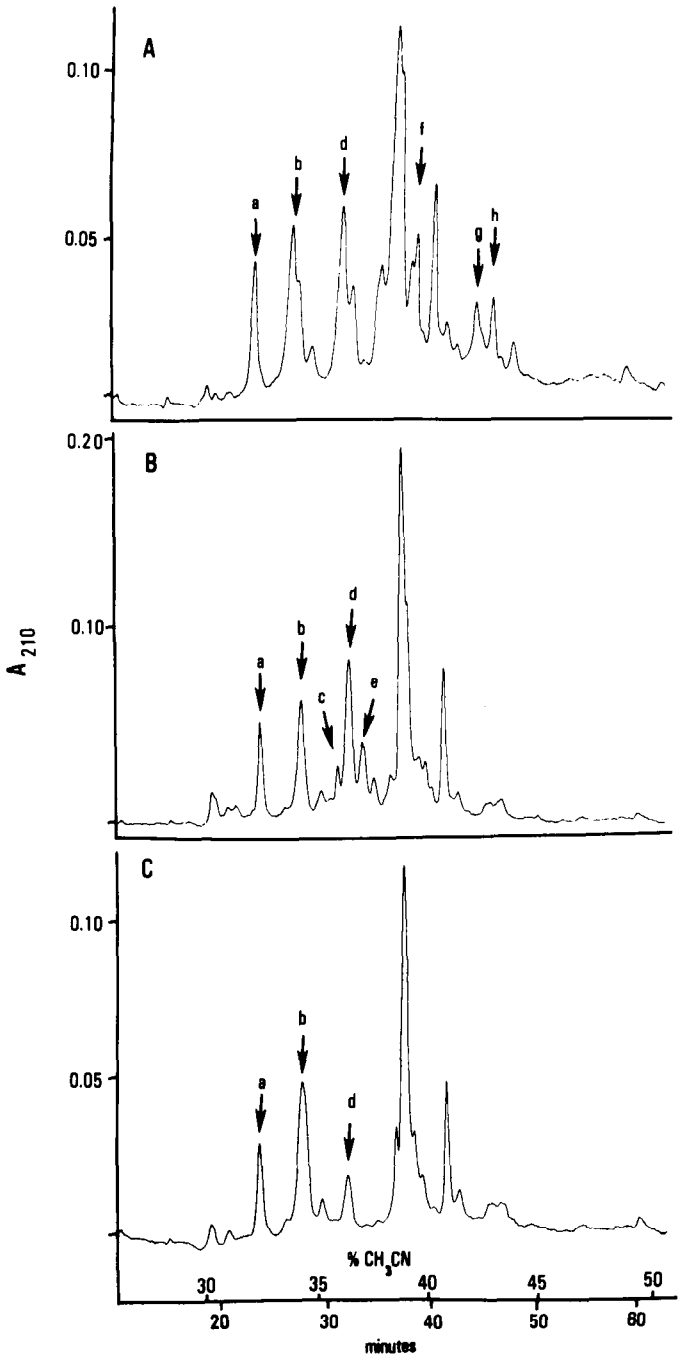

Fig. 7. RP-HPLC of PE-glutenins from bread wheat varieties (A) Marquis, (B) Hardi, and (C) Maris-Huntsman. Conditions are as in Fig. 6. Arrows indicate chromatographic peaks differing between samples.

ing that RP-HPLC of glutenin can reveal differences among varieties and be used for varietal identification.

Major differences occur between early eluting Hardi and Maris-Huntsman PE-glutenins (Fig. 7, B and C). Peaks a and b are common to these varieties, but c and e are present only in Hardi, and peak d is much larger in Hardi. Since Hardi and Maris-Huntsman differ greatly in breadmaking quality (Hardi is very good, while Maris-Huntsman is unsuitable) and since quality of varieties may be related to their HMW glutenin subunits[10], our results indicate that RP-HPLC can be used, as is electrophoresis, to study relationships between HMW glutenin subunit composition and the technological properties of flour.

DISCUSSION

Native glutenin cannot at present be analyzed by RP-HPLC because of its high molecular weight (up to 20,000,000)[38], but after reduction of disulfide bonds, its subunits, which range in molecular weight from 10,000 to about 150,000[6–8], are amenable to RP-HPLC fractionation. We have shown that reduction can be done with either 2-ME or DTT. We used 2-ME in most of our experiments but DTT which has a lower oxidation–reduction potential than does 2-ME[39] can be used at much lower concentration, so subsequent alkylation requires less alkylating agent.

Choice of dissociating agent can even be more critical. SDS forms strong complexes with proteins, but it is not clearly understood how it modifies their surface hydrophobicities[26]. Furthermore, since RP-HPLC facilitates polypeptide purification, the presence of SDS, which is difficult to remove from proteins, may be undesirable. GuHCl may be advantageous because it is a stronger dissociating agent than urea, but urea is considerably less hazardous and expensive than GuHCl. Fresh urea solution is preferable to avoid formation of cyanate, which could react with susceptible amino groups of proteins and modify RP-HPLC separation.

In strongly denaturing conditions, some soluble starch may be extracted from the glutenin-containing residue and injected onto columns along with glutenin subunits. When samples are transferred from a urea to an CH_3CN environment upon injection, polysaccharides can precipitate and partially block the column. We have noticed constantly increasing pressure upon repeated analyses of PE-glutenins on a Bakerbond column; backflushing the column (0.2 ml/min, 50°C) with 90% DMSO, a good solvent for starch, restored normal operating pressure. However, SynChropak and Brownlee columns, used for numerous analyses of glutenin subunits, operated at constant pressure. Differences in bonded phases, carbon loading, silica type, packing methods or particle size of packing materials may explain differences between columns.

RP-HPLC resolution of glutenin subunits improves significantly upon alkylation. This improvement may have two origins. First, after injection of samples, sulfhydryl groups are no longer protected against reoxidation since 2-ME and urea are rapidly eliminated, so polypeptides may reassociate to higher-molecular-weight polymers. Indeed, HMW glutenin subunits coded by chromosomes 1D (peaks a and b in Fig. 6) can form oligomers in a medium poor in 2-ME[40]. However, formation of intermolecular disulfide bonds during RP-HPLC may be limited by the rapid separation of polypeptides which is achieved. The improved resolution noted for

reduced-alkylated glutenin may also be attributed to the alkylation reaction itself, which introduces new chemical groups to the molecules and may therefore modify surface hydrophobicity. PE-glutenins may, because of additional positive charge under acidic conditions, elute earlier than underivatized glutenins, in contrast to CN-derivatives of proteins, whose charge at acidic pH is not altered. Retention times of reduced glutenins decreased after derivatization with iodoacetic acid, although carboxymethyl groups are not charged under acidic conditions: this suggests that alkylation itself may significantly alter protein conformations and resulting surface hydrophobicities. In addition, location of modified cysteine residues may affect interactions with polar or non-polar functional groups at the silica surface or with the eluent itself, thus modifying resolution.

Resolution of reduced-alkylated glutenin subunits was best when 4-vinylpyridine was used. Similar observations were made by Huebner and Wall[22] for electrophoretic separations of derivatized glutenin subunits. Alkylation with 4-vinylpyridine under mild conditions (short reaction time and a 1:1 ratio of alkylating agent to total sulfhydryl groups[33]) is highly specific for cysteine residues; with acrylonitrile, however, secondary reactions with lysine may be more likely. It is also possible that pyridylethylation may facilitate RP-HPLC separation of subunits having similar hydrophobicities but different cysteine contents, since subunits rich in sulfhydryl groups could elute sooner after derivatization.

C_{18} and C_8 columns from different manufacturers give similar elution profiles of PE-glutenins, and elution times do not differ significantly. Similar resolution by C_{18} and C_8 columns may be due to folding of C_{18} chains under aqueous conditions or to a lack of protein hydrophobic sites large enough to discriminate between these two bonded phases[41]. Also, both C_8 and C_{18} chains are long and may occupy a large fraction of pore volume; this may inhibit accessibility to any surface silanol groups, thus avoiding different retention behavior observed with stationary phases having shorter alkyl groups[42].

Elution times of proteins on diphenyl columns may be delayed relative to aliphatic columns because of aromatic stacking interactions between proteins and the bonded phase. However, PE-glutenin subunits eluted sooner from a Bakerbond diphenyl column than from aliphatic columns, indicating weaker hydrophobic interactions with the diphenyl surface. In addition, overall elution profiles were extremely similar, suggesting no aromatic interactions. By contrast, resolution differed greatly on the Whatman diphenyl column, from which PE-glutenin eluted later than from other bonded phases, revealing that aromatic stacking effects could have occurred. Similar findings were obtained when chromatographing gliadins on these two diphenyl columns[43]. The reason why these two columns resolve protein so differently is unclear; resolution may be linked to the silica used, as shown for C_8 bonded-phases[44], to silanol content[45], to spatial configurations of binding sites, or to pore morphology[42]. Differences in surface area (100 m^2/g for the Bakerbond column and 250 for the Whatman column[35]) may affect the number of reactive groups on the stationary phase, and efficiency of end-capping may also differ between these columns. Finally, the Whatman column has a higher carbon loading (8% as compared to 4%[35]), which may explain higher retention times of proteins and diminish mass transfer between mobile and stationary phases. Cyanopropyl columns are less hydrophobic than the other columns used, leading to decreased retention times; such differences in selectivity may be useful in protein purification.

Analysis of aneuploid lines was useful to determine which chromosomes contain genes coding PE-glutenin subunits and to recognize which peaks contain HMW and LMW subunits. HMW subunits elute earlier than LMW subunits, indicating that they have lower surface hydrophobicities. This observation agrees with predictions of total hydrophobicity from amino acid analyses: HMW subunits are much richer in glycine, whereas LMW subunits contain more phenylalanine, valine, leucine, isoleucine, and methionine[3,9]. In RP-HPLC, the HMW glutenin subunits from Chinese Spring separate into four major peaks (a–d in Fig. 6), two coded by chromosomes 1D and two by 1B. Thus, RP-HPLC and electrophoretic studies[6] are in good agreement: SDS-PAGE revealed four major HMW bands, two (MW 133,000 and 86,000) coded by 1D (allele Glu-Dla[46]) and two (MW 104,000 and 93,000) by 1B (allele Glu-B1b[46]). Although the two 1B subunits have MWs between those of the 1D subunits, both 1D-coded PE-glutenins eluted earlier upon RP-HPLC than the 1B-coded PE-glutenins (Fig. 6), indicating lower surface hydrophobicities for the 1D subunits. Alternatively the 1D-coded subunits may have higher cysteine contents than 1B subunits, making them more polar after pyridylethylation.

RP-HPLC indicates significant variation among glutenin subunits of bread wheat varieties, confirming analyses by SDS-PAGE[7,10,11]. Quality-protein associations often involve subunits coded by genes which are allelic, but which have opposite effects on quality. Further RP-HPLC analyses will reveal the extent of glutenin subunit polymorphism, and determine whether allelic subunits have different surface hydrophobicities. If so, RP-HPLC of glutenin subunits could potentially be used as a screening tool for breadmaking quality. In addition, variation in glutenin subunit composition could be exploited for varietal identification purposes, especially when gliadin patterns do not differentiate closely related varieties[29,30].

In conclusion, RP-HPLC is an excellent new method for analysis of glutenin subunits. Preliminary experiments suggest that their resolution is further improved at increased temperature. Separation by surface hydrophobicity complements methods of separation by molecular weight or charge and reveals new information on the biochemistry of these proteins. RP-HPLC of glutenin subunits has high potential as a tool for flour quality investigation, for screening in breeding programs, and for genetic analyses. In addition, our study should promote the use of RP-HPLC as a preparative method for purification of these polypeptides, which have been difficult to purify by classical means[22].

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REFERENCES

1 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.
2 P. I. Payne and K. G. Corfield, *Planta*, 145 (1979) 83.
3 J. A. Bietz and J. S. Wall, *Cereal Chem.*, 50 (1973) 537.
4 J. A. Bietz and J. S. Wall, *Cereal Chem.*, 57 (1980) 415.

5 E. A. Jackson, L. M. Holt and P. I. Payne, *Theor. Appl. Genet.*, 66 (1983) 29.
6 J. A. Bietz, K. W. Shepherd and J. S. Wall, *Cereal Chem.*, 52 (1975) 513.
7 P. I. Payne, K. G. Corfield and J. A. Blackman, *Theor. Appl. Genet.*, 55 (1979) 153.
8 P. I. Payne, L. M. Holt and G. J. Lawrence, *J. Cereal Sci.*, 1 (1983) 3.
9 F. R. Huebner, G. L. Donaldson and J. S. Wall, *Cereal Chem.*, 51 (1974) 240.
10 T. Burnouf and R. Bouriquet, *Theor. Appl. Genet.*, 58 (1980) 107.
11 G. J. Lawrence and K. W. Shepherd, *Aust. J. Biol. Sci.*, 33 (1980) 221.
12 P. I. Payne, L. M. Holt and C. N. Law, *Theor. Appl. Genet.*, 60 (1981) 229.
13 B. J. Miflin, J. M. Field and P. R. Shewry, in J. Daussant, J. Mossé and J. Vaughan (Editors), *Seed Proteins*, Academic Press, London, 1983, pp. 253–319.
14 P. I. Payne, K. G. Corfield, L. M. Holt and J. A. Blackman, *J. Sci. Food Agric.*, 32 (1981) 51.
15 J. H. E. Moonen, A. Scheepstra and A. Graveland, *Euphytica*, 31 (1982) 677.
16 P. I. Payne, C. N. Law and E. E. Mudd, *Theor. Appl. Genet.*, 58 (1980) 113.
17 G. J. Lawrence and K. W. Shepherd, *Theor. Appl. Genet.*, 60 (1981) 333.
18 T. Burnouf, A. Le Blanc, P. Poullard and R. Bouriquet, *Bull. Soc. Bot. Nord. Fr.*, 34 (1981) 67.
19 T. Burnouf, R. Bouriquet and P. Poullard, *Theor. Appl. Genet.*. 64 (1983) 103.
20 J. A. Bietz and J. S. Wall, *Cereal Chem.*, 49 (1972) 416.
21 Z. I. Hamauzu, T. Arakawa and D. Yonezawa, *Agric. Biol. Chem.*, 36 (1972) 1829.
22 F. R. Huebner and J. S. Wall, *Cereal Chem.*, 51 (1974) 228.
23 R. A. Orth and W. Bushuk, *Cereal Chem.*, 50 (1973) 191.
24 J. W. S. Brown, C. N. Law, A. J. Worland and R. B. Flavell, *Theor. Appl. Genet.*, 59 (1981) 361.
25 L. M. Holt, R. Astin and P. I. Payne, *Theor. Appl. Genet.*, 60 (1981) 237.
26 J. A. Bietz, *J. Chromatogr.*, 255 (1983) 219.
27 T. Burnouf and J. A. Bietz, *J. Cereal Sci.*, 2 (1984) 3.
28 J. A. Bietz, T. Burnouf, L. A. Cobb and J. S. Wall, *Cereal Chem.*, 61 (1984) 124.
29 J. A. Bietz, T. Burnouf, L. A. Cobb and J. S. Wall, *Cereal Chem.*, 61 (1984) 129.
30 T. Burnouf, J. A. Bietz, L. A. Cobb and J. S. Wall, *C.R. Acad. Sci. Paris*, Ser. III, 297 (1983) 377.
31 E. R. Sears, *University of Missouri Research Bulletin*, 572 (1954) 3.
32 W. W. Cleland, *Biochemistry*, 3 (1964) 480.
33 M. Friedman, L. H. Krull and J. F. Cavins, *J. Biol. Chem.*, 245 (1970) 3868.
34 J. F. Cavins and M. Friedman, *J. Biol. Chem.*, 243 (1968) 3357.
35 C. T. Wehr, in W. S. Hancock (Editor), *Handbook of HPLC for the Separation of Amino Acids, Peptides and Proteins*, Vol. 1, CRC Press, Boca Raton, FL, 1984, pp. 31–57.
36 T. Burnouf and J. A. Bietz, in preparation.
37 P. I. Payne, L. M. Holt, E. A. Jackson and C. N. Law, *Phil. Trans. Roy. Soc. London*, B, 304 (1984) 359.
38 F. R. Huebner and J. S. Wall, *Cereal Chem.*, 53 (1976) 258.
39 W. Konigsberg, in C. H. W. Hirs and S. N. Timasheff (Editors), *Methods in Enzymology, Vol. XXV, Enzyme Structure Part B*, Academic Press, New York, 1972, pp. 185–188.
40 G. J. Lawrence and P. I. Payne, *J. Exp. Bot.*, 34 (1983) 254.
41 R. V. Lewis and D. Dewald, *J. Liquid Chromatogr.*, 5 (1982) 1367.
42 Cs. Horváth, *LC*, 1 (1983) 552.
43 J. A. Bietz, T. Burnouf, L. A. Cobb and J. S. Wall, *Cereal Foods World*, 28 (1983) 555 (Abstract).
44 J. D. Pearson, N. T. Lin and F. E. Regnier, in M. T. W. Hearn, F. E. Regnier and C. T. Wehr (Editors), *High-Performance Liquid Chromatography of Proteins and Peptides*, Academic Press, New York, 1983, pp. 81–94.
45 W. S. Hancock and J. T. Sparrow, in Cs. Horváth (Editor), *High-Performance Liquid Chromatography, Advances and Perspective*, Vol. 3, Academic Press, New York, 1983, pp. 49–85.
46 P. I. Payne and G. J. Lawrence, *Cereal Res. Commun.*, 11 (1983) 29.