Analysis of Protein Hydrolysates. 1. Use of Poly(2-hydroxyethylaspartamide)–Silica Column in Size Exclusion Chromatography for the Fractionation of Casein Hydrolysates

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A poly(2-hydroxyethylaspartamide)–silica column (PHEA), with a 0.05 M formic acid mobile phase, was tested for the size exclusion chromatography (SEC) of amino acids and small peptides (molecular mass lower than 1000 Da). The PHEA shows good SEC properties, although some nonideal size exclusion behavior suggests it has a low cationic character. On the other hand, the separation of peptides is affected by the sample salt concentration. The technique was applied to the analysis of several casein hydrolysates, and the mean chain length of peptides in the collected fractions indicates a good separation of peptides on the basis of their size.

Keywords: *Size exclusion chromatography; peptides; casein hydrolysates*

INTRODUCTION

The nutritional quality of protein hydrolysates is related to their small peptide contents. It has been shown that the di- and tripeptides are absorbed from the small intestine more rapidly than are free amino acids. Thus, small peptide formulas are used more effectively than amino acid mixtures and have higher nutritive value (Fairclough et al., 1980; Rees et al., 1984; Hara et al., 1984; Keohane et al., 1985; Webb, 1990).

Therefore, the development of a method capable of separating the small peptides from protein hydrolysates would be a valuable tool in characterizing such preparations. Size exclusion chromatography (SEC) is one of the most widely used techniques for the fractionation of proteins and peptides. However, the different supports, previously described, were not successful in separating complex mixtures of peptides owing to low resolution (Barth, 1982; Lemieux and Amiot, 1989) and to interactions of solutes with the stationary phase resulting in nonideal size exclusion behavior (Ui, 1979; Himmel and Squire, 1981; Barth, 1982; Kopaciewicz and Regnier, 1982; Irvine and Shaw, 1986; Vijayalakshmi et al., 1986; Wahl et al., 1986; Lemieux and Amiot, 1989; Lemieux et al., 1991).

The aim of this work was to test the performance of the poly(2-hydroxyethylaspartamide) phase in size exclusion chromatography of amino acids and small peptides and to apply this technique to casein hydrolysates.

MATERIALS AND METHODS

Materials. The absorbance measurements for the trinitrobenzenesulfonic acid (TNBS) method were made with a Titertek Multiskan Plus MK II spectrophotometer (Flow lab.,

Boggio Lugano, Switzerland), using Nunc 96 P-ELISA plates (Poly-Labo, Strasbourg, France). Amino acid analyses were performed with a Biotronik LC 3000 analyzer (Biotronik, Maintal, Germany). The HPLC system consisted of two M 510 pumps, a Wisp 710 B injector, and a Lambda Max M 481 spectrophotometer (Waters, Milford, MA). The system was coupled to a computer equipped with Baseline 810 software (Waters). A poly(2-hydroxyethylaspartamide)–silica (PHEA) column, 250 × 9.4 mm, 5 μm, 200 Å pore size (PolylC, Columbia, MD), was used for HPLC.

Pancreatin (grade VI, activity = 4 × USP), trypsin (type XIII, TPCK treated, from bovine pancreas), TNBS reagent, peptides, and amino acids were purchased from Sigma (St. Louis, MO). Hydrochloric and formic acids (98–100%, analytical grade) were obtained from Merck (Darmstadt, Germany). The commercial casein hydrolysates were provided by Nutripharm-Gallia (Steenvoorde, France). The other reagents (analytical grade) were obtained from Prolabo (Paris, France).

For HPLC, water was purified by passage through a Milli-Q water purification system (Millipore, Belford, MA). All solvents used for the HPLC were carefully degassed by sonication for 10 min before use.

Methods. *Preparation of Casein Hydrolysates.* Bovine whole casein was precipitated from fresh skim milk by the addition of 1 N HCl to pH 4.6 (approximately 48 mL of 1 N HCl for 1 L of milk). Four successive precipitations were carried out. Between each, the precipitate was washed and dissolved in water (half of the original volume of milk) by slow addition of 1 N NaOH to pH 7 (around 20 mL of 1 N NaOH for 1 L of original milk). After the fourth precipitation, the solution of casein at pH 7 was dialyzed against distilled water and then lyophilized.

A 1% solution of whole casein in 0.01 M phosphate buffer, pH 7.5, was submitted to proteolysis by pancreatin and trypsin, at 37 °C, using the following enzyme/substrate ratios (E/S) and hydrolysis times: with pancreatin, E/S = 1/25, 6 h (P1), E/S = 1/200, 6 h (P2), E/S = 1/200, 2 h (P3); and with trypsin E/S = 1/25, 15 h (T1), E/S = 1/200, 2 h (T2). The reactions were stopped by decreasing the pH to 2.0 with formic acid, and these preparations, named standard hydrolysates, were freeze-dried.

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High-Performance Size Exclusion Chromatography. Casein hydrolysates, peptides, and amino acids were dissolved in 0.05 M formic acid and chromatographed at room temperature under isocratic conditions (0.05 M formic acid in water, pH near 2.5). Flow rate was 0.5 mL/min. The injection volumes of each individual amino acid and peptide solution ranged from 20 to 200 μL, and injection amounts ranged from 2 to 300 μg. For the mixture containing 350 nmol/mL of various peptides and amino acids, the injection volume was 50 μL. Standard hydrolysates were dissolved to a concentration of 0.4% so that the phosphate concentration of these solutions was lower than 0.005 M. Fifty microliters of these solutions was injected on the column. The same volume of a 0.8% solution was used for the commercial hydrolysates. Chromatographic peaks were detected at 230 nm. For each sample, four fractions (F1–F4) were collected: F1, from 13.5 to 18 min; F2, from 18 to 21.5 min; F3, from 21.5 to 22.5 min; and F4, from 22.5 to 32 min. Five runs were done, the fractions were pooled, and the solvent was removed using a Speed-vac evaporator.

Estimation of the Mean Chain Length of Peptides by the TNBS Method. Fractions collected from casein hydrolysates (standard and commercial) were analyzed for their free amino group contents, before and after acid hydrolysis (5.7 N HCl, 110 °C, 24 h). Samples (hydrolyzed and nonhydrolyzed fractions) were dissolved in water, and 10 μL of the solution was placed on an ELISA plate; 100 μL of 1 M borate buffer, pH 9.2, and 40 μL of the TNBS reagent (1/42 dilution in water) were added. After incubation for 1 h at 37 °C, 10 μL of the stopping solution made up daily (0.02 M Na_2SO_3, 2 M NaH_2PO_4) was added and the absorbance was measured at 420 nm. Two or three replicates were made for each sample. For every assay, a standard curve was made with glycine solutions (0–1.5 mM) to check the linearity between absorbance and free amino group content. The mean chain length of the peptides in a fraction was estimated by calculating the ratio between the free amino group contents of the hydrolyzed and nonhydrolyzed fractions.

RESULTS AND DISCUSSION

Study of Some PHEA Characteristics in SEC of Amino Acids and Peptides. *1. Fractionation Range.* A set of standard amino acids and peptides containing 2–10 amino acid residues, pure or mixed, was used to evaluate the performance of the PHEA support in SEC mode (Table 1). Most of the small molecules (MW < 200) were eluted between 23 and 31 min, those with MW of 200–500 between 20 and 23 min, those with MW of 500–900 between 18 and 20 min, and the large ones (MW of 900–1300) around 15 min.

These results indicate that it is possible to separate amino acids and peptides with MW ranging from 80 to 900 with this support. This fractionation range covers MWs much smaller than previously described for SE-HPLC of peptides. With other packings, separations can be made down to a MW of 200, but the smallest size previously noted for total exclusion was 10 000 (Barth, 1982; Shioya et al., 1982; Bennett et al., 1983; Irvine and Shaw, 1986; Lemieux et al., 1991; Ahmed and Modrek, 1992; Visser et al., 1992).

The low exclusion size of this support with 200 Å pore size would be due to the use of formic acid as mobile phase. According to Andrews and Alpert (1990), the coating swells in formic acid to the point that it fills the pores completely and sieving occurs between the polymer chains of the coating, whose distance is about 15–20 Å. In fact, formic acid competes with adjacent ligands of the support for dipole–dipole interactions, such as hydrogen bonding, increasing the mobility of the chains as well as the permeability of the coating.

2. Retention Mechanism. The results with the PHEA support demonstrate mainly a size exclusion mecha-

Table 1. Retention Times (RT) and Molecular Weights (MW) of Amino Acids and Peptides Used for the SEC Performance Test of the Poly(hydroxyethylaspartamide) Column[a]

product	MW	RT (min)
G	75.0	24.9
A	89.0	23.3
S	105.1	24.3
P	115.1	24.9
V	117.1	23.2
T	119.1	23.9
C-SO₃H	169.2	30.0
L	131.2	24.2
I	131.2	23.8
N	132.1	23.9
K	146.2	14.2
E	147.1	27.4
M	149.2	23.8
F	165.2	25.1
R	174.2	14.5
Y	181.2	25.5
W	204.2	30.5
L-S	218.3	20.5
S-N	219.2	22.5
D-E	262.2	30.7
S-Y	268.3	21.7
M-E	278.3	21.6
L-W	317.4	22.2
F-Y	328.4	22.1
F-G-G	279.3	20.9
W-G-G	318.3	21.9
Y-V-G	337.4	20.6
Y-Y-L	457.5	21.3
F-G-F-G	426.5	20.2
Y-G-G-F-L	555.6	18.3
W-H-W-L-Q-L	882.0	18.4
M-E-H-F-R-W-G	962.1	14.6
R-P-P-G-F-S-P-F-R	1060.5	14.6
P-H-P-F-H-F-F-V-Y-K	1318.5	14.5

[a] Mobile phase, 0.05 M formic acid; flow rate, 0.5 mL/min; column, 250 × 9.4 mm, 5 μm, 200 Å.

nism. However, other retention mechanisms have some effect resulting in nonideal size exclusion behavior. In fact, basic amino acids were eluted more quickly, while tryptophan and small acidic peptides, *e.g.* Asp-Glu, were eluted later than expected.

Generally, hydrophobic or electrostatic interactions between solute molecules and the matrix occur because the stationary phases used in SEC have some anionic character (Kopaciewicz and Regnier, 1982; Irvine and Shaw, 1986). In the case of poly(methyl methacrylate)-based support, this charge is the result of residual carboxyl groups on that support. With silica-based supports, the residual negative charge is the result of underivatized surface silanols (Kopaciewicz and Regnier, 1982). The occurrence of both ion exclusion and ion exchange effects was previously described with different supports (Golovchenko et al., 1992; Kopaciewicz and Regnier, 1982).

Under acidic conditions used here the silanol groups are not charged ($pK = 3.5–4.0$), but the faster elution of basic amino acids and the delayed elution of small acidic peptides suggest that PHEA has some cationic character. This could be due to the occurrence of underivatized amino groups, since the preparation of the matrix involves the covalent binding of polysuccinimide to an aminopropyl silica (Alpert, 1990). The same low cationic character was previously reported when the PHEA was used with a mobile phase of low ionic concentration and in the absence of organic solvents (Alpert, 1990). However, it seems that these electrostatic interactions mainly occur with amino acids and
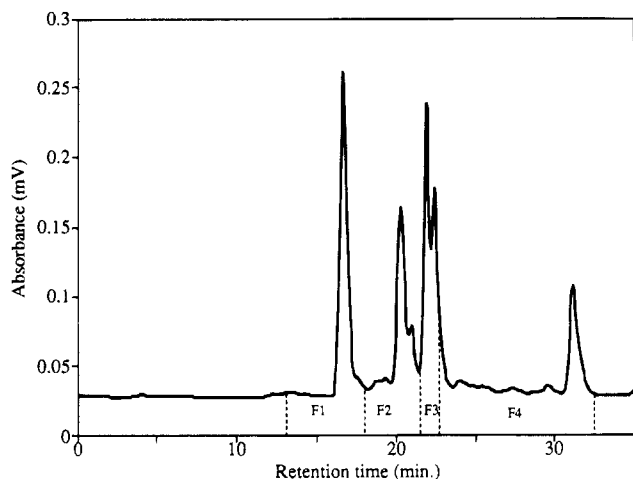
Figure 1. SEC of a mixture of amino acids and peptides on a PHEA column: mobile phase, 0.05 M formic acid; flow rate, 0.5mL/min. The mixture contained M-E-H-F-R-W-G, Y-P-F-P-G-P-I, R-P-P-G-F-S-P-F-R, and P-H-P-F-H-F-F-V-Y-K identified in fraction F1 (13.5–18 min); F-G-F-G and F-L-E-E-I identified in fraction F2 (18–21.5 min); E-K, L-W, M-E, L-S, W-G-G, and Y-V-G identified in fraction F3 (21.5–22.5 min); and D-E, W, E, F, I, and A identified in fraction F4 (22.5–32 min).

very small peptides, since the elution of an acidic pentapeptide F-L-E-E-I was not delayed (Figure 1). This is probably related to steric obstruction.

As previously described for other SEC supports (Inouye, 1991; Golovchenko et al., 1992; Iliev and Tchorbanov, 1992), the elution of tryptophan from this support was also delayed. Interaction of the aromatic amino acids with the matrix is normally expected to be of a hydrophobic type. However, since PHEA is a very polar coating, a hydrogen bond between the NH group of the lateral amino acid chain and the OH groups of the support could be involved (Inouye, 1991). In that case, one might expect elution of L-W to be delayed compared with F-Y. Judging from Table 1, it is not. W-G-G also compares well with other tripeptides. This suggests that the anomalous behavior of Trp reflects a property found in the amino acid but not when it is part of a peptide.

Since the aim of the technique reported here is the separation of peptides and amino acids from casein hydrolysates, the delayed elution of tryptophan is not really a problem, because it is eluted in the amino acid fraction. Moreover, this nonideal size exclusion behavior may be useful when tryptophan determination is required (Iliev and Tchorbanov, 1992).

3. Resolution. The theoretical plate number of this support, determined at the flow rate used for SE-HPLC analysis (0.5 mL/min), was 8303. From this result the peak capacity (the maximum number of peaks separated on a given column), calculated as reported by Hagel (1992), was 7 or 10, for a 1.5 or 1.0 resolution (R_s), respectively ($R_s = 2$ (spacing of the center of two peaks/sum of two peak widths)). Although this peak capacity is similar to that predicted for prepacked SEC columns (Hagel, 1992), the fractionation range is narrower than those of other SEC columns, resulting in better resolution within the range. In fact, theoretically, peptides having MW differences of 100 can be separated with 1.0 resolution.

A mixture of standard amino acids and peptides was submitted to SEC on this column. Its components were resolved in four fractions (Figure 1). For identification,

each fraction was collected and analyzed, before and after acid hydrolysis, for the amino acid contents. Results (data not shown) complement and support the experiments with pure peptides and amino acids; *i.e.*, F1 contains the large peptides (seven or more residues); F2, the medium peptides (four to six residues); F3, the small peptides (two or three residues); and F4, the amino acids. The basic amino acids were eluted with the large peptides (F1), and the small acidic peptide was in F4.

The sharpness of the chromatographic peaks of the peptide and amino acid mixture on the PHEA column was similar to that reported by Irvine and Shaw (1986), using a TSK-G2000-SW column, but their MW range (574–66 000) was greater than that used in this study.

4. Recovery. For the study of the PHEA phase with respect to recovery of peptides, 100–300 μg of the standard and commercial hydrolysates was injected on the column. The recovery of the peptide mass (determined by amino acid analysis) ranged from 93 to 100%. These results are better than the values reported for other commercial SEC columns (Ui, 1979; Inouye, 1991; Ahmed and Modrek, 1992).

5. Coating Stability. To determine the origin of an abnormally large amount of aspartic acid (Asp) in the amino acid analysis of fractions collected after passage through the column, eluent from a blank injection (formic acid) was analyzed, after acid hydrolysis, for its amino acid composition. The occurrence of some Asp in the eluent suggests an elution of a coating excess, the aspartamide group giving rise to aspartic acid after acid hydrolysis. This checking was made throughout the study and showed that at the initial use of the column, the Asp concentration of the eluent was 13 nmol/mL and progressively decreased to 3 nmol/mL. However, the column was used for more than 400 separations without deterioration of resolution. These results were considered to determine the Asp contents of all hydrolyzed fractions. For a precise determination of amino acids in the eluent it will be safer to determine Asp concentration in a blank eluent.

Effect of Sample Salt Concentration. Since the preparation of casein hydrolysates is usually performed in a buffer solution, the effect of sample salt content on the chromatographic separation of peptides needed to be studied. A casein hydrolysate was prepared in a 0.01 M phosphate buffer, pH 7.5 (HS2), and then formic acid was added to pH 2. Two other hydrolysates were prepared from the first one by salt addition (sodium mono- and dihydrogen phosphate) or by dilution, to achieve final concentrations of 0.1 (HS3) and 0.005 M (HS1).

The chromatographic patterns of these hydrolysates (Figure2) showed a peak shifting toward higher retention times with increasing salt concentrations. In fact, with 0.01 M salt, the large peptides are eluted a little later than with 0.005 M, while with 0.1 M most of the peptides are eluted as the di- and tripeptides were with 0.005 M salt. The same behavior was observed with NaCl. Although this behavior suggests an increasing of the fractionation range of the support, it remains to be explained.

Analysis of Casein Hydrolysates. After the conditions were set to operate in SEC mode with the PHEA support, this technique was applied to the separation of peptides and amino acids from casein hydrolysates. Five standard (P1, P2, P3, T1, T2) and three commercial (H1, H2, H3) hydrolysates were SE-HPLC analyzed. As
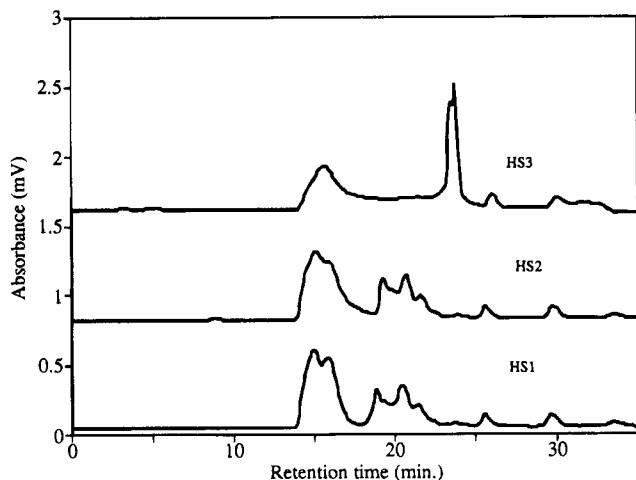
Figure 2. Effect of sample salt concentration (sodium mono- and dihydrogen phosphate) on peptide separation from pancreatin casein hydrolysates: salt concentrations, (HS1) 0.005 M, (HS2) 0.01 M, and (HS3) 0.1 M; PHEA column, mobile phase, 0.05 M formic acid; flow rate, 0.5 mL/min.
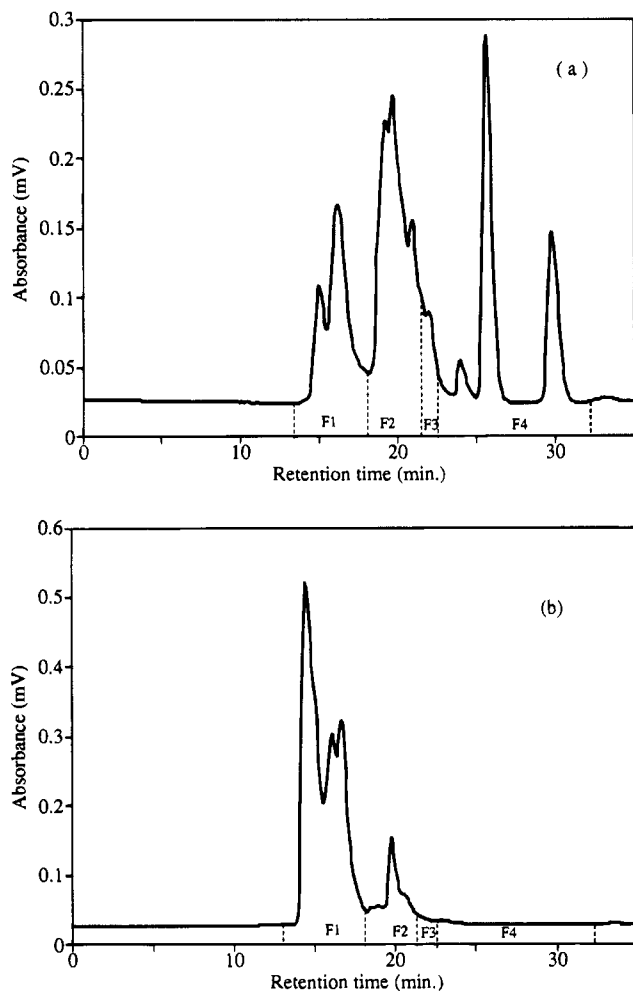
Figure 3. SEC of (a) pancreatin casein hydrolysate P1 and (b) tryptic casein hydrolysate T2: PHEA column; mobile phase, 0.05 M formic acid; flow rate, 0.5 mL/min; collected fractions, F1 (13.5−18 min), F2 (18−21.5 min), F3 (21.5−22.5 min), and F4 (22.5−32 min).

an illustration, the chromatographic patterns of two hydrolysates, P1 and T2, are shown in Figure 3. As the mixture of peptides and amino acids, the hydrolysates were resolved in four fractions that were analyzed for estimating the mean chain length of peptides (Table 2).

Table 2. Mean Chain Length of Peptides of SE-HPLC Fractions from Casein Hydrolysates[a]

| hydrolysate | mean chain length (amino acid residue number) | | | |
	F1	F2	F3	F4
P1	7.5	4.7	2.1	1.4
P2	9.0	6.7	1.7	1.5
P3	8.5	7.5	2.8	1.5
T1	8.1	7.7	3.4	1.3
T2	8.4	6.1	2.1	1.7
H1	9.2	5.5	2.2	1.4
H2	7.8	3.7	3.2	1.0
H3	8.5	5.0	3.2	1.2

[a] The average peptide size of the four SE-HPLC fractions from the casein hydrolysates was determined by TNBS assay of the free amino group contents. The ratio between hydrolyzed and nonhydrolyzed fractions gives the mean chain length of peptides. PHEA column: mobile phase, 0.05 M formic acid; flow rate, 0.5 mL/min.

The mean number of amino acid residues of peptides in fractions 1 varied from 7.5 to 9.2, in fractions 2 from 3.7 to 7.7, in fractions 3 from 1.7 to 3.4, and in fractions 4 from 1.0 to 1.7. These values correlated rather well with the size of peptides expected in each fraction.

However, the values higher than 1 found for the fourth fraction were not expected and could be explained by the presence of both small acidic peptides and tryptophan. In fact, this amino acid is destroyed by the acid hydrolysis, but its degradation products reacted with the TNBS reagent giving a 1.8 free amino group content ratio, between hydrolyzed and nonhydrolyzed solution.

The close values obtained for the first fraction from different hydrolysates were not expected either, because of the occurrence of all the excluded peptides. For instance, in the T1 hydrolysate, considering the amino acid sequences of caseins and the specifity of trypsin, the average size of all peptides longer than seven residues can be estimated to 16. However, the estimation by the TNBS method was 8.1 residues. This indicates that, for this fraction, the values obtained by this assay are underestimated, partially due to the presence of free basic amino acids. Moreover, as the ε-amino groups of lysyl residues react with TNBS reagent (Polychroniadou, 1988), the free amino group contents of nonhydrolyzed fractions are proportionally more overestimated than that of hydrolyzed fractions, leading to a decrease of the TNBS estimation.

Although the mean chain length does not indicate the MW range of peptides, the result of this analysis indicates that the separation of peptides by size from complex mixtures was fairly good. This is probably due to the good resolution of the PHEA column when compared to other matrices (Lemieux and Amiot, 1989; Lemieux et al., 1991).

CONCLUSIONS

The PHEA support used here for the SE-HPLC of amino acids and small peptides showed good SEC properties, *i.e.*, low nonspecific interactions with the solutes, high recovery, and good resolution.

Sample salt concentrations higher than 0.005 M were shown to affect the peptide separation on the PHEA support with formic acid as mobile phase.

The chromatographic method described here appears to be an advance in the search for new techniques for characterizing protein hydrolysates, particularly when the fractionation of peptides of molecular mass lower than 1000 Da is required.

LITERATURE CITED

Ahmed, F.; Modrek, B. Biosep-SEC-S high-performance size exclusion chromatography columns for proteins and peptides. *J. Chromatogr.* **1992,** *599,* 25–33.

Alpert, A. J. Hydrophilic-interaction chromatography for separation of peptides, nucleic acids and other polar compounds. *J. Chromatogr.* **1990,** *499,* 177–196.

Andrews, P. C.; Alpert, A. J. Size exclusion chromatography (SEC) of small peptides and other small solutes. Poster 110, 10th ISPPP convention, Wiesbaden, Germany, 1990.

Barth, H. G. High-performance size exclusion chromatography of hydrolyzed plant proteins. *Anal. Biochem.* **1982,** *124,* 191–200.

Bennett, H. P. J.; Browne, C. A.; Solomon, S. α-N-acetyl-β-endorphin₁-26 from the neurointermediary lobe of the rat pituitary: isolation, purification, and characterization by high-performance liquid chromatography. *Anal. Biochem.* **1983,** *128,* 121–129.

Fairclough, P. D.; Hegarty, J. E.; Silk, D. B. A.; Clark, M. L. Comparison of the absorption of two protein hydrolysates and their effects on water and electrolyte movements in the human jejunum. *Gut* **1980,** *21,* 829–834.

Golovchenko, N. P.; Kataeva, I. A.; Alimenko, V. K. Analysis of pH-dependent protein interactions with gel filtration medium. *J. Chromatogr.* **1992,** *591,* 121–128.

Hagel, L. Peak capacity of columns for size exclusion chromatography. *J. Chromatogr.* **1992,** *591,* 47–54.

Hara, H.; Funabiki, R.; Iwata, M.; Yamazaki, K-I. Portal absorption of small peptides in rats under unrestrained conditions. *J. Nutr.* **1984,** *114,* 1122–1129.

Himmel, M. E.; Squire, P. G. High pressure gel permeation chromatography of native proteins on TSK-SW columns. *Int. J. Pept. Protein Res.* **1981,** *17,* 365–373.

Iliev, I.; Tchorbanov, B. Determination of tryptophan in protein hydrolysates and fermentation media using gel chromatography. *Biotechnol. Appl. Biochem.* **1992,** *16,* 29–33.

Inouye, K. Chromatographic behaviors of proteins and amino acids on a gel filtration matrix, TSK-GEL toyopearl. *Agric. Biol. Chem.* **1991,** *55,* 2129–2139.

Irvine, G. B.; Shaw, C. High-performance gel permeation chromatography of proteins and peptides on columns of TSK-G200-SW and TSK-G3000-SW: a volatile solvent giving separation based on charge and size of polypeptides. *Anal. Biochem.* **1986,** *155,* 141–148.

Keohane, P. P.; Grimble, G. K.; Brown, B.; Spiller, R. C.; Silk, D. B. A. Influence of protein composition and hydrolysis method on intestinal absorption of protein in man. *Gut* **1985,** *26,* 907–913.

Kopaciewicz, W.; Regnier, F. E. Nonideal size exclusion chromatography of proteins: effects of pH at low ionic strength. *Anal. Biochem.* **1982,** *126,* 8–16.

Lemieux, L.; Amiot, J. Application of reversed-phase high-performance liquid chromatography to the separation of peptides from phosphorylated and dephosphorylated casein hydrolysates. *J. Chromatogr.* **1989,** *473,* 189–206.

Lemieux, L.; Piot, J.-M.; Guillochon, D.; Amiot, J. Study of the efficiency of a mobile phase used in size exclusion HPLC for the separation of peptides from a casein hydrolysate according to their hydrodynamic volume. *Chromatographia* **1991,** *32,* 499–504.

Polychroniadou, A. J. A. A simple procedure using trinitroben-zenesulfonic acid for monitoring proteolysis in cheese. *J. Dairy Res.* **1988,** *55,* 585–596.

Rees, R. G.; Grimble, G. K.; Keohane, P. P.; Higgins, B. E.; West, M.; Spiller, R. C.; Silk, D. B. A. Peptide chain length of protein hydrolysates influences jejunal nitrogen absorption. *Gut* **1984,** *25,* 547–552.

Shioya, Y.; Yoshida, H.; Nakajima, T. Estimation of molecular weigths of peptides by determination of heigth equivalent to a theoretical plate in size exclusion chromatography. *J. Chromatogr.* **1982,** *240,* 341–348.

Ui, N. Rapid estimation of the molecular weights of protein polypeptide chains using high-pressure liquid chromatography in 6M guanidine hydrochloride. *Anal. Biochem.* **1979,** *97,* 65–71.

Vijayalakshmi, M. A.; Lemieux, L.; Amiot, J. High performance size exclusion liquid chromatography of small molecular weight peptides from protein hydrolysates using methanol as a mobile phase additive. *J. Liq. Chromatogr.* **1986,** *9,* 3559–3576.

Visser, S.; Slangen, C. J.; Robben, J. P. M. Determination of molecular mass distributions of whey protein hydrolysates by high-performance size exclusion chromatography. *J. Chromatogr.* **1992,** *599,* 205–209.

Wahl, R.; Maasch, H. J.; Geissler, W. High-performance liquid chromatographic molecular weight determination of allergen extracts. *J. Chromatogr.* **1986,** *351,* 39–46.

Webb, K. E., Jr. Intestinal absorption of protein hydrolysis products: a review. *Anim. Sci.* **1990,** *68,* 3011–3022.