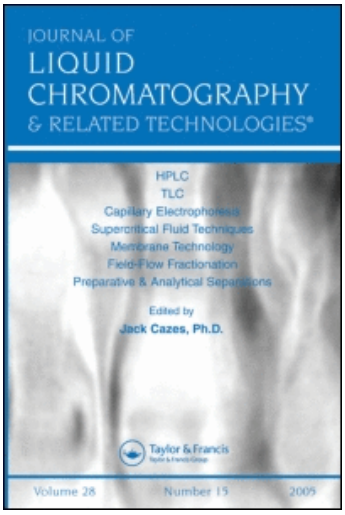

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article O'Callaghan, D. M. , Donnelly, W. J. , Slattery, H. M. and Mulvihill, D. M.(1995) 'Non-Size Exclusion Effects During Gel Permeation Chromatography of Milk Protein Hydrolysates on an FPLC Superose 12 Column', Journal of Liquid Chromatography & Related Technologies, 18: 8, 1543 — 1562
To link to this Article: DOI: 10.1080/10826079508009294
URL: http://dx.doi.org/10.1080/10826079508009294

JOURNAL OF LIQUID CHROMATOGRAPHY, 18(8), 1543-1562 (1995)

NON-SIZE EXCLUSION EFFECTS DURING GEL PERMEATION CHROMATOGRAPHY OF MILK PROTEIN HYDROLYSATES ON AN FPLC SUPEROSE 12 COLUMN

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ABSTRACT

Hydrophobic peptides and aromatic amino acids adsorbed to the matrix of a Superose 12 FPLC column during gel permeation chromatography (GPC) of milk protein hydrolysates. The adsorption phenomenon was most obvious when hydrolysates were prepared using enzyme mixtures which contained exopeptidases. The elution areas of peaks for tryptophan and tyrosine from a Superose 12 column was linear with concentration in the range 0 to 1 μmol. ml^{-1}. Amino acid analysis confirmed that a strongly adsorbed peak in the elution profile, measured by absorbance at 280 nm, of an extensively hydrolysed rennet casein contained tryptophan. Individual hydrophobic peptides, used as molecular weight markers, also interacted with the column, eluting later than expected. Nitrate in a general water supply used for the production of a whey protein hydrolysate on a pilot-scale was responsible for the appearance of an additional peak in the elution profiles from a Superose 12 column, as measured by absorbance at 214 nm.

INTRODUCTION

Proteins and peptides can be separated according to differences in molecular size using gel permeation chromatography (GPC), otherwise referred to as size (steric) exclusion chromatography (SEC) or gel filtration. Theoretical and practical aspects of GPC have been discussed (Cooper and Matzinger, 1977; Pharmacia, 1979; Yau *et al.*,

1979). A variety of gel permeation media and pre-packed columns covering a wide fractionation range are available (Pfannkoch *et al.*, 1980). The Sephadex range, G15 to G50 (Pharmacia), has been used to estimate molecular weight profiles of hydrolysed casein (Hernandez and Asenjo, 1982; Umetso *et al.*, 1983), casein-derived peptides in cheese (Reville and Fox, 1978), whey protein hydrolysates (Kuehler and Stine, 1974; Monti and Jost, 1977) and soy protein hydrolysates (Vallejo-Cordoba *et al.*, 1986). High performance liquid chromatography (HPLC) and fast protein liquid chromatography (FPLC) have now largely replaced the traditional low-pressure columns which were time-consuming and cumbersome to use. Developments in the analysis of milk proteins and peptides by HPLC and FPLC have been reviewed (Gonzalez-Llano *et al.*, 1990). The Toya Soda (TSK) range of HPLC gel permeation columns was discussed by Kato (1984). TSK 2000 SW columns were used to monitor proteolysis in cheese (Lemieux *et al.*, 1989) and to study casein hydrolysates (Mahmoud *et al.*, 1992; Lemieux and Amiot, 1990) and Biosil TSK 20 and Biogel TSK 125 columns (BioRad, Munich, Germany) were used for molecular weight determination of peptides (Richter *et al.*, 1983).

A column of Superose 12 gel (a cross-linked agarose-based matrix suitable for operation at low pressures) was used in an FPLC system to analyse whey proteins (Andrews *et al.*, 1986), milk protein hydrolysates (Thibault, 1990; Chobert *et al.*, 1988a, b) and to monitor proteolysis in cheese (Wilkinson *et al.*, 1992).

Proteins and peptides should, ideally, not interact with the column matrix during GPC. However, in practice, such conditions are difficult to achieve. Examples of non-size exclusion effects include ionic and hydrophobic interactions between GPC media and proteins, peptides and/or amino acids (Gelotte, 1960; Porath, 1960; Eaker and Porath, 1967; Belew *et al.*, 1978; Tchorbanov *et al.*, 1991; Ilieu and Tchorbanov, 1992; Wallace, 1992). Inorganic ions also interact with GPC media (Neddermeyer and Rogers, 1968; Yoza, 1973; Sinibaldi and Lederer, 1975). A useful discussion on non-size exclusion effects was presented by Barth (1980).

Non-size exclusion phenomena are often ignored in reports on size analysis of peptides in protein hydrolysates. Tryptophan and tryptophan-containing peptides were adsorbed onto polyacrylamide Biogel P2 during chromatography of protein hydrolysates and fermentation media (Tchorbanov *et al.*, 1991). A technique for quantifying tryptophan, exploiting this phenomenon, was described by Ilieu and Tchorbanov (1992).

This chapter describes anomalous elution patterns observed during chromatography of milk protein hydrolysates on a Pharmacia FPLC Superose 12 column and, in particular, interactions of amino acids, peptides, $NaNO_3$ and $NaNO_2$ with the gel matrix.

MATERIALS AND METHODS

Gel permeation chromatography

Chromatography was performed using a fast protein liquid chromatograph (FPLC) fitted with a Superose 12 gel permeation column, two ultraviolet single-path monitors (UV-1) set to operate at 214 or 280 nm (in that order), an autosampler (ACT 100) and fraction collector (FRAC 100) from Pharmacia LKB Biotechnology, Uppsala, Sweden. The analogue outputs of the UV-1 monitors were interfaced with a Minichrom data handling package (V.G. Data Systems, Altrincham, Manchester, UK) for data collection and peak integration. The elution buffer (pH 7.0) was 0.1 M Tris-HCl - 0.1 M NaCl - 10% methanol, at a flow rate of 0.5 ml min^{-1}. Protein hydrolysates were diluted to the equivalent of 0.25% protein (N x 6.38) in the elution buffer and 100 µl applied to the column. When fractions were recovered, 6 mg protein, disolved in 200 µl of elution buffer, was applied to the column and 1 ml fractions collected.

Calibration standards

The calibration standards used were obtained from Sigma Chemical Co. Ltd., Gillingham, Dorset, UK; Serva Feinbiochemica, GmbH and Co., Heidelberg, Germany; Pharmacia LKB Biotechnology, Uppsala, Sweden or BDH Laboratory Supplies, Merck Ltd., Poole, Dorset, UK.

Amino acid analysis

Amino acid analysis was performed on a Beckman 6300 autoanalyser (Beckman Instruments Ltd., High Wycombe, UK) using a cation exchange column, (Na form, 12 cm x 4 mm i.d.). A standard amino acid mixture

(Sigma, A-9781) and a tryptophan standard (Sigma, T-1029) were used to calibrate the column. Norleucine (Sigma, N-8513) was used as internal standard. Samples and standards were diluted in 0.2 M sodium citrate buffer, pH 2.2; 50 μl were applied to the column and eluted with sodium citrate buffers as described in Table 1. Amino acids were post-column derivitized with ninhydrin and detected by absorbance at 570 and 440 nm. Data collection and integration was with a V.G. Minichrom system, as described above.

Protein hydrolysates

The milk protein hydrolysates were prepared as described by O'Callaghan (1994).

Protein determination

The nitrogen content of protein solutions was determined in duplicate by the micro-Kjeldahl procedure (AOAC, 1980) and converted to protein by multiplying by 6.38.

RESULTS

Elution pattern of standard proteins, peptides and amino acids

Protein-containing eluates from chromatographic columns are generally detected by measuring absorbance at 280 nm which relies on the absorbance of the aromatic residues, tryptophan and tyrosine (Sober, 1968). However, in the case of small peptides, detection at 280 nm may be unsuitable, as all peptides may not contain these aromatic residues; therefore, wavelengths in the range 205-220 nm are more suitable for peptides as the amide bond between residues absorbs in this region (Segal, 1976).

The elution data for all the standards, their molecular weights (M_r) and elution volumes (V_e), as measured by absorbance at 214 and 280 nm, are shown in Table 2. The void volume of the column (V_o) was estimated to be 7.7 ml (see elution volume for blue dextran) and the

TABLE 1

Elution programme for analysis of amino acids on the Beckman 6300 autoanalyser using a cation exchange column (Na form, 12 cm x 4 mm i.d.).

(a) Buffer (Na citrate) changes

Conc., M	pH	Time, min
0.2	3.28	0-17.8
0.2	4.25	17.8-32.0
1.0	6.40	32-62.0
0.2	3.28	62-75*

(b) Temperature changes

Temp. °C	
47	0-11
70	11-28
77	28-60
47	60-70

*Prior to regeneration, the column was washed for 1 min with 1 M NaOH

total column volume (*Vt*) to be 19.5 ml (see elution volume for NaN_3 and average elution volume of the non-aromatic amino acids).

The elution plots (log_{10} molecular weight *vs* elution time, min), as measured by absorbance at 214 and 280 nm are shown in Figure 1. For the sake of clarity, all the points are not shown in the 214 nm plot as the elution time of some of the low molecular weight standards overlapped. All the protein standards eluted as single peaks in order of decreasing molecular weight, as expected; however, some peptides and aromatic amino acids showed anomalous elution patterns. Angiotensinogen, a hydrophobic plasma peptide (M_r, 1759) consisting of 14 residues (Mahler and Cordes, 1968), eluted as two peaks of equal absorbance at 20 ml and 22 ml at 280 nm but when assayed at 214 nm, the peak at 22 ml dominated. Actinomycin, an antibiotic (M_r, 1280) from *Streptomyces spp.* which is structurally a complicated molecule possessing two short chains (5 residues each) linked to a dicarboxylic acid derivative of a phenoxazone (Stryer, 1988), had similar elution patterns at both wavelengths, i.e., a major peak at 22.2 ml, preceded by

TABLE 2

Elution volume, ml, as determined by absorbance at 280 and 214 nm, of molecular weight standards used to determine the elution behaviour from a Superose 12 FPLC gel permeation column. The source, molecular weight, daltons, and $\log_{10}$ molecular weight of standards are shown

No.	Standard	Source	Code	Molecular weight (daltons)	$\log_{10}$	Elution volume, ml 280 nm	214 nm
1	Blue dextran	Pharmacia	17-0442-01	2,000, 000	6.301	7.87	7.69
2	Ferritin	Serva	21318	455, 000	5.658	10.93	10.93
3	IgG	Sigma	I-5506	150, 000	5.176	12.08	12.07
4	BSA	Pharmacia	17-0442-01	67, 500	4.829	13.00	13.00
5	Ovalbumin	Pharmacia	17-0442-01	43, 000	4.633	13.92	13.77
6	β-Lactoglubulin	Sigma	L-0130	36, 000	4.556	14.35	14.23
7	α-Lactalbumin	Sigma	L-6010	14, 400	4.158	15.58	15.47
8	Cytochrome C	Serva	18020	12, 300	4.090	16.20	16.07
9	Insulin chain B	Serva	52190	3, 494	3.543	18.00	17.87
10	Angiotensinogen	Serva	51295	1, 759	3.245	19.83/22.56*	18.58/22.13*
11	Actinomycin	Serva	10708	1, 280	3.107	19.77/22.95*	18.93/22.75*
12	Pz-Pro-Leu-Gly-Pro-D-Arg	Serva	52268	777	2.890	24.72	24.58
13	Suc-Ala-Ala-Ala-$_p$NA	Serva	51053	451	2.654	22.57	22.42
14	Riboflavin	Sigma	R-450	377	2.576	24.27	24.13
15	DL-Tryptophan	Sigma	T-0129	204	2.310	28.65	28.50
16	L-Tyrosine	BDH	37156	181	2.258	21.75	21.65
17	L-Arginine	Sigma	A-5131	174	2.241	-	20.00
18	DL-Phenylalanine	BDH	2496090	165	2.218	-	20.38
19	L-Histidine	Sigma	H-8000	155	2.190	-	19.41
20	L-Lysine	BDH	C010980	146	2.164	-	19.50
21	L-Aspartic acid	Sigma	A-9256	133	2.124	-	18.77
22	L-Leucine	Sigma	L-8000	131	2.118	-	19.80
23	L-Isoleucine	Sigma	I-2752	131	2.118	-	19.40
24	L-Cysteine	Sigma	C-7880	121	2.069	-	19.80
25	L-Valine	Sigma	V-0500	117	2.062	-	19.41
26	Proline	Sigma	P-0380	115	2.061	-	19.10
27	Serine	Sigma	S-4500	105	2.022	-	19.40
28	Alanine	Sigma	A-7627	89	1.950	-	19.37
29	$NaNO_3$	BDH	10255	85	1.929	-	22.22
30	Glycine	BDH	10119	75	1.876	-	19.41
31	NaN_3	BDH	10369	56	1.748	-	19.50

*indicates that more than one peak was detected.

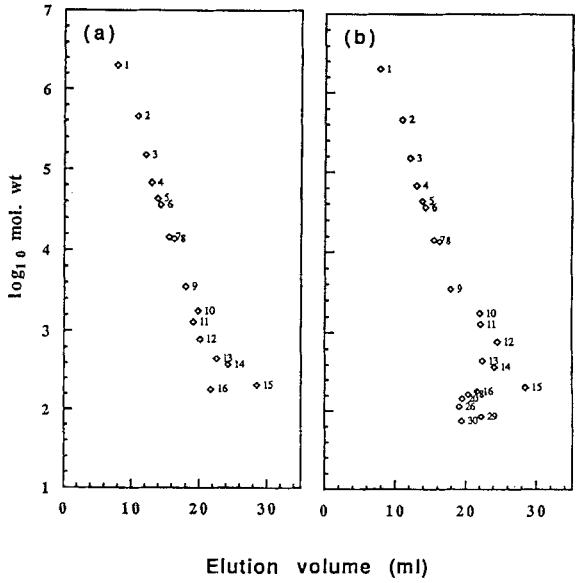

FIGURE 1. Elution pattern of standard proteins, peptides, amino acids and inorganic salts from an FPLC Superose 12 gel permeation column (Pharmacia) assayed by absorbance at: a) 280 and b) 214 nm. The elution buffer (pH 7.0) was 0.1M Tris-HCl - 0.1M NaCl - 10% methanol; flow rate, 0.5 ml min^{-1}. Standards are identified in Table 2.

a broad skewed peak at 19 ml. The synthetic peptides, PZ-Pro-Leu-Gly-Pro-D-Arg (M_r, 777) and Suc-Ala-Ala-Ala (M_r, 451) eluted as single peaks at 25 and 22 ml, respectively. The hydrophobic nature of these peptides was assumed to be responsible for their interaction with the column and they were excluded from the calibration plots used to estimate molecular size distribution of peptides in protein hydrolysates (Fig. 2). Wallace (1990) showed that single peptides also eluted later than expected from a Sephadex G-25 column. The aromatic amino acids, tryptophan (Trp) and tyrosine (Tyr) were adsorbed strongly on the Superose 12 column, eluting at volumes of 28.5 and 22 ml, respectively. Phenylalanine (Phe) showed little interaction with the column, eluting close to V_t at 20.35 ml. Of all the amino acids, Phe, Tyr and Trp are the most hydrophobic, with side chain hydrophobicities of 11.1, 12 and 12.5 $kJ.mol^{-1}$, respectively (Fennema, 1985), which corresponded to the order in which they eluted from the column. Tyr

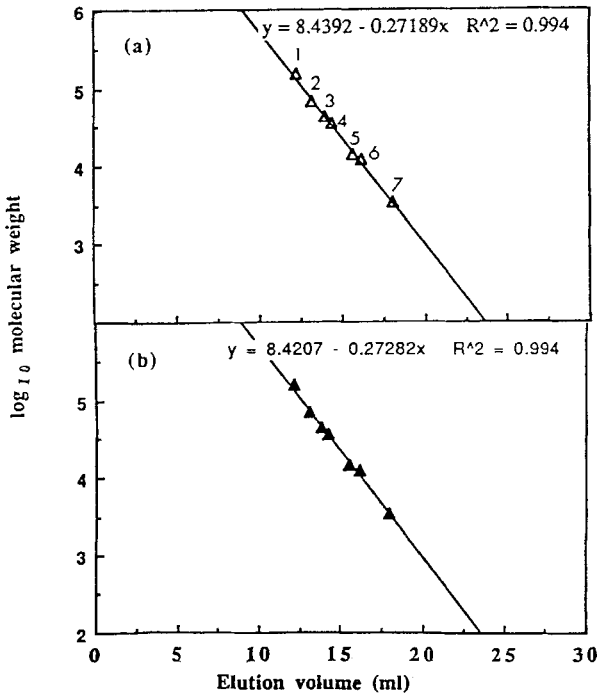

FIGURE 2. Calibration plots for the FPLC Superose 12 column, determined by absorbance at: a) 280 and b) 214 nm, used to estimate molecular size distribution of peptides in protein hydrolysates; 1, IgG (150,000); 2, BSA (67,500); 3, ovalbumin (43,000); 4, b-lactoglobulin (38,000); 5, a-lactalbumin (14,700); 6, cytochrome C (12,300); 7, insulin chain B (3,494).

was less interactive than Trp. All other amino acids eluted close to V_t, including Leu and Ile, which have side chain hydrophobicities of 10.1 and 12.4 kJ. mol-1, respectively. The aromatic side chains of Phe, Tyr and Trp are most likely to be responsible for interaction with the gel matrix.

Calibration plots for estimating molecular weight profiles of peptides in protein hydrolysates

Calibration plots were prepared using elution data for the seven standard proteins, ranging in molecular weights from 150, 000 to

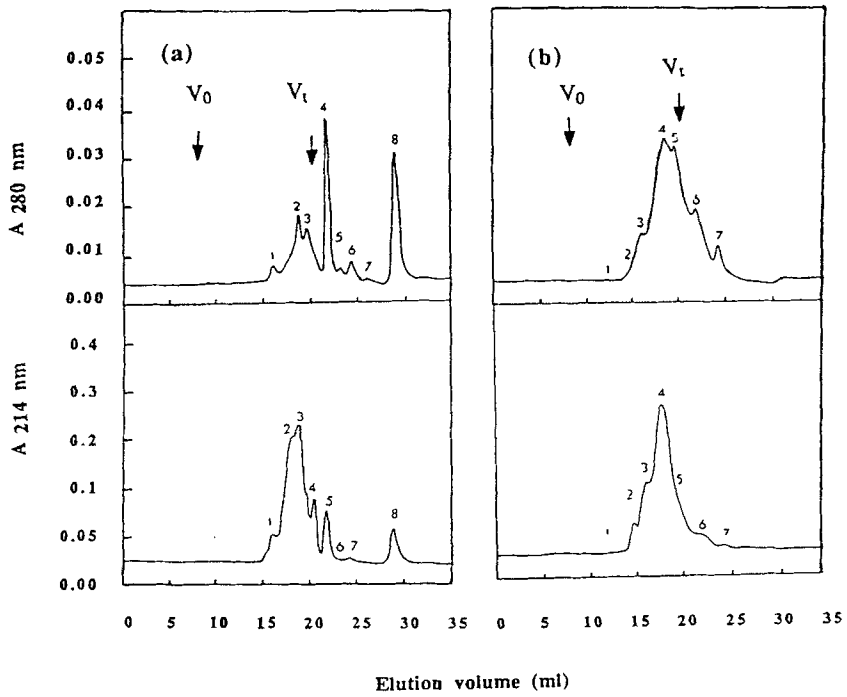

FIGURE 3. Elution profiles of casein hydrolysates from an FPLC Superose 12 column assayed by absorbance at: a) 280 and b) 214 nm. Elution conditions were as in Fig. 1. Sample volume: 100 µl of 0.25%, w/v, protein solution; (a) rennet casein hydrolysed with a mixed enzyme preparation, BN2001 (DH, 23%); (b) rennet casein hydrolysed with a specific proteinase preparation, Profix (Papain, DH, 10%).

3,494 daltons and are shown in Fig. 2. The elution volumes of the adsorbing, low molecular weight peptides were not included in the calibration plots because of their anomalous elution pattern.

The regression equations for the calibration plots had high r^2 values (0.994) and from the regression lines, the lower end of the fractionation range was estimated to be approx. 1000 daltons.

Hydrophobic interactions during GPC of protein hydrolysates

During GPC of milk protein hydrolysates, a number of peaks were observed to elute after V_t, in particular when the hydrolysates were prepared using enzyme mixtures containing exopeptidases (Chapters 3

to 5). Figure 3 shows the elution profiles, as measured by absorbance at 280 and 214 nm, of two casein hydrolysates, one prepared using a mixed enzyme preparation (BN2001, Imperial Biotechnology, London, UK) which contains pancreatic exopeptidases and the other prepared using a single proteinase, papain (Profix, Quest-Biocon, Cork, Ireland). In the hydrolysate prepared using the mixed enzyme preparation (Fig. 3a) two major peaks (4 and 8) and some minor peaks eluted after V_t (which corresponded to peak 3). However, in the hydrolysate produced with papain (Fig. 3b) there was little adsorption since little material eluted after V_t.

Isolation and identification of material present in adsorbed peak 8 from a chromatogram of casein hydrolysate, prepared using proteinase BN2001, on Superose 12

Casein hydrolysate (6 mg) prepared using the mixed proteolytic enzyme preparation, BN2001, was applied to the Superose 12 FPLC column and eluted as described previously. The elution profile was monitored at 280 nm and the eluate corresponding to peak 8 collected. Some of the eluate (100 μl) was rechromatographed on the same column; a tryptophan solution (100 μl) was also chromatographed on the column under similar conditions. The elution profile of the casein hydrolysate, the rechromatographed peak and tryptophan are shown in Fig. 4. The adsorbed peak (8) and the tryptophan standard had the same elution volume (28.5 ml). A sample (50 μl) of eluate corresponding to peak 8, a standard Sigma amino acid mixture and a tryptophan standard were analysed separately on a Beckman 6300 amino acid analyser. The chromatogram for the standard Sigma amino acid mixture is shown in Fig. 5a. Peaks 1 to 20 were identified based on retention times for individual amino acids (Table 3). Peak 18 corresponded to Trp which eluted as a broad peak. This is attributed to its bulky nature which causes a molecular sieving effect during ion exchange chromatography (Bech-Anderson, 1992, National Institute of Animal Science, Tjele, Denmark, personal communication). Peak 19 was identified as ammonia and appeared as a contaminant in all chromatograms, including blank runs. Its appearance has been attributed to absorption of atmospheric ammonia by the acidic citrate buffers (Brown, 1991, Beckman Instruments, High Wycombe, UK). Norleucine (peak 13) was included as an internal standard in the dilution buffer.

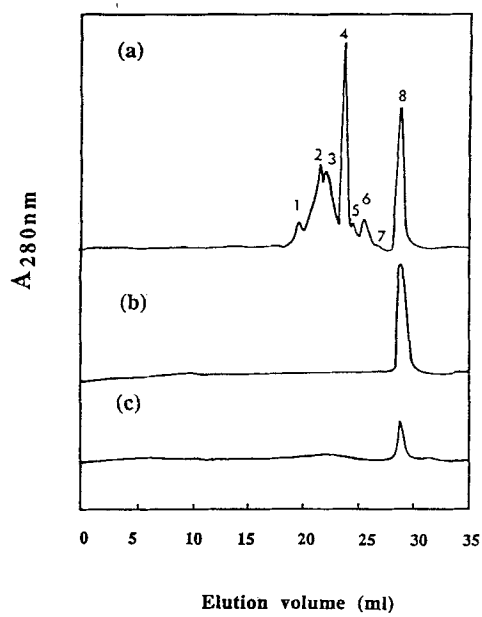

FIGURE 4. Chromatograms from the Superose 12 FPLC column, assayed by absorbance at 280 nm, of a) BN2001/casein hydrolysate; b) tryptophan standard, 80 μmol. ml-1; c) eluate (100 μl) corresponding to adsorbed peak 8 reapplied to column.

The ninhydrin derivative of proline (peak 5) absorbed weakly at 570 nm and was detected separately at 440 nm. Changes in baseline absorbances were due to buffer and temperature changes during the elution programme (Table 2). Figure 5b shows the chromatogram of the Trp standard. The chromatogram of the eluate containing the material in the 'adsorbed' peak (8) from the FPLC column showed four ninhydrin-positive components (Fig. 5c), two of which corresponded to peaks 13 and 19, i.e., norleucine and ammonia, respectively; one of the other ninhydrin-positive compounds corresponded to Trp (peak 19). The elution profile obtained for a 1:1 mixture of the FPLC buffer and dilution buffer containing the internal standard showed two peaks, one corresponding to norleucine (19), the other (T) to Tris-hydroxymethylamine from the FPLC buffer. These results confirm that peak 8 in the FPLC chromatogram (Figs. 3 and 4) contained tryptophan which interacts hydrophobically with the Agarose moiety

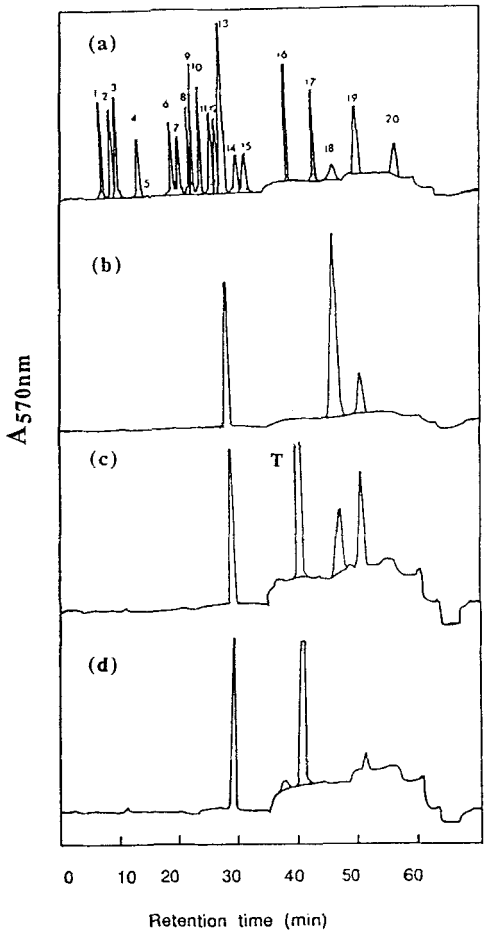

FIGURE 5. Chromatograms of amino acids obtained from a Beckman 6300 AAA assayed by absorbance at 570 nm after post-column derivitisation with ninhydrin. Elution conditions are in Table 1 and peaks were identified according to retention times of individual amino acids (Table 3). a) standard amino acid mixture, 25 nmoles ml^{-1}; b) tryptophan standard, 125 nmoles ml^{-1}; c) adsorbed peak 8 from the Superose 12 FPLC column; d) FPLC elution buffer (pH 7.0), Tris-HCl - 0.1M NaCl - 10% methanol.
The peak labelled 'T' corresponds to Tris-hydroxymethylamine

TABLE 3

Identification of ninhydrin-positive compounds in a standard amino acid mixture (Sigma A-9781), analysed on a Beckman 6300 autoanalyser (cation exchange column, Na form, 12 cm x 4 mm i.d.), as shown in the chromatogram in Figure 5a

Peak No.	Amino acid	Abbreviation	retention time (min)
1	Aspartic acid	Asp	8.80
2	Threonine	Thr	10.45
3	Serine	Ser	11.38
4	Glutamic acid	Glu	14.92
5	Proline	Pro	16.34
6	Glycine	Gly	20.53
7	Alanine	Ala	21.83
8	Cysteine	Cys	23.51
9	Valine	Val	24.03
10	Methionine	Met	25.37
11	Isoleucine	Ile	27.08
12	Leucine	Leu	28.00
13	Norleucine[1]	Nor	29.15
14	Tyrosine	Tyr	31.39
15	Phenylalanine	Phe	32.94
16	Histidine	His	39.86
17	Lysine	Lys	44.33
18	Tryptophan	Trp	47.41
19	Ammonia	NH_3	51.72
20	Arginine	Arg	58.17

1. Norleucine was used as internal standard.

of Superose 12. Tryptophan has also been observed to bind to the polyacrylamide gel medium, Biogel P2, during chromatography of commercial protein hydrolysates (soy and casein) and fermentation media (Tchorbanov *et al.*, 1991; Ilieu and Tcharbanov, 1992).

Adsorption of inorganic salts to the Superose 12 matrix

During elution of a whey protein hydrolysate, produced on a pilot-scale, from the Superose 12 column, an additional peak (V_e ~22 ml) was observed after V_t in the elution profile assayed at 214 nm (Fig. 6c), which was not evident in the chromatograms of laboratory-prepared hydrolysates assayed at 214 nm (Fig. 6b) or in the chromatogram of any hydrolysate assayed at 280 nm. It was assumed initially that this adsorbed peak was due to an additional peptide. Analysis of the chromatograms of the materials (WPC, enzymes, neutralising agents, etc) used in the preparation of the hydrolysate showed no peak eluting in this position. When whey protein hydrolysates prepared in the laboratory under various conditions were chromatographed on the Superose 12 column this additional peak was absent. In the pilot-scale process used for the production of whey protein hydrolysates, a general water supply was used to prepare the whey solution and when 100 µl of this water were chromatographed on the FPLC column, a peak with an elution volume corresponding to that of the additional peak (V_e, ~22 ml) was evident (Fig. 6d). Furthermore, the material responsible for this additional peak was removed by passing the water through an anion exchange resin, while passage through a cation exchanger or activated carbon did not remove the material, suggesting that the material adsorbing on the column was anionic.

When aqueous solutions (0.2%, w/v) of $MgCl_2$, Na_2HPO_4, K_2SO_4, Na_2SO_4, KCl, NaCl and $CaCO_3$ were chromatographed on the Superos 12 column, no absorbance at 214 nm in the elution region of interest was observed. However, when solutions of $NaNO_3$ and $NaNO_2$ (0.2%, w/v) were chromatographed on the column a large absorbance at 214 nm in the region of 22 ml elution was evident, which corresponded in elution volume to the 'additional' peak seen in the pilot-scale whey protein hydrolysate and in the general water supply (Fig. 6d). Analysis on the general water supply showed that it contained a high level of nitrate (22 mg.kg^{-1} NO_3). After deionisation, NO_3 levels in the water were neglible and when this deionised water was used, the FPLC

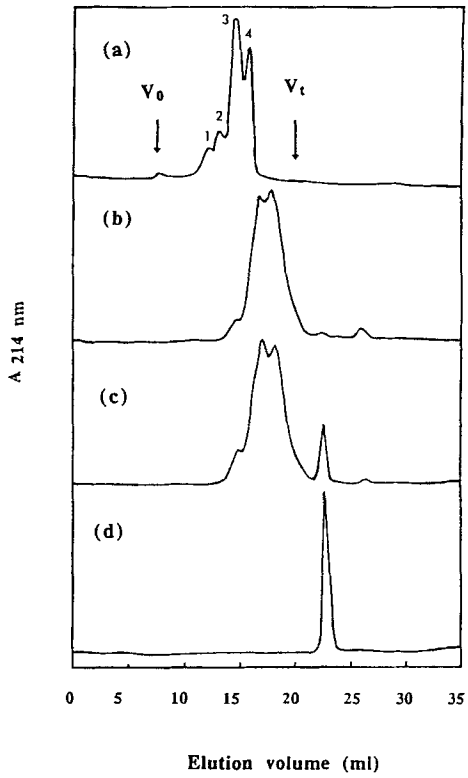

FIGURE 6. Elution profiles of whey protein hydrolysates from an FPLC Superose 12 column, assayed by absorbance at 214 nm. Elution conditions were as in Fig. 1. Sample volume, 100 µl of 0.25% (w/v) protein solution; (a) whey protein concentrate; 1, IgG; 2, BSA; 3, b-Lg, 4, a-La; (b) laboratory-prepared whey protein hydrolysate; (c) pilot-scale whey protein hydrolysate showing an additional peak at 22 ml; (d) 100 µl of general water supply used to prepare pilot-scale hydrolysate, $NaNO_3$ (20 $mg.kg^{-1}$) eluted in exactly the same position. The void volume (V_0) and total column volumn (V_t) are indicated by arrows.

chromatograms of later pilot-scale hydrolysates were similar to those of laboratory-produced hydrolysates.

Quantitative adsorption of Tyrosine, Tryptophan and NaNO3 to the Superose 12 column matrix

When standard solutions (0-1 µmole ml^{-1}) of tyrosine and tryptophan were chromatographed on the Superose 12 column and the

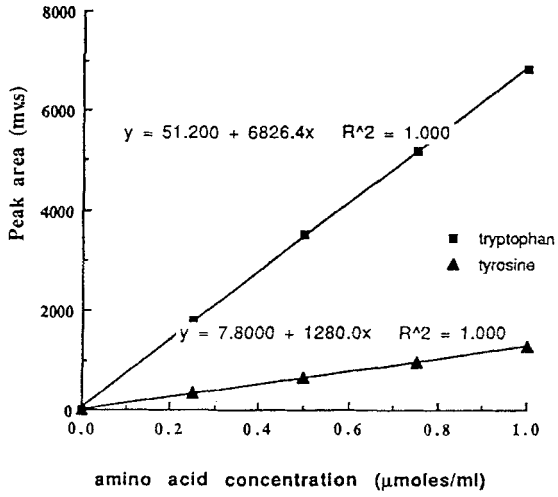

FIGURE 7. Relationship between peak area (mv.s) and concentration of applied tryptophan and tyrosine standard solutions (0-1 μmol. ml^{-1}), assayed by absorbance at 280 nm, of eluate from the FPLC Superose 12 column. Regression analysis showed $r^2 = 1.00$

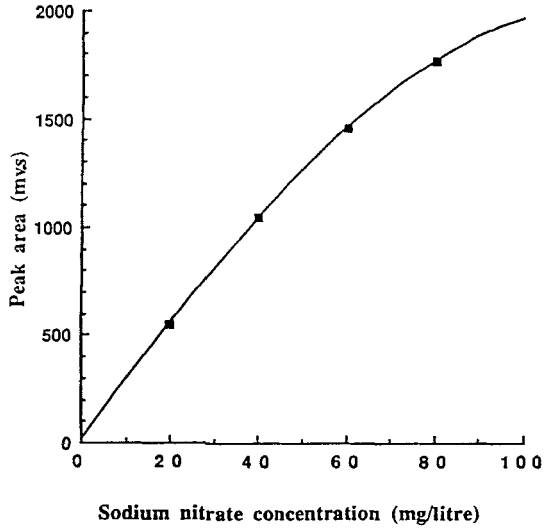

FIGURE 8. Relationship between peak area (mv.s) and concentration of applied $NaNO_3$ solutions (0-80 mg.l^{-1}), assayed by absorbance at 214 nm, of eluate from the FPLC Superose 12 column. Regression analysis showed $r^2 = 1.00$

absorbance at 280 nm measured, a linear relationship (r^2 =1 .00) was found between peak area, as measured in millivolt seconds (mv.s), and the concentration of applied amino acid solutions (Fig. 7). When standard solutions of $NaNO_3$ (0-80 $mg.kg^{-1}$) were chromatographed on the column, a curvilinear response (r^2 =1.00) was observed between peak area (mv.s), assayed by absorbance at 214 nm, and concentration of applied solutions (Fig. 8).

DISCUSSION

When reporting on the molecular size distribution of protein hydrolysates, as determined by gel permeation chromatography, it is necessary to keep in mind that elution is not necessarily governed by differences in molecular size only. The gross shape of the molecule and its physicochemical characteristics, especially net charge and hydrophobicity, may also play a role in separation. Hydrophobic amino acids and pure peptides containing hydrophobic residues interacted with the Agarose matrix of the Superose 12 column. Gelotte (1960) was one of the first to observe the phenomenon of hydrophobic interaction of aromatic and heterocyclic compounds with Sephadex. Wallace (1990) found a poor relationship between molecular weight and elution volume of pure peptides, which eluted later than expected from a Sephadex G-25 column.

During GPC of milk protein hydrolysates on a Superose 12 FPLC column, a number of peaks eluted after V_t, one of which contained the aromatic amino acid, tryptophan. The elution of hydrophobic amino acids from the Superose 12 column was in the order of increasing hydrophobicity, i.e., Phe, Tyr and Trp. Adsorption of tryptophan to Biogel-P2 was observed previously during GPC of commercial protein hydrolysates and fermentation media (Tchorbanov *et al.*, 1991; and Ilieu and Tcharbanov, 1992). These authors proposed that the adsorption of tryptophan to the Biogel P2 medium could be exploited for quantifying tryptophan in extensively hydrolysed protein samples. The adsorption of tryptophan and tyrosine on Superose 12 matrix was also quantitative and can be exploited to monitor exopeptidase activity during enzymatic hydrolysis of milk proteins (Chapter 5). The inorganic salts, $NaNO_3$ and $NaNO_2$, also interacted with the Superose 12 column matrix, giving rise to peaks when assayed at 214 nm but not at 280 nm.

A salt exclusion effect was observed previously with Sephadex (Neddermeyer and Rogers, 1960) where anions were excluded from the

gel bed and eluted earlier than expected. This 'ion exclusion' effect arises when the surface of a gel matrix has a net charge and solutes of similar charge become excluded from the pores because of electrostatic repulsion. However, this type of interaction does not explain the elution of NO_3^- and NO_2^- from the Superose 12 column as these ions eluted later than expected from the column. An 'ion inclusion effect' was reported by Stenlund (1976) during gel chromatography, and was explained on the basis of a Donnan membrane equilibrium between ions and the gel surface.

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Received: November 5, 1994
Accepted: November 18, 1994