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APPLICATION OF REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY TO THE SEPARATION OF PEPTIDES FROM PHOSPHORYLATED AND DEPHOSPHORYLATED CASEIN HYDROLYSATES

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SUMMARY

Peptides from phosphorylated and dephosphorylated casein hydrolysates were fractionated on a TSK G2000SW size-exclusion column. The fractionated peptides were separated by reversed-phase high-performance liquid chromatography on a C_{18} column using aqueous trifluoroacetic acid as the mobile phase and acetonitrile as the mobile phase modifier in the linear gradient elution system. The separation of the dephosphorylated and phosphorylated hydrolysates gave 213 and 187 peptides, respectively, of which 116 and 99, respectively, were reported. A study of their composition and retention times verified that the peptide separation mechanism includes ionic interactions, hydrogen bonding and peptide characteristics, in addition to overall peptide hydrophobicity.

INTRODUCTION

Reversed-phase high-performance liquid chromatography (RP-HPLC) is a method for resolving complex peptide mixtures from protein cleavage reaction, especially when only small amounts are available for amino acid sequencing studies[1]. Unlike size-exclusion and ion-exchange chromatographies, with RP-HPLC there is no obvious correlation between retention and molecular size and no apparent relationship between retention and acidity[2]. Retention of peptides on a reversed-phase column has been shown to be largely dependent on their amino acid composition but not on their sequence, and to be primarily determined by the ionization state, the location of charges and the hydrophobicity[3]. Many authors have predicted the retention times of different peptides with fewer than 29 amino acid residues, under particular sets of chromatographic conditions[4–6].

The work reported here aimed to separate and identify peptides from casein hydrolysates previously fractionated on a TSK G2000SW size-exclusion column. Separation was achieved by RP-HPLC and identification by Pico-Tag (Waters) amino acid analysis. N- and C-terminal amino acid analyses were also performed on a C_{18} Pico-Tag column. The peptide separation is discussed on the basis of their hydro-

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phobicity, conformation, amino acid composition, hydrogen bonding and ionic interactions with the stationary phase.

EXPERIMENTAL

Materials

Phosphorylated and dephosphorylated casein hydrolysates (CHPS and CHDS) were kindly provided by Laboratoire Sopharga (France). A controlled hydrolysis of whole casein by physiological enzymes (chymotrypsin and trypsin) was carried out in a continuous-flow membrane enzymic reactor, according to the procedure developed by Maubois and Brulé[7], and led to the following molecular weight distribution of peptides: mol.wt. > 5000 daltons, 3%; 1000 < mol.wt. < 5000 daltons, 28%; mol.wt. < 1000 daltons, 69% including 8% of free amino acids[8]. The resultant hydrolysate was ultrafiltered with a membrane, to retain the enzyme and to obtain a permeate that contained phosphorylated and dephosphorylated peptides. The permeate was acidified, and a divalent cation salt was added to aggregate the phosphopeptides. Thereafter, fractionation of the two groups of peptides was performed by ultrafiltration and diafiltration with water so as to remove the whole dephosphorylated peptide fraction (CHDS). The diafiltrated concentrate obtained corresponded to the phosphopeptidic fraction (CHPS). HPLC-grade water and methanol, and monobasic and dibasic sodium phosphate, were obtained from Fisher Scientific (Quebec, Canada). Trifluoroacetic acid (TFA) was supplied by Pierce (U.S.A.).

Triethylamine (TEA), sequanal grade, TFA, phenyl isothiocyanate (PITC) and amino acid standard mixture H were obtained from Pierce. Absolute ethanol, sodium acetate trihydrate and hydrochloric acid (R. P. Normapur) were supplied by Prolabo (Paris, France). Acetonitrile "Baker Analyzed" reagent for chromatography was purchased from J. T. Baker (Deventer, The Netherlands). HPLC-grade acetic acid, ammonia (Suprapur) and 2-propanol (LiChrosolv) were obtained from Merck (Socolab, France). Double-distilled water was purified by passing it through a Milli-Q water purification system (Millipore, Bedford, MA, U.S.A.).

Methods

Size-exclusion chromatography. High-performance size-exclusion chromatography (HPSEC) was carried out on a TSK G2000SW column (600 × 7.5 mm I.D.) with a guard column (60 × 7.5 mm I.D.) (Toyo Soda, Tokyo, Japan) using an LKB HPLC system equipped with a Model 2150 pump, a Model 2152 controller, a Model 2151 variable-wavelength monitor, a Rheodyne M7010 sample injection valve with a 20-μl loop, and a Hewlett-Packard 3390 A integrator. The mobile phase was 0.1% TFA, 0.05 *M* phosphate buffer (pH 5.0) and 35% methanol. The system was run isocratically at a flow-rate of 0.75 ml/min under constant temperature. Polypeptides were monitored at 214 nm with an absorbance scale of 0.05[9]. The mobile phase was filtered through a 0.45-μm filter (Millipore) and sonicated before use. Hydrolysate fractions were collected manually and pooled.

Reversed-phase chromatography. Peptides from each fraction obtained by HPSEC were separated on a Waters μBondapak C_{18} reversed-phase column (10 μm, 300 × 3.9 mm I.D.) with a Waters HPLC system equipped with two F-6000A pumps, an M720 solvent programmer, a WISP automated sample injector, an M441

fixed-wavelength detector (214 nm) and an M730 two-channel chart recorder. A water-bath was used to keep the column at 40°C. After equilibration of the column with 0.115% TFA (solvent A) at a flow-rate of 2 ml/min, peptides were eluted by linearly increasing the concentration of solvent B [60% (v/v) acetonitrile in 0.1% TFA] as follows: 0–24 min, 0–48% B; 24–25 min, 48–100% B; 25–25.5 min, 100–0% B. The time necessary from one run to the other, including the equilibration time, was 33 min. Prior to use, the mobile phases were degassed with helium. Peptides were monitored at 214 nm with an absorbance scale of 0.1. Portions of the RP-HPLC column fractions were evaporated in a Speed-Vac Concentrator (Savant, Hicksville, NY, U.S.A.).

Peptides that coeluted were isolated by utilization of a second solvent system: (A) 25 m*M* ammonium acetate (pH 6.0) and (B) 60% (v/v) acetonitrile in 50 m*M* ammonium acetate (pH 6.0); all other conditions were the same. The absorbance scale was increased to 0.2 and the flow-rate was decreased to 1 ml/min.

Peptide identification. Peptides were hydrolysed with 5.7 *M* triple-distilled hydrochloric acid in evacuated sealed tubes for 24 h at 110°C. The amino acid analyses were then performed on a Waters Pico-Tag amino acid analysis system according to the manufacturer's instructions. Prior to hydrolysis, Pyrex tubes were heated at 500°C for 16 h to eliminate any contamination.

The identity of each peptide was established by comparison of its amino acid composition with that of α_{s1}-, α_{s2}-, β- and κ-caseins according to the Petrilli's program[10], using an Apple IIE computer, and confirmed by N-terminal and C-terminal analysis according to the methods of Tarr[11] and Ribadeau-Dumas[12], respectively. The chromatography of phenylthiohydantoin (PTH) and phenylthiocarbamyl (PTC) derivatives was then carried out on a Pico-Tag column (Waters) using the LKB system. For PTC derivatives, 1 m*M* EDTA (Fisher Scientific) was added to solvent A, and the gradient was modified by increasing the solvent B concentration as follows: 0–4 min, 0–30% B; 4–12 min, 30–45% B; 12–12.5 min, 45–100% B; 14–15 min, 100–0% B. Injections could be performed every 21 min. The gradient for the identification of PTH derivatives was modified; solvents A and B were the same, and concentration of eluent B increased in the following manner: 0–3.5 min, 0–2% B; 3.5–6 min, 2–36% B; 6.0–7.0 min, 36–40% B; 10.5–10.7 min, 40–60% B; 11.0–11.5 min, 60–0% B. Between two injections, the time required for the analysis and equilibration was 20 min. In each case the flow-rate was kept constant at 1.0 ml/min, and norleucine was used as internal standard.

The hydrophobicity of peptides (kcal/mol) was calculated according to the values given by Cheftel *et al.*[13]. The total number of hydrophobic amino acid residues was determined by the presence of tryptophan, phenylalanine, leucine, isoleucine, tyrosine, valine, methionine and proline in the peptide.

RESULTS AND DISCUSSION

The elution profiles obtained for CHDS and CHPS on a TSK G2000SW HPSEC column are given in Fig. 1A and B. The retention times for each fraction collected were different for the two hydrolysates since the chromatograms showed few distinctive differences. Ten and eight fractions (as indicated by numbers) were obtained for CHDS and CHPS, respectively. Fractions 7 and 8 of CHDS were combined for further studies. HPSEC fractions 4 (550–840 daltons) and 7–8 (210–410 daltons), respectively,

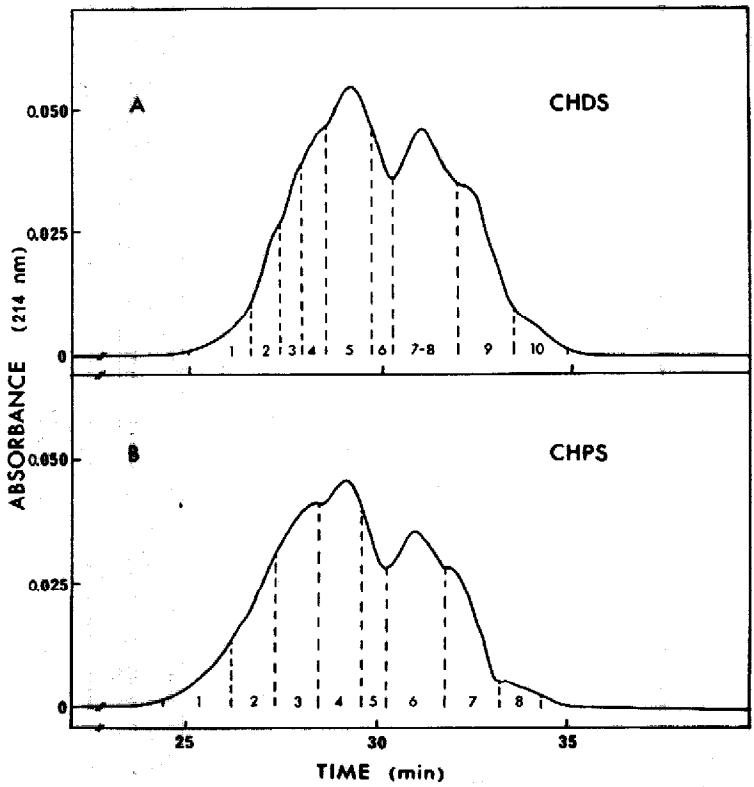

Fig. 1. Elution profiles of tryptic and chymotryptic digests of casein on a TSK-G2000 SW column: (A) CHDS, (B) CHPS. Fractions 1–10 were collected manually. Each hydrolysate (2 mg) was dissolved in 1 ml of mobile phase [0.1% TFA, 0.05 *M* phosphate buffer (pH 5.0) and 35% methanol]. The injection volume was 20 μl.

from CHPS and CHDS, contained more peptides that were identified in each hydrolysate. The total numbers of peptides that were isolated and identified in CHDS and CHPS were 213 and 187, respectively. This difference between the two hydrolysates could be explained by the irreversible binding of phosphopeptides at low concentration by the C_{18} column.

The chromatogram of fraction 5 of a CHPS hydrolysate obtained from HPSEC and injected on a μBondapak reversed-phase column is shown in Fig. 2 as an example. The straight line represents the gradient used. The first peak was eluted in the isocratic mode, as the gradient was effective at 2.5 min; it contained salts from the phosphate buffer used on the size-exclusion column. Free amino acids, including phenylalanine and tyrosine and some small peptides with polar amino acids, were present in the peak juxtaposed to the first peak.

The identified peptides, 116 for CHDS and 99 for CHPS, with their RP elution times, hydrophobicities (kcal/mol), percentages of eluent B in mobile phase for a specific elution time, numbers of hydrophobic residues[14] over total residues and sequences are given in Tables I and II. The elution times obtained with the first RP

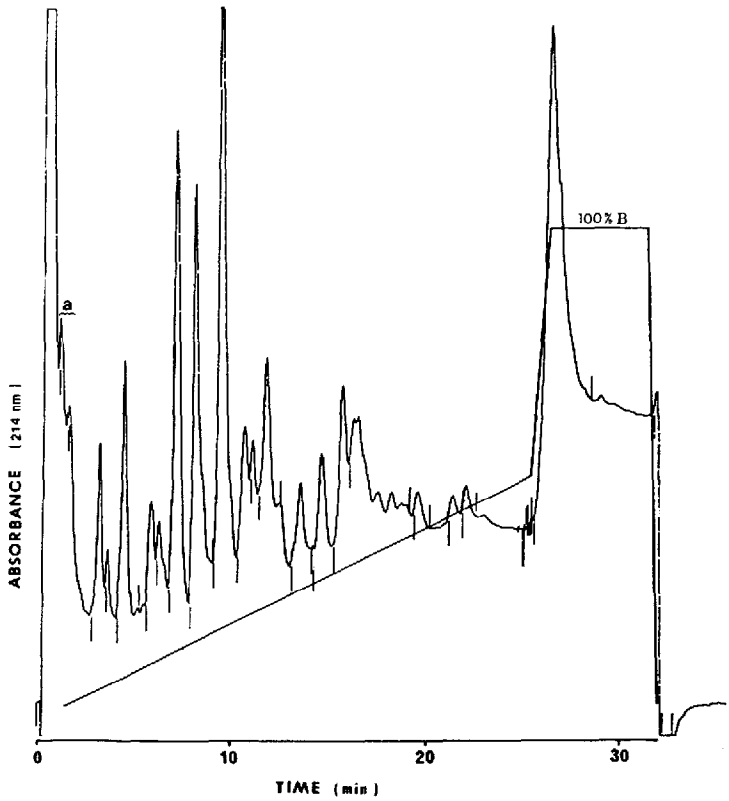

Fig. 2. Reversed-phase peptide mapping on a Waters μBondapak C_{18} column of fraction CHPS-5. Each fraction from HPSEC was diluted with 500 μl of solvent A, filtered through a VH 0.45 μm (Millipore). The injection volume was 100 μl. Chromatography was performed as described under Experimental.

system were used for the peptides that were rechromatographed in a second system for further purification. An example of such a chromatogram is shown on Fig. 3.

Comparison of the results from Tables I and II indicates that many peptides are common to both digests and that they elute at the same time, even though one protein hydrolysate is phosphorylated and the other one is not.

Two or more adjacent fractions obtained by HPSEC had the same peptide but in different concentrations. This may be due to the low resolution of the HPSEC column. The molar composition of the peptide could be used to predict in which HPSEC fraction the purified peptide should elute[15].

Generally peptides in the same HPSEC fraction appeared to separate by RP chromatography according to their hydrophobicity. However, peptides eluted during the first part of the gradient were more polar and less strongly adsorbed. Janssen *et al.*[16] reported that short hydrophilic peptides with basic character are poorly adsorbed owing to minor interaction with the support, and thus cannot be determined under the chromatographic conditions used for longer and more hydrophobic peptides. Addition of an anionic reagent such as TFA to the mobile phase was helpful to increase

TABLE I

IDENTIFICATION OF PEPTIDES FROM A CASEIN HYDROLYSATE (CHPS)

The single-letter code for amino acids is used.

Hydrolysate fraction Nb	*Casein fragment*	*Elution time (min)*	*Hydrophobicity (kcal/mol)*[a]	*Eluent B (%)*[b]	*Hydrophobic residues*[c]	*Sequence*
CHPS-2	κ : 41–42	2.71	2.86	0.42	1/2	NY
	κ : 146–156	<21.64	16.51	<38.28	7/11	QPHQPLPPTVM
	κ : 144–163	21.64–22.18	30.20	38.28–39.36	13/20	MHQPHQPLPPTVMFPPQSVL
CHPS-3	α_{s1}: 125–132	4.34	6.06	3.68	1/8	EGIHAQQK
	α_{s2}: 126–137[d]	5.95	7.50	6.90	1/12	EQLSTSEENSKK
	β : 89–97	5.95	9.25	6.90	4/9	QPEVMGVSK
	κ : 61–68	7.10	11.59	9.20	3/8	YAKPAAVR
	α_{s1}: 80–90	7.60	11.55	10.20	3/11	HIQKEDVPSER
	α_{s2}: 200–205	9.76	12.53	14.52	5/6	VIPYVR
	α_{s2}: 115–125	12.82	14.59	20.64	5/11	NAVPITPTLNR
	α_{s1}: 106–119[d]	14.42	17.00	23.84	6/14	VPQLEIVPNSAEER
	α_{s1}: 174–193	16.52	21.34	28.04	6/20	TDAPSFSDIPNPIGSENSEK
	α_{s1}: 8–22	18.20	16.05	31.40	6/15	NQGLPQEVLNENLLR
	β : 49–68	19.88	32.66	34.76	12/20	IHPFAQTQSLVYPFPGPIPN
	β : 144–163	21.42–22.08	30.20	37.84–39.16	13/20	MHQPHQPLPPTVMFPPQSVL
CHPS-4	α_{s1}: 1–3	1.70–2.76	4.82	0–0.52	1/3	RPK
	α_{s1}: 55–58		5.54		1/4	EDIK
	β : 29–32		6.50		1/4	KIEK
	κ : 62–65		5.56		1/4	AKPA
	α_{s2}: 42–45		4.66		2/4	EVVR
	β : 164–169		3.93		1/6	SLSQSK
	α_{s2}: 77–80		4.76		1/4	HYQK
	α_{s1}: 80–83		4.86		1/4	HIQK
	β : 100–105	4.20	7.42	3.40	2/6	EAMAPK
	κ : 80–86		7.52		2/7	SNTVPAK
	α_{s1}: 125–132		6.06		1/8	EGIHAQQK
	α_{s1}: 120–124		5.75		2/5	LHSMK
	α_{s1}: 37–42	4.60	6.17	4.20	2/6	VNELSK
	β : 89–97	5.47	9.25	5.94	4/9	QPEVMGVSK
	β : 177–183	8.14	11.12	11.28	4/7	AVPYPQR
	β : 170–176	9.51	13.01	14.02	5/7	VLPVPQK

	β : 33–48[d]	10.50	9.84	16.00	2/16	FQSEEQQQTEDELQDK
	α_{s2}: 138–150[d]		13.31		4/13	TVDMESTEVFTKK
	β : 194–202	11.02	11.51	17.04	5/9	QEPVLGPVR
	α_{s2}: 81–89		12.71		4/9	ALNEINQFY
	α_{s2}: 115–125	12.79	14.59	20.58	5/11	NAVPITPTLNR
	α_{s1}: 106–119[d]	14.44	17.00	23.88	6/14	VPQLEIVPNSAEER
	α_{s1}: 105–119[d]		18.48		6/15	KVPQLEIVPNSAEER
	α_{s1}: 104–119[d]		21.34		7/16	YKVPQLEIVPNSAEER
	β : 192–202		16.79		7/11	LYQEPVLGPVR
	α_{s1}: 174–193	16.50	21.34	28.00	6/20	TDAPSFSDIPNPIGSENSEK
	β : 33–52[d]	17.16–17.51	18.54	29.32–30.02	5/20	FQSEEQQQTEDELQDKIHPF
	α_{s1}: 170–193		24.55		7/24	GIQYTDAPSFSDIPNPIGSENSEK
	α_{s1}: 25–34		15.99		6/10	VAPFPQVFGK
CHPS-5	β : 98–99	1.55	3.18	0	1/2	VK
	α_{s2}: 204–205		2.42		1/2	VR
	α_{s2}: 171–173		4.26		1/3	YQK
	β : 94–97	2.27–2.72	3.22	0–0.44	1/4	GVSK
	α_{s2}: 42–45		4.66		2/4	EVVR
	β : 164–169		3.93		1/6	SLSQSK
	α_{s2}: 194–197		6.95		2/4	IQPK
	β : 100–105	4.32	7.42	3.64	2/6	EAMAPK
	α_{s1}: 37–42	4.75	6.17	4.50	2/6	VNELSK
	β : 89–97	5.56	9.25	6.12	4/9	QPEVMGVSK
	β : 177–183	8.26	11.12	11.52	4/7	AVPYPQR
	β : 49–52	9.24	8.70	13.48	3/4	IHPF
	β : 108–113	10.62	11.18	16.24	4/6	EMPFPK
	α_{s1}: 146–154	12.90	17.23	20.80	6/9	YPELFRQFY
	α_{s1}: 106–119[d]	14.56	17.00	24.32	6/14	VPELEIVPNSAEER
	α_{s2}: 100–109	15.64	18.00	26.28	7/10	YQGPIVLNPW
	β : 134–139	16.75	12.92	28.50	5/6	HLPLPL
	α_{s1}: 25–34	17.54	15.99	30.08	6/10	VAPFPEVFGK
CHPS-6	α_{s2}: 171–173	1.86	4.26	0	1/3	YQK
	β : 126–127	4.04	2.85	3.08	1/2	TL
	α_{s2}: 183–184		4.55		2/2	VY
	α_{s1}: 91 92	5.00	5.28	5.00	2/2	YL
	κ : 17–21	6.95	7.37	8.90	2/5	FFSDK
	β : 193–198		9.36		4/6	YQEPVL
	β : 177–183	7.47	11.12	9.94	2/7	AVPYPQR

(Continued on p. 196)

TABLE I *(continued)*

Hydrolysate fraction Nb	*Casein fragment*	*Elution time (min)*	*Hydrophobicity (kcal/mol)*[a]	*Eluent B (%)*[b]	*Hydrophobic residues*[c]	*Sequence*
	α_{s2}: 200–207	8.26–9.19	17.81	11.52–13.38	7/8	VIPYVRYL
	β : 191–193		7.69		3/3	LLY
	β : 49–52		8.70		3/4	IHPF
	κ : 44–50	9.98–10.58	8.73	14.96–16.16	3/7	QQKPVAL
	α_{s1}: 146–150		11.07		3/5	YPELF
	α_{s2}: 142–147		5.92		2/6	ESTEVF
	α_{s2}: 100–106		12.42		4/7	YQGPIVL
	β : 184–190	11.26–12.24	10.69	17.52–19.48	4/7	DMPIQAF
	α_{s1}: 166–173		12.77		5/8	YVPLGTQY
	β : 114–119	13.15	12.29	21.30	5/6	YPVEPF
	β : 184–190	14.44	10.69	23.88	4/7	DMPIQAF
	α_{s1}: 133–144	15.56	15.55	26.12	6/12	EPMIGVNQELAY
	α_{s1}: 152–164	16.75	18.25	28.50	6/13	QFYQLDAYPSGAW
	α_{s2}: 97–109	17.72	23.18	30.44	9/13	QYLYQGPIVLNPW
	α_{s1}: 25–32	18.51	14.50	32.02	6/8	VAPFPQVF
	β : 203–209	19.16	15.44	33.32	6/7	GPFPIIV
CHPS-7	α_{s1}: 23	1.83	2.65	0	1/1	F
	α_{s1}: 104		2.87		1/1	Y
	α_{s2}: 206–207	4.94	5.28	4.88	2/2	YL
	α_{s2}: 99–100	9.00	5.28	13.00	2/2	LY
	α_{s1}: 152–154		6.07		2/3	QFY
	β : 184–190	12.06–13.27	10.69	19.12–21.54	4/7	DMPIQAF
	β : 114–119		12.29		5/6	YPVEPF
	α_{s1}: 166–173		12.77		5/8	YVPLGTQY
	κ : 26–30		11.28		4/5	IPIQY
	β : 184–190	14.59	10.69	24.18	4/7	DMPIQAF
	α_{s2}: 92–96		10.42		4/5	FPQYL
	α_{s1}: 145–150	17.86	13.72	30.72	5/6	FYPELF
CHPS-8		3.02		1.04	0/1	N or D
	α_{s1}: 23–24	8.55	5.30	12.10	2/2	FF
	α_{s1}: 152–154	8.96	6.07	12.92	2/3	EFY

[a] From ref. 13.
[b] Eluent B: 60% (v/v) acetonitrile in 0.1% TFA.
[c] From ref. 14.
[d] Ser (P) peptides.

TABLE II

IDENTIFICATION OF PEPTIDES FROM A DEPHOSPHORYLATED CASEIN HYDROLYSATE (CHDS)

The single-letter code for amino acids is used.

Hydrolysate fraction Nb	*Casein fragment*	*Elution time (min)*	*Hydrophobicity (kcal/mol)[a]*	*Eluent B (%)[b]*	*Hydrophobic residues[c]*	*Sequence*
CHDS-1		1.75		0	0/1	N or D
CHDS-2		1.66		0	0/1	N or D
	β : 53–68	21.08	23.97	37.16	9/16	AQTQSLVYPFPGPIPN
CHDS-3		1.63		0	0/1	N or D
	κ : 98–102	2.78	6.69	0.56	2/5	HPHPH
	α_{s1}: 125–132	4.11	6.06	3.22	1/8	EGIHAQQK
	α_{s1}: 106–119	14.46	17.00	23.92	6/14	VPQLEIVPNSAEER
	α_{s1}: 174–193	16.62	21.34	28.24	6/20	TDAPSFSDIPNPIGSENSEK
	α_{s1}: 8–22	18.18	15.51	31.36	6/15	HQGLPQEVLNENLLR
	β : 144–163	21.84	30.20	38.68	13/20	MHQPHQPLPPTVMFPPQSVL
CHDS-4		1.66	2.87	0	1/1	Y
			1.49		0/1	K
	α_{s1}: 35–36		2.04		0/2	EK
	α_{s1} 4–7	2.30	7.55	0	2/4	HPIK
	κ : 98–102	2.79	6.69	0.58	2/5	HPHPH
	α_{s1}: 125–132	4.12	6.06	3.24	1/8	EGIHAQQK
	β : 89–97	5.64–5.87	9.25	6.28–6.74	4/9	QPEVMGVSK
	α_{s1}: 106–119	13.68–14.51	17.00	22.36–24.02	6/14	VPQLEIVPNSAEER
	α_{s1}: 174–193	16.63	21.34	28.26	6/20	TDAPSFSDIPNPIGSENSEK
	α_{s1}: 8–22	18.22	15.51	31.44	6/15	HQGLPQEVLNENLLR
	β : 49–68	19.50–21.03	32.66	34.00–37.06	12/20	IHPFAQTQSLVYPFPGPIPN
CHDS-5	α_{s1}: 35–36	<2.59	2.04	<0.18	0/2	EK
	β : 30–32		4.36		1/3	IQK
	α_{s1}:100–102		4.65		1/3	RLK
	β : 94–97		3.22		1/4	GVSK
	α_{s1}: 55–58		5.54		1/4	EDIK
	κ : 62–63		2.23		0/2	AK
	κ : 64–65		3.33		1/2	PA
	α_{s2}: 42–45		4.66		2/4	EVVR
	β : 164–169		3.93		1/6	SLSQSK
	β : 100–105	3.80	7.42	2.60	2/6	EAMAPK

(Continued on p. 198)

TABLE II *(continued)*

Hydrolysate fraction Nb	*Casein fragment*	*Elution time (min)*	*Hydrophobicity (kcal/mol)[a]*	*Eluent B (%)[b]*	*Hydrophobic residues[c]*	*Sequence*
	α_{s1}: 37–42	4.16	6.17	3.32	2/6	VNELSK
	α_{s1}: 125–132		6.06		1/8	EGIHAQQK
	α_{s1}: 84–90	5.12	6.69	5.24	2/7	EDVPSER
	β : 108–113	7.75	11.18	10.50	4/6	EMPFPK
	β : 177–183		11.12		4/7	AVPYPQR
	β : 170–176	9.07	13.01	13.14	5/7	VLPVPQK
	β : 33–48	10.10	9.84	15.20	2/16	FQSEEQQQTEDELQDK
	α_{s2}: 138–150	10.55	13.31	16.10	4/13	TVDMESTEVFTKK
	α_{s2}: 81–89	11.23	12.71	17.46	4/9	ALNEINQFY
	β : 194–202	12.35	11.51	19.70	5/9	QEPVLGPVR
	β : 193–202		14.38		6/10	YQEPVLGPVR
	α_{s1}: 106–119	14.07	17.00	23.14	6/14	VPQLEIVPNSAEER
	β : 134–139	16.12	12.92	27.24	5/6	HLPLPL
CHDS-6	α_{s2}: 110–113	<2.56	3.62	<0.12	1/4	DQVK
	α_{s2}: 180–181		3.91		1/2	LK
	β : 30–32		4.36		1/3	IEK
	α_{s2}: 171–173		4.26		1/3	YQK
	κ : 62–63		2.23		0/2	AK
	κ : 64–65		3.33		1/2	PA
	α_{s2}: 42–45		4.66		2/4	EVVR
	α_{s2}: 167–170		3.65		1/4	ISQR
	β : 164–169		3.93		1/6	SLSQSK
	α_{s2}: 194–197		6.95		2/4	IQPK
	β : 100–105	3.79	7.42	2.58	2/6	EAMAPK
	α_{s1}: 37–42	4.14	6.17	3.28	2/6	VNELSK
	α_{s1}: 84–90	5.08	6.69	5.16	2/7	EDVPSER
	κ : 1–10	6.26–6.86	7.65	7.52–8.72	2/10	EEQNQEQPIR
	κ : 17–21		7.37		2/5	FFSDK
	α_{s2}: 162–165		6.55		2/4	NFLK
	β : 108–113	6.86–7.70	11.18	8.72–10.40	4/6	EMPFPK
	β : 177–183		11.12		4/7	AVPYPQR
	β : 49–52	8.47	8.70	11.94	3/4	IHPF
	β : 170–176	9.38	13.01	13.76	5/7	VLPVPQK
	β : 33–48	10.04	9.84	15.08	2/16	FQSEEQQQTEDELQDK
	β : 114–123	12.28	13.22	19.56	5/10	YPVEPFTESQ
	β : 194–202		11.51		5/9	QEPVLGPVR

	β : 193–202		14.38		6/10	YQEPVLGPVR
	α_{s1}: 106–119	14.04	17.00	23.08	6/14	VPQLEIVPNSAEER
	β : 134–139	16.03	12.92	27.06	5/6	HLPLPL
CHDS-7-8	α_{s2}: 171–173	<3.79	4.26	<2.58	1/3	YQK
	α_{s1}: 170–173		3.21		1/4	GTQY
	α_{s2}: 182–188	3.79	6.80	2.58	2/7	TVYQHQK
	α_{s1}: 157–159	4.56–4.82	4.14	4.12–4.64	1/3	DAY
	β : 120–125		3.39		1/6	TESQSL
	β : 53–58		3.44		1/6	AQTQSL
	α_{s1}: 91–92		5.28		2/2	YL
	κ : 1–10	5.48–7.20	7.65	5.96–9.40	2/10	EEQNQEQPIR
	κ : 39–42		5.27		2/4	GLNY
	β : 193–198		9.36		4/6	YQEPVL
	α_{s2}: 174–181		15.05		5/8	FALPQYLK
	κ : 17–21		7.37		2/5	FFSDK
	κ : 35–38	7.20–8.42	8.36	9.40–11.84	3/4	YPSY
	β : 108–113		11.18		4/6	EMPFPK
	β : 177–183		11.12		4/7	AVPYPQR
	β : 191–193		7.69		3/3	LLY
	α_{s2}: 200–207	8.91	17.81	12.82	7/8	VIPYVRYL
	α_{s2}: 138–149	9.79	11.82	14.58	4/12	TVDMESTEVFTK
	α_{s2}: 101–109	10.42	15.13	15.84	6/9	QGPIVLNPW
	β : 184–190	11.44–11.74	10.69	17.88–18.48	4/7	DMPIQAF
	α_{s1}: 166–173		12.77		5/8	YVPLGTQY
	α_{s1}: 133–142	12.46–12.98	11.94	19.92–20.96	5/10	EPMIGVNQEL
	α_{s1}: 133–144		15.55		6/12	EPMIGVNQELAY
	β : 194–202		11.51		5/9	QEPVLGPVR
	β ; 114–119		12.29		5/6	YPVEPF
	α_{s1}: 92–98		10.56		4/7	LGYLEQL
	α_{s1}: 154–159		9.32		3/6	YQLDAY
	κ : 25–30		14.15		5/6	YIPIQY
	β : 184–190	14.44	10.69	23.88	4/7	DMPIQAF
	α_{s2}: 92–96		10.42		4/5	FPQYL
	β : 59–68	17.58	20.52	30.16	8/10	VYPFPGPIPN
	α_{s1}: 25–32	18.40	14.50	31.80	6/8	VAPFPQVF
	β : 203–209	19.04	15.44	33.08	6/7	GPFPIIV

(Continued on p. 200)

TABLE II *(continued)*

Hydrolysate fraction Nb	*Casein fragment*	*Elution time (min)*	*Hydrophobicity (kcal/mol)*[a]	*Eluent B (%)*[b]	*Hydrophobic residues*[c]	*Sequence*
CHDS-9		<3.82	2.87	<2.64	1/1	Y
			2.65		1/1	F
	α_{s1}: 93–94		2.87		1/2	GY
	α_{s2}: 206–207	4.63	5.28	4.26	2/2	YL
	α_{s1}: 92–94	5.31	5.28	5.62	2/3	LGY
	κ : 103–105	5.91	5.10	6.82	2/3	LSF
	β : 120–127	7.08	6.24	9.16	2/8	TESQSLTL
	α_{s1}: 146–149	7.82–8.78	8.42	10.64–12.56	3/4	YPEL
	α_{s1}: 152–154		5.42		2/3	QFY
	β : 191–193		7.69		3/3	LLY
	α_{s1}: 23–24		5.30		2/2	FF
	β : 184–191	11.56	13.10	18.12	5/8	DMPIQAFL
	α_{s2}: 100–106	12.34	12.42	19.68	5/7	YQGPIVL
	β : 114–119	13.08	12.29	21.16	5/6	YPVEPF
	β : 184–190	14.59–15.11	10.69	24.18–25.22	4/7	DMPIQAF
	α_{s2}: 92–96		10.42		4/5	FPQYL
	α_{s2}: 7–20		12.67		4/14	VSSSEESIISQETY
	α_{s1}: 145–150	17.68	13.72	30.36	5/6	FYPELF
CHDS-10		<2.86	2.87	<0.72	1/1	Y
			2.65		1/1	F
	α_{s1}: 152–154	7.88–8.75	5.42	10.76–12.50	2/3	QFY
	κ : 17–18		5.30		2/2	FF

[a] From ref. 13.
[b] Eluent B: 60% (v/v) acetonitrile in 0.1% TFA.
[c] From ref. 14.

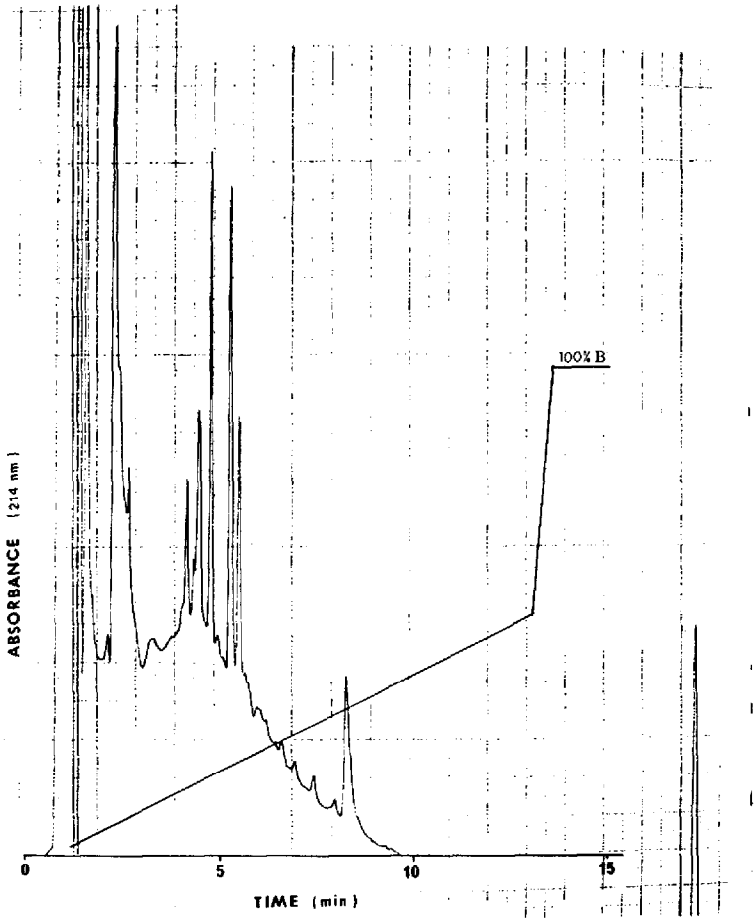

Fig. 3. Elution profile of peak a from Fig. 2 (CHPS-5), obtained as described under Experimental on a Waters μBondapak C_{18} reversed-phase column.

the elution time of small peptides such as α_{s2}: 204–205 (Val-Arg), which could be well separated and identified. The addition of perfluorinated organic acid, TFA, to the mobile phases has been justified due to its characteristics[17,18]. Elution conditions using TFA in the RP system had been previously studied with casein digests[19,20] and found to be suitable for peptide separation.

Changes in selectivity from the first mobile phase to the second could explain the variations in hydrophobicity between peptides eluted from the second RP chromatography. Solute–solute interactions in the mobile phase and at the surface of the stationary phase could be responsible for the elution of peptides with different hydrophobicities[21].

The elution times of peptides (α_{s1}: 106–119, α_{s2}: 126–137, α_{s2}: 138–150, and β: 33–48) with one or two phosphorylated or dephosphorylated serines are identical in both casein digests. This finding does not agree with previous results obtained by

Grego *et al.*[22], who observed higher retention times for corresponding dephosphorylated peptides. This result could be explained by the possible contamination of CHPS by dephosphorylated peptides[23]. The elution times of peptides with one or two dephosphorylated serines were shorter than that of the peptide with four dephosphorylated serines (α_{s2}: 7–20) and with lower hydrophobicity. Carles[24] has also reported that the retention times of peptides β: 1–25 and β: 2–25, which have four phosphorylated serines, increased with dephosphorylation.

The fraction CHPS-4 contained three peptides, α_{s1}: 106–119, α_{s1}: 105–119 and α_{s1}: 104–119, in the proportion 1.0, 0.25 and 0.10, respectively. The low molar concentration of these peptides could be explained by the low probability that the enzyme will hydrolyse the peptide bond of the first amino acid in sequences such as K-V-P-Q-L and Y-K-V-P-Q-L. Trypsin cleaves mainly peptide bonds on the carboxyl side of lysine and arginine, whereas chymotrypsin cleaves on the carboxyl side of aromatic amino acids, such as tyrosine, tryptophan and phenylalanine, and other peptide bonds more slowly[25]. Although the hydrophobicity of these three peptides is different, they eluted together in the first RP-HPLC solvent system and had to be chromatographed in a second solvent system to be separated for identification.

The peptides (α_{s1}: 25–32; α_{s1}: 166–173; α_{s2}: 92–96, α_{s2}: 142–147 and β: 203–209) with aromatic amino acids, such as tyrosine and phenylalanine, eluted later than expected. This observation indicates that as well as hydrophobic interactions, which make the dominant contribution to peptide chromatography, multiple retention processes could be involved in the binding of peptides to the stationary phase. Wehr and Correia[26] reported that endcapping the free silanols with trichloromethylsilane did not significantly decrease retention times of peptides. Specific hydrogen-bonding interactions, due to the low pH of the mobile phase, with silanol groups may therefore be suspected[20]. The longer elution time may be explained by bond-breaking at higher concentrations in acetonitrile. Since it is a weak dipolar base, it is more difficult for acetonitrile to manipulate the hydrogen-bonding characteristics of the stationary phase than 2-propanol[22].

The percentage of eluent B in the gradient is given in column 5 of Tables I and II; it was found that the best resolution was obtained between 15% and 40%[27] of eluent B, corresponding to 7.5 and 22.5 min. The amount of acetonitrile [60% (v/v) acetonitrile in 0.1% TFA] in eluent B was found to be right for casein digests.

Ionic interactions between ionized silanols and cationic solutes (containing arginine, lysine and histidine residues) were suspected to occur with peptides β: 134–139 and β: 194–202. Hearn and Grego[28] reported that the interaction of peptides with alkylsilica involves both a hydrophobic and a silanophilic component in the retention mechanism. Peptides can interact with accessible silanol groups at the surface of the stationary phase if complete ionization of the surface silanols on the column is not prevented by TFA. Dipeptide (β: 126–127) Thr-Leu eluted at a higher retention time than expected from its hydrophobicity. The presence of TFA in this system should not be neglected; being an anionic counter-ion it might pair with a positively charged peptide to form a complex and thus increase the retention time. Since TFA has amphiphilic properties, it can also serve as a hydrophilic pairing agent with hydrophobic residues[29]. Some peptides, such as α_{s1}: 25–34, α_{s2}: 162–165, β: 33–52, β: 49–52, β: 144–163 and β: 192–202, with one or many basic residues and aromatic amino acids (tyrosine or phenylalanine), elute later than expected from their

hydrophobicity. Their binding to the stationary phase may be due to multiple retention processes, including ionic interactions and hydrogen bonding.

Peptides with a number of aspartic and glutamic acid residues, such as α_{s1}: 8–22, α_{s1}: 133–144 and β: 33–48, were retained longer in the column. Similarly, peptides such as α_{s1}: 92–98; α_{s1}: 154–159, α_{s2}: 81–89 and β: 33–52 were delayed much longer owing to the presence of aspartic, glutamic and aromatic amino acid residues in their sequences. Although aspartic and glutamic acids are very polar and have low hydrophobicity, the peptides with these amino acids at pH 7.4 had higher retention in the column[30]. Carles[24] observed a positive contribution of aspartic and glutamic acids to the peptide retention of a β-casein trypsin digest with a slightly acidic mobile phase (pH 6.5).

Peptide β: 184–190 was found at two different elution times in the first RP chromatography; this can be explained by the oxidation of the methionine residue to the more polar sulphoxide or sulphone[31]; the two forms, which represent different oxidation states of sulphur in methionine, were identified from the PTC-amino acid analysis chromatograms: methionine sulphoxide was eluted between arginine and threonine; methionine sulphone was eluted after proline. The presence of an aspartic acid residue could also contribute to the delay of the peptide.

Addition of one hydrophobic residue, leucine, to the latter peptide (β: 184–191) made it elute sooner than expected. Other peptides, β: 59–68, β: 100–105, α_{s2}: 200–207, and α_{s1}: 133–144, were also eluted earlier from the column. The latter had twelve amino acid residues and the highest hydrophobicity (15.55 kcal/mol); such peptides may have conformations in which hydrophobic residues are located inside and hydrophilic residues outside[3].

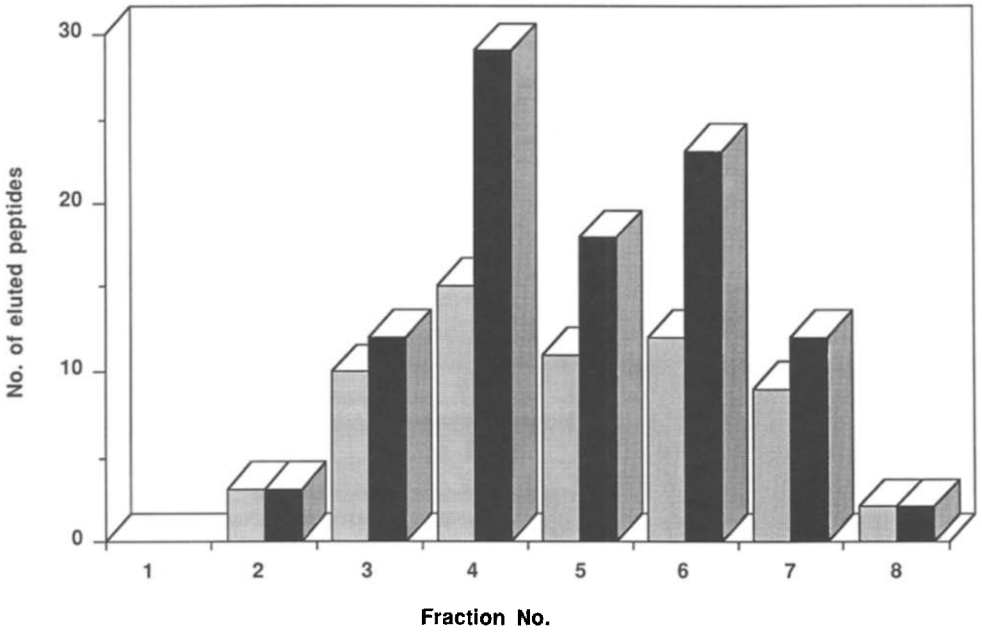

Fig. 4. Separation of CHPS peptides on a Waters μBondapak C_{18} reversed-phase column as described under Experimental. Peptides eluted according to their hydrophobicity (dotted areas) beside total number of peptides (black areas) eluted from each fraction obtained by HPSEC for CHPS.

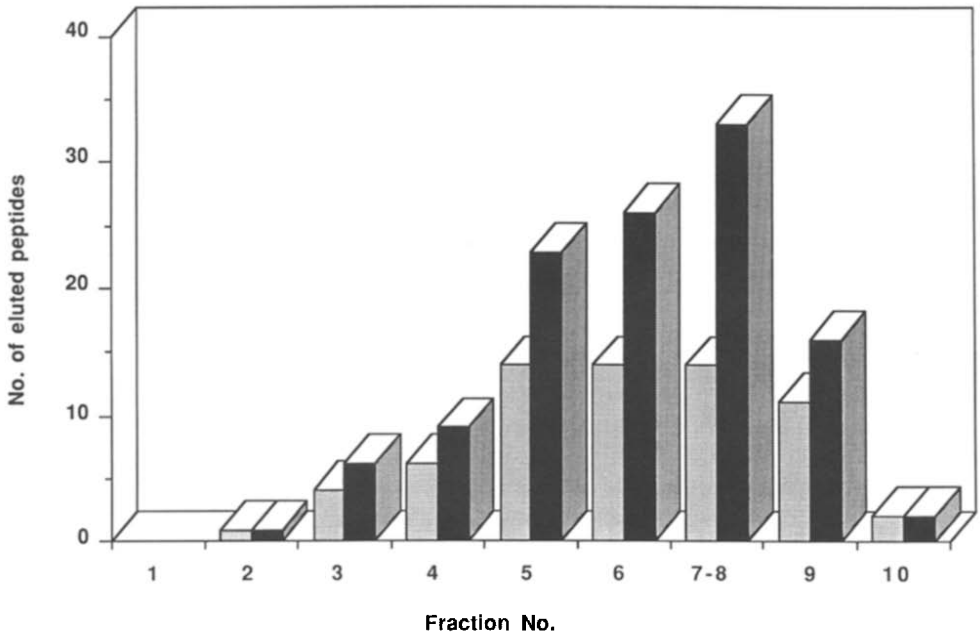

Fig. 5. Separation of CHDS peptides on a Waters μBondapak C_{18} reversed-phase column as described under Experimental. Peptides eluted according to their hydrophobicity (dotted areas) beside total number of peptides (black areas) eluted from each fraction obtained by HPSEC for CHDS.

Peptides obtained from CHDS (116) and CHPS (99) were eluted and identified for their amino acid sequences (Figs. 4 and 5). Out of these, the total numbers of peptides eluted according to their hydrophobicity were 65 and 62, respectively. This means that only 60% of the peptides were eluted from the RP-HPLC column according to their hydrophobicity. The separation of casein peptides was largely dependent on the overall peptide hydrophobicity, in addition to ionic interactions, hydrogen bonding and peptide characteristics.

The interactions occurring in RP-HPLC were taken into account in the methods recently developed for the prediction of retention data; however, some modifications to the predicted values might be needed[32,33], except when an internal peptide standard is chromatographed along with the peptides under investigation[34]. Taneja *et al.*[35] reported that since the peptides are retained much more tenaciously on a new column, the age of the column is important.

CONCLUSION

Being the most versatile and most widely used HPLC mode, RP chromatography has proved to be a reliable method with a high resolving power for the separation of peptides from casein hydrolysates. Peptides that elute together in the first mobile phase are easily separated by a second RP chromatography. RP-HPLC is considered to be a hydrophobic process in which the interaction between the peptide

and the stationary phase is regarded as being controlled by the net repulsions between the aqueous eluent and both the bonded phase and the non-polar part of the peptides. However, besides hydrophobicity, ionic interactions of the peptide side-chain with the free silanols of the stationary phase, hydrogen bonding with silica, and peptide characteristics such as the length of the peptide chain and the nature of the individual amino acid residues[36], might also contribute to peptide chromatography.

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REFERENCES

1 B. L. Jones and G. L. Lookhart, *Cereal Chem.*, 62 (1985) 89.
2 J. M. DiBussolo, in J. C. MacDonald (Consulting Editor), *HPLC: Instrumentation and Applications*, International Scientific Communications, Fairlfield, CT, 1986, p. 47.
3 T. Imoto and H. Yamada, *Molec. Cell. Biochem.*, 51 (1983) 111.
4 T. Sasagawa, T. Okuyama and D. C. Teller, *J. Chromatogr.*, 240 (1982) 329.
5 J. L. Meek and Z. L. Rossetti, *J. Chromatogr.*, 211 (1981) 15.
6 K. J. Wilson, A. Honegger, R. P. Stotzel and G. J. Hughes, *Biochem. J.*, 199 (1981) 31.
7 J. L. Maubois and G. Brulé, *Lait*, 62 (1982) 484.
8 Reabilan, Scientific Dossier, DS/FT, Sopharea Laboratories, Creuly, July, 1984.
9 M. A. Vijayalakshmi, L. Lemieux and J. Amiot, *J. Liq. Chromatogr.*, 9 (1986) 3559.
10 P. Petrilli, *Italian J. Biochem.*, 31 (1982) 391.
11 G. E. Tarr, in M. Elzinga (Editor), *Methods in Protein Sequence Analysis*, Humana Press, Clifton, NJ, 1982, p. 223.
12 B. Ribadeau-Dumas, *Biochim. Biophys. Acta*, 168 (1968) 274.
13 J.-C. Cheftel, J. L. Cuq and D. Lorient, *Protéines Alimentaires Technique et Documentation*, Lavoisier, Paris, 1985.
14 J. M. Di Bussolo and J. R. Gant, *J. Chromatogr.*, 327 (1985) 67.
15 L. Lemieux and J. Amiot, *J. Chromatogr.*, submitted for publication.
16 P. S. L. Janssen, J. W. van Nispen, R. L. A. E. Hamelinck, P. A. T. A. Melgers and B. C. Goverde, *J. Chromatogr. Sci.*, 22 (1984) 234.
17 S. Y. M. Lau, A. K. Taneja and R. S. Hodges, *J. Chromatogr.*, 317 (1984) 129.
18 W. S. Hancock, *Chem. N.Z.*, 47 (1983) 145.
19 C. Carles and B. Ribadeau-Dumas, *J. Dairy Res.*, 53 (1986) 595.
20 M. Yvon, *Thèse de Docteur d'Université*, Université de Paris-VII, Paris, 1985.
21 M. T. W. Hearn and B. Grego, *J. Chromatogr.*, 282 (1983) 541.
22 B. Grego, G. S. Baldwin, J. A. Knessel, R. J. Simpson, F. J. Morgan and M. T. W. Hearn, *J. Chromatogr.*, 297 (1984) 21.
23 L. Roger, personal communication.
24 C. Carles, *Thèse de Docteur Ingénieur "Sciences Agronomiques"*, Institut National Agronomique de Paris-Grignon, Paris-Grignon, 1983.
25 Y. B. Keil, in M. Elzinga (Editor), *Methods in Protein Sequence Analysis*, Humana Press, Clifton, NJ, 1982, p. 291.
26 C. T. Wehr and L. Correia, *Analytika (Johannesburg)*, Feb. (1982) 17.
27 M. Hermodson and W. C. Mahoney, *Methods Enzymol.*, 91 (1983) 352.
28 M. T. W. Hearn and B. Grego, *J. Chromatogr.*, 218 (1981) 497.
29 X. Geng and F. E. Regnier, *J. Chromatogr.*, 296 (1984) 15.
30 J. L. Meek, *Proc. Natl. Acad. Sci. U.S.A.*, 77 (1980) 1632.

31 P. M. Yuan, H. Pande, B. R. Clark and J. E. Shively, *Anal. Biochem.*, 120 (1982) 289.
32 C. A. Browne, H. P. J. Bennett and S. Solomon, *Anal. Biochem.*, 124 (1982) 201.
33 I. Molnar and M. Schoeneshoefer, in F. Lottspeich, A. Henschen, K.-P. Hupe (Editors), *High-Performance Liquid Chromatography in Protein and Peptide Chemistry; Proceedings International Symposium*, Walter de Gruyter, Berlin, New York, 1981, p. 97.
34 D. Guo, C. T. Mant, A. K. Taneja, J. M. R. Parker and R. S. Hodges, *J. Chromatogr.*, 359 (1986) 499.
35 A. K. Taneja, S. Y. M. Lau and R. S. Hodges, *J. Chromatogr.*, 317 (1984) 1.
36 D. Guo, C. T. Mant, A. K. Taneja and R. S. Hodges, *J. Chromatogr.*, 359 (1986) 519.