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High-performance liquid chromatography of casein hydrolysates phosphorylated and dephosphorylated

I. Peptide mapping

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ABSTRACT

A mixture of small peptides of molecular weight averaging 1000 daltons, obtained by controlled hydrolysis of casein with proteases, chymotrypsin and trypsin, was separated by size-exclusion and reversed-phase high-performance liquid chromatography. Peptides were identified and located in the known casein structures from their amino acid content and their N- and C-terminal amino acid analyses. The primary structure of peptides identified from casein hydrolysate phosphorylated and casein hydrolysate dephosphorylated is presented.

INTRODUCTION

Milk is the natural food for all newborn mammals, including humans, and it is a good source of protein and various nutrients. Caseins, accounting for about 80% of milk proteins [1], are phosphoproteins, α_{s1}-, α_{s2}-, β- and κ-, which are present in the micelles in the proportions 38, 11, 38 and 13%, respectively [2]. One approach to obtain casein hydrolysates is by *in vitro* digestion using multiple enzymes [3].

Casein digestion in an enzymic membrane reactor has been proposed as a means of producing food ingredients. This product has been shown to have a nutritive content similar to that found in the intestine after digestion of milk proteins [4]. As *in vitro* hydrolysis of casein simulates gastric and pancreatic digestion, the hydrolysates are better absorbed than a mixture of free amino acids [5], which may be due to the size and nature of the peptides produced during the digestive process.

Phosphorylated or dephosphorylated casein hydrolysates have been used as nutritional food ingredients for people suffering from severe gastrointestinal diseases [6,7] as they have favourable proportions of small peptides and amino acids.

Some of the peptides obtained after the hydrolysis of milk proteins have been reported to have physiological and biological functions [8]. These peptidic fragments and others can be identified and located in the phosphoproteins, as the amino acid sequences of these proteins have been completely elucidated [9–12]. If the amino acid

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sequences of the peptides are known, it would be possible to synthesize peptides with dietetic and pharmaceutical properties.

Casein hydrolysates obtained by proteases, trypsin and chymotrypsin contain more than 200 peptides of different sizes. It has been proposed that these peptides could be separated by a size-exclusion high-performance liquid chromatography (HPSEC) method [13] according to their hydrodynamic volume. This study was conducted to verify if peptides obtained from casein hydrolysates phosphorylated and dephosphorylated could be efficiently separated, according to their size, by the HPSEC method. Further separations of peptides and amino acids, including C- and N-terminal amino acids, were performed by reversed-phase high-performance liquid chromatography (RP-HPLC). The identification and location of the peptides in the known casein structures were determined.

EXPERIMENTAL

Materials

Commercial casein hydrolysates phosphorylated and dephosphorylated (CHPS and CHDS) were provided by Laboratoire Sopharga (France). In the abbreviations used we have included the letter "S" to denote that it is a gift from Sopharga. Casein hydrolysates were prepared according to the procedure developed by Maubois and Brulé [14], which involves a controlled hydrolysis of casein by proteases, chymotrypsin and trypsin in a continuous-flow membrane enzymatic reactor. The molecular weight distribution profile of the hydrolysates were as follows: >5000 daltons, 3%; 1000–5000 daltons, 28%; and <1000 daltons, 69%, which includes 8% of free amino acids [4]. Isolation of phosphoserine residues from peptidic hydrolysates was possible after aggregation in the presence of added calcium and phosphate ions; phosphorylated peptides, which formed aggregates in this solution, could not pass through the ultrafiltration membrane (CHPS) whereas non-phosphorylated peptides could do so (CHDS).

Sequanal-grade triethylamine (TEA), trifluoroacetic acid (TFA), phenyl isothiocyanate (PITC) and amino acid standard mixture H were obtained from Pierce (U.S.A.). 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). Doubly distilled water was purified by passing it through a Milli-Q water purification system (Millipore, Bedford, MA, U.S.A.).

Size-exclusion HPLC

Primary separation of CHPS and CHDS was performed by HPSEC on a TSK G2000 SW column (600 × 7.5 mm I.D.) with a guard column (60 × 7.5 mm I.D.) (Toyo Soda, Tokyo, Japan) according to the method of Vijayalakshmi *et al.* [13] for protein hydrolysates. An LKB HPLC system equipped with a Model 2150 pump, a Model 2152 controller, a Model 2151 variable-wavelength monitor, a Rheodyne M 7010 sample injection valve with a 20-μl loop, and a Model 3390A integrator (Hewlett-Packard) was used. The mobile phase consisted of 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 at constant temperature. Polypeptides were monitored at 214 nm with an absorbance scale of 0.05. The mobile phase was filtered through a 0.45-μm filter (Millipore) and sonicated before use.

Reversed-phase HPLC

Fractions obtained by HPSEC were rechromatographed to separate the peptides on a Waters μBondapak C_{18} (10 μm) reversed-phase column (300 × 3.9 mm I.D.) according to the method of Yvon [3] for casein hydrolysates. A Waters Assoc. HPLC system equipped with two Model F-6000A pumps, a Model M 720 solvent programmer, a WISP automated sample injector, a Model M 441 fixed-wavelength detector (214 nm) and a Model M 730 two-channel chart recorder was used. The column was maintained at 40°C in a water-bath. 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 required for one complete run, 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. The fractions obtained from the RP-HPLC column were evaporated in a Speed-Vac concentrator (Savant, Hicksville, NY, U.S.A.).

Peptides that coeluted were isolated by utilizing 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 as for RP-HPLC. 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* triply 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 [15] 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 using Petrilli's program [16] on an Apple IIE computer, and confirmed by N- and C-terminal analyses following the methods of Tarr [17] and Ribadeau-Dumas [18], respectively.

The identification of phenylthiocarbamyl (PTC) and phenylthiohydantoin (PTH) derivatives was carried out on an LKB HPLC system using a Pico-Tag column (Waters). For PTC derivatives, 1 m*M* EDTA (Fisher Scientific) was added to the Waters Pico-Tag eluent A and the gradient was modified by increasing the concentration of the Waters Pico-Tag eluent B 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 were performed every 21 min. When PTC derivatization of a blank hydrochloric acid was performed, values of about 10 pmol were obtained for serine and glycine. This "background" effect was previously reported by Stone and Williams [19]. These blank values were subtracted from the amino acid analysis of the samples. The identification of PTH derivatives [20] was performed by using solvent A [35 m*M* sodium acetate (pH

5.0) (500 ml)–acetonitrile (100 ml)] and by increasing the concentration of solvent B (2-propanol, 60%) in the following manner: 0–3.5 min, 0–2% B; 3.5–6.0 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. The time required for the analysis and equilibration was 20 min. In each instance the flow-rate was kept constant at 1.0 ml/min and norleucine was used as an internal standard.

RESULTS

According to the HPSEC elution conditions described above, 40 μg of hydrolysate could be injected per run. Fig. 1A and B represent the chromatographic patterns of casein digests CHDS and CHPS, respectively. Forty-five injections were performed for each hydrolysate of CHDS and CHPS; various fractions, as indicated in the Fig. 1A and B, were collected manually and pooled. After evaporation under nitrogen, the fractions were freeze-dried. These were solubilized in 0.115% TFA and injected onto a μBondapak C_{18} reversed-phase column. As an example, the RP-HPLC

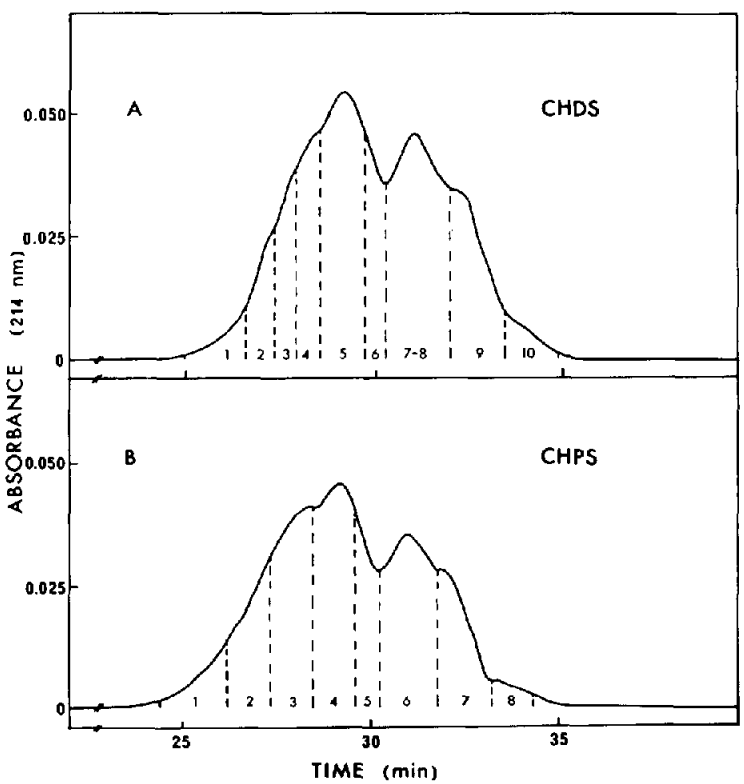


Fig. 1. Elution profiles of tryptic and chymotryptic digests of casein on a TSK G2000SW column. Each hydrolysate (2 mg) was dissolved in 1 ml of mobile phase [0.1% TFA–0.05 *M* phosphate buffer (pH 5.0)–35% methanol]. Injection volume, 20 μl. (A) CHDS (casein hydrolysate dephosphorylated); (B) CHPS (casein hydrolysate phosphorylated). 1–10, Fractions collected manually.

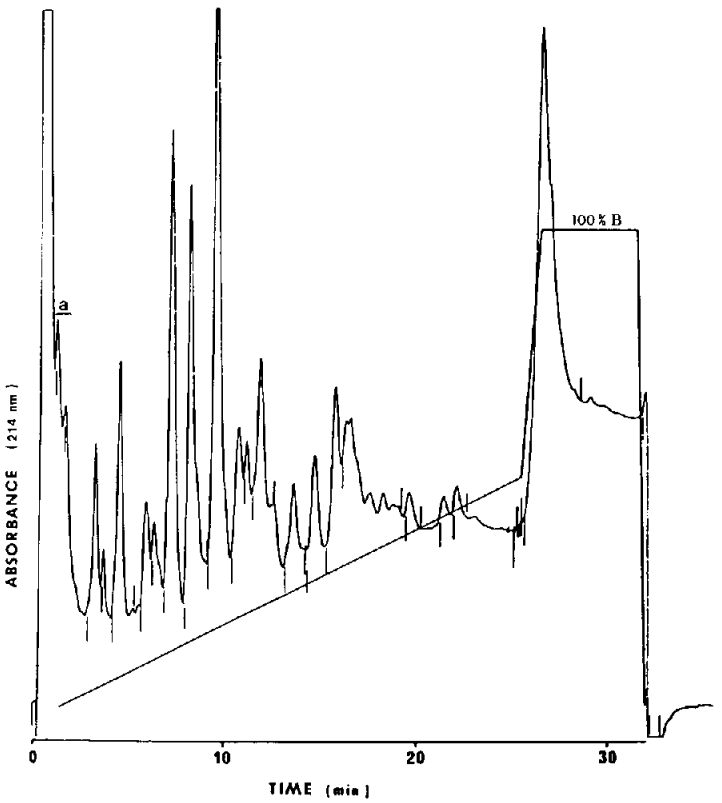


Fig. 2. Reversed-phase peptide mapping on a μBondapak C_{18} column of fraction CHPS-5. Each fraction from HPSEC was diluted with 500 μl of solvent A and filtered through an HV 0.45-μm filter (Millipore). The injection volume was 100 μl. Chromatography was performed as described under Experimental.

elution profile for fraction 5 obtained from the CHPS hydrolysate by HPSEC is shown in Fig. 2; the first peak which eluted in the isocratic mode contained salts from the phosphate buffer used in the HPSEC system, however, it was found to be devoid of peptides on further analysis. We observed that the gradient mode was effective only at 2.5 min. Four injections of the same product were performed and individual fractions were collected in separate test-tubes and evaluated for purity on the basis of symmetry and narrowness of the peak. Large peaks or those having a shoulder were further isolated by using a second RP-HPLC step as described. As the second RP-HPLC step was able, for example, to separate peak a obtained from the first RP-HPLC step (Fig. 2) into five peptides (Fig. 3), it confirms that the second solvent system was necessary for complete separation.

Seventy fractions collected from the first RP-HPLC step which were not pure were rechromatographed with a second RP-HPLC step before amino acid analysis. A picomole amino acid analysis of the peptide κ: 98–102, from fraction CHDS-4, as an example, was performed after converting its amino acids into their PTC derivatives (Fig. 4). The amino acid analysis and C- and N-terminal residue analyses were also performed for 223 pure peptides isolated from the first RP-HPLC step.

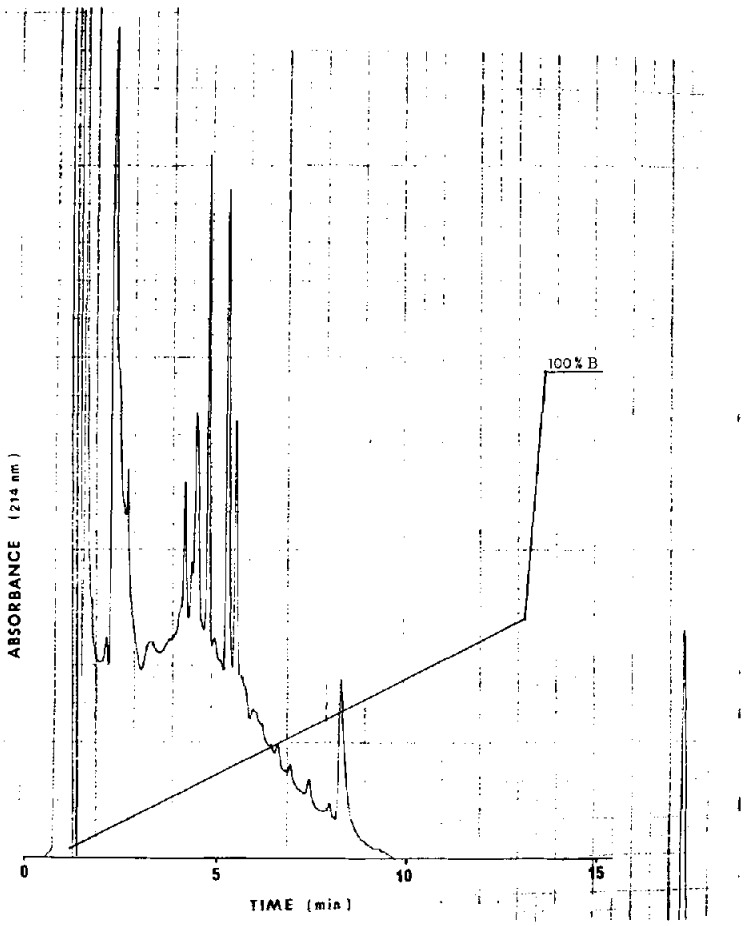


Fig. 3. Elution profile of peak a from Fig. 2 (CHPS-5), obtained as described under Experimental on a Waters μBondapak C_{18} reversed-phase column.

The identified sequences of peptides isolated from each HPSEC fraction of both casein hydrolysates are presented in Tables I and II. The sequences of α_{s1}-, α_{s2}-, β- and κ-caseins [21–23] are shown in Figs. 5 and 6. The underlined fragments indicate where the identified peptides are located in caseins and the numbers indicate in which fraction the peptides were eluted in the HPSEC for CHPS and CHDS.

Casein fragments obtained from CHDS and CHPS contained 1–24 amino acid residues with molecular weights ranging from 132 to 2600 daltons. Peptide molar composition values (Tables I and II) were determined by Pico-Tag amino acid analyses. The amounts injected onto the Pico-Tag column were determined from the peptide molar composition values and found to be 500 and 800 pmol for CHPS and CHDS, respectively (Tables I and II). As trypsin was used for hydrolysis, small peptides obtained by this method should contain only one arginine or lysine. If the peptides were devoid of arginine or lysine, these peptides would have come from the

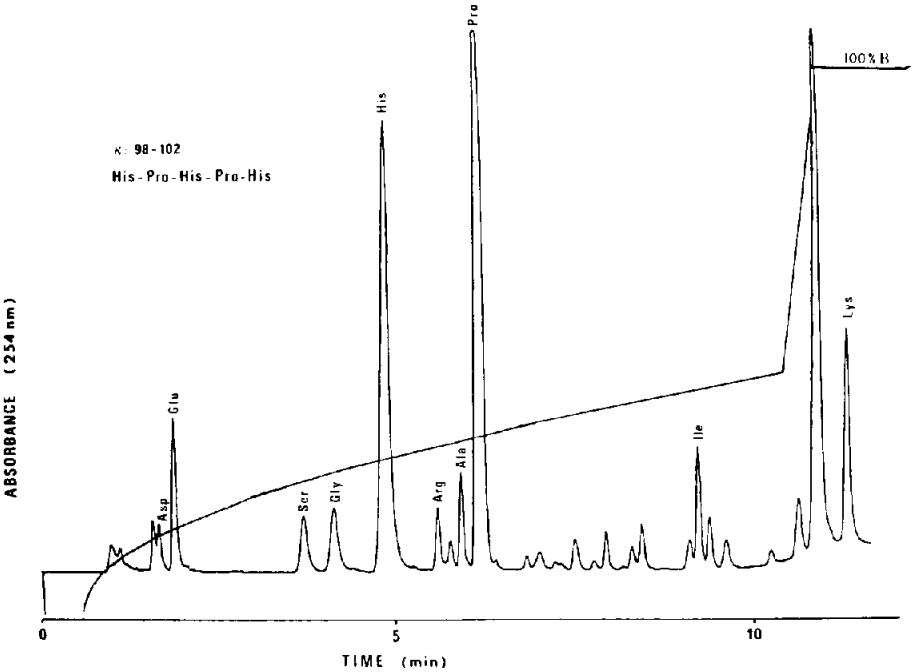


Fig. 4. Picomole chromatography of PTC-amino acids for the peptide κ: 98–102 (His–Pro–His–Pro–His). Peptide amino acids were converted into their PTC derivatives and then separated by RP-HPLC on a Pico-Tag column according to the manufacturer's instructions.

TABLE I

IDENTIFICATION OF PEPTIDES FROM A DEPHOSPHORYLATED CASEIN HYDROLYSATE (CHDS) ISOLATED BY SIZE-EXCLUSION HPLC FOLLOWED BY REVERSED-PHASE HPLC

The single-letter code for amino acids is used.

Fraction No. from HPSEC[a]	Corresponding casein fragment	Molar composition (nmol per 10 μl)[b]	Sequence
1			N
			G
2			N
	β: 53–68	0.20	AQTQSLVYPFPGPIPN
3			N
	κ: 98–102	0.20	HPHPH
	α_{s1}: 125–132	0.50	EGIHAQQK
	α_{s1}: 106–119	0.20	VPQLEIVPNSAEER
	α_{s1}: 174–193	0.60	TDAPSFSDIPNPIGSENSEK
	α_{s1}: 8–22	0.30	HQGLPQEVLNENLLR
	β: 144–163	0.40	MHQPHQPLPPTVMFPPQSVL

(*Continued on p. 306*)

TABLE 1 (*continued*)

Fraction No. from HPSEC[a]	Corresponding casein fragment	Molar composition (nmol per 10 μl)[b]	Sequence
4			Y
			K
	α_{s1}: 35–36[c]	0.25	EK
	α_{s1}: 4–7	2.00	HPIK
	κ: 98–102	0.70	HPHPH
	α_{s1}: 125–132	1.80	EGIHAQQK
	β: 89–97	0.20	QPEVMGVSK
	α_{s1}: 106–119	0.50	VPQLEIVPNSAEER
	α_{s1}: 174–193	1.50	TDAPSFSDIPNPIGSENSEK
	α_{s1}: 8–22	0.40	HQGLPQEVLNENLLR
	β: 49–68	0.20	FFVAPFPQVFGKEKVNEL
5	α_{s1}: 35–36	1.10	EK
	β: 30–32[c]	0.80	IQK
	α_{s1}: 100–102[c]	0.50	RLK
	β: 94–97[c]	2.00	GVSK
	α_{s1}: 55–58[c]	1.30	EDIK
	κ: 62–63[c]	1.60	AK
	κ: 64–65[c]	1.60	PA
	α_{s2}: 42–45[c]	0.50	EVVR
	β: 164–169[c]	1.40	SLSQSK
	β: 100–105	3.00	EAMAPK
	α_{s1}: 37–42	2.00	VNELSK
	α_{s1}: 125–132[c]	0.40	EGIHAQQK
	α_{s1}: 84–90	3.00	EDVPSER
	β: 108–113	2.00	EMPFPK
	β: 177–183[c]	3.00	AVPYPQR
	β: 170–176	2.00	VLPVPQK
	β: 33–48	0.80	FQSEEQQQTEDELQDK
	α_{s2}: 138–150	0.40	TVDMESTEVFTKK
	α_{s2}: 81–89	0.40	ALNEINQFY
	β: 194–202	1.00	QEPVLGPVR
	β: 193–202[c]	1.00	YQEPVLGPVR
	α_{s1}: 106–119	1.00	VPQLEIVPNSAEER
	β: 134–139	1.50	HLPLPL
6	α_{s2}: 110–113	0.60	DQVK
	α_{s2}: 180–181[c]	0.70	LK
	β: 30–32[c]	0.70	IEK
	α_{s2}: 171–173[c]	0.60	YQK
	κ: 62–63[c]	0.80	AK
	κ: 64–65[c]	0.50	PA
	α_{s2}: 42–45[c]	0.40	EVVR
	α_{s2}: 167–170[c]	0.50	ISQR
	β: 164–169[c]	0.50	SLSQSK
	α_{s2}: 194–197[c]	0.30	IQPK
	β: 100–105	0.35	EAMAPK
	α_{s1}: 37–42	1.00	VNELSK
	α_{s1}: 84–90	2.00	EDVPSER
	κ: 1–10	0.70	EEQNQEQPIR
	κ: 17–21[c]	0.17	FFSDK
	α_{s2}: 162–165[c]	0.30	NFLK

TABLE I (*continued*)

Fraction No. from HPSEC[a]	Corresponding casein fragment	Molar composition (nmol per 10 μl)[b]	Sequence
	β: 108–113	3.00	EMPFPK
	β: 177–183[c]	2.00	AVPYPQR
	β: 49–52	2.00	IHPF
	β: 170–176	0.30	VLPVPQK
	β: 33–48	1.00	FQSEEQQQTEDELQDK
	β: 114–123	0.25	YPVEPFTESQ
	β: 194–202[c]	1.00	QEPVLGPVR
	β: 193–202[c]	0.20	YQEPVLGPVR
	α_{s1}: 106–119	0.60	VPQLEIVPNSAEER
	β: 134–139	0.70	HLPLPL
7–8	α_{s2}: 171–173	0.30	YQK
	α_{s1}: 170–173[c]	0.40	GTQY
	α_{s2}: 182–188	0.50	TVYQHQK
	α_{s1}: 157–159	0.30	DAY
	β: 120–125[c]	1.00	TESQSL
	β: 53–58[c]	0.50	AQTQSL
	α_{s1}: 91–92[c]	1.20	YL
	κ: 1–10	0.35	EEQNQEQPIR
	κ: 39–42[c]	0.30	GLNY
	β: 193–198[c]	0.50	YQEPVL
	α_{s2}: 174–181[c]	0.20	FALPQYLK
	κ: 17–21[c]	0.20	FFSDK
	κ: 35–38	0.20	YPSY
	β: 108–113[c]	0.80	EMPFPK
	β: 177–183[c]	0.21	AVPYPQR
	β: 191–193[c]	0.55	LLY
	α_{s2}: 200–207	1.00	VIPYVRYL
	α_{s2}: 138–149	0.75	TVDMESTEVFTK
	α_{s2}: 101–109	0.40	QGPIVLNPW
	β: 184–190	4.00	DMPIQAF
	α_{s1}: 166–173[c]	1.30	YVPLGTQY
	α_{s1}: 133–142	0.20	EPMIGVNQEL
	α_{s1}: 133–144[c]	0.70	EPMIGVNQELAY
	β: 194–202[c]	0.60	QEPVLGPVR
	β: 114–119[c]	3.50	YPVEPF
	α_{s1}: 92–98[c]	0.20	LGYLEQL
	α_{s1}: 154–159[c]	0.15	YQLDAY
	α_{s1}: 166–173[c]	0.20	YVPLGTQY
	κ: 25–30[c]	0.50	YIPIQY
	α_{s1}: 133–144[c]	0.24	EPMIGVNQELAY
	β: 184–190	1.00	DMPIQAF
	α_{s2}: 92–96[c]	0.50	FPQYL
	β: 59–68	0.20	VYPFPGPIPN
	α_{s1}: 25–32	0.60	VAPFPQVF
	β: 203–209	0.60	GPFPIIV
9			Y
			F
	α_{s1}: 93–94[c]	0.20	GY
	α_{s2}: 206–207	0.85	YL
	α_{s1}: 92–94	0.40	LGY

(*Continued on p. 308*)

TABLE I (*continued*)

Fraction No. from HPSEC[a]	Corresponding casein fragment	Molar composition (nmol per 10 μl)[b]	Sequence
	κ: 103–105	0.30	LSF
	β: 120–127	0.50	TESQSLTL
	α_{s1}: 146–149	0.15	YPEL
	α_{s1}: 152–154[c]	3.00	QFY
	β: 191 193[c]	0.50	LLY
	α_{s1}: 23 24[c]	2.00	FF
	κ: 17 18	2.00	FF
	β: 184–191	0.40	DMPIQAFL
	α_{s2}: 100–106	0.18	YQGPIVL
	β: 114–119	0.32	YPVEPF
	β: 184 190	0.35	DMPIQAF
	α_{s2}: 92–96[c]	0.60	FPQYL
	α_{s2}: 7–20[c]	0.25	VSSSEESIISQETY
	α_{s1}: 145–150	0.12	FYPELF
10			Y
			F
	α_{s1}: 152–154	0.50	QFY
	α_{s1}: 23–24	0.50	FF

[a] Fig. 1A.
[b] Peptide molar composition determined with an injection ion volume of 10 μl.
[c] Peptides identified from the second RP system.

TABLE II

IDENTIFICATION OF PEPTIDES FROM A PHOSPHORYLATED CASEIN HYDROLYSATE (CHPS) ISOLATED BY SIZE-EXCLUSION HPLC FOLLOWED BY REVERSED-PHASE HPLC

The single-letter code for amino acids is used.

Fraction No. from HPSEC[a]	Corresponding casein fragment	Molar composition (nmol per 10 μl)[b]	Sequence
1	Nothing		
2	κ: 41–42	0.40	NY
	β: 146–156	0.12	QPHQPLPPTVM
	β: 144–163	0.17	MHQPHQPLPPTVMFPPQSVL
3	α_{s1}: 125–132	0.70	EGIHAQQK
	α_{s2}: 126 137	0.20	EQLSTSEENSKK
	β: 89–97	0.20	QPEVMGVSK
	κ: 61–68	0.15	YAKPAAVR
	α_{s1}: 80–90	0.20	HIQKEDVPSER
	α_{s2}: 200–205	0.25	VIPYVR
	α_{s2}: 115 125	0.15	NAVPITPTLNR
	α_{s1}: 106–119	0.25	VPQLEIVPNSAEER
	α_{s1}: 174–193	1.20	TDAPSFSDIPNPIGSENSEK
	α_{s1}: 8–22	0.42	HQGLPQEVLNENLLR
	β: 49–68	0.08	IHPFAQTQSLVYPFPGPIPN
	β: 144–163	0.65	MHQPHQPLPPTVMFPPQSVL

TABLE II (*continued*)

Fraction No. from HPSEC[a]	Corresponding casein fragment	Molar composition (nmol per 10 μl)[b]	Sequence
4	α_{s1}: 1–3	0.80	RPK
	α_{s1}: 55–58[c]	0.80	EDIK
	β: 29–32[c]	0.80	KIEK
	κ: 62–65[c]	0.50	AKPA
	α_{s2}: 42–45[c]	0.30	EVVR
	β: 164–169[c]	1.60	SLSQSK
	α_{s2}: 77–80[c]	0.40	HYQK
	α_{s1}: 80–83[c]	0.40	HIQK
	β: 100–105	3.00	EAMAPK
	κ: 80–86[c]	1.50	SNTVPAK
	α_{s1}: 125–132[c]	1.00	EGIIIAQQK
	α_{s1}: 120–124[c]	0.90	LIISMK
	β: 100–105	0.50	EAMAPK
	α_{s1}: 37–42	0.50	VNELSK
	β: 89–97	0.70	QPEVMGVSK
	β: 177–183	1.40	AVPYPQR
	β: 170–176	1.00	VLPVPQK
	β: 33–48	0.50	FQSEEQQQTEDELQDK
	α_{s2}: 138–150[c]	0.50	TVDMESTEVFTKK
	β: 194–202	0.50	QEPVLGPVR
	α_{s2}: 81–89[c]	0.50	ALNEINQFY
	α_{s2}: 115–125	0.50	NAVPITPTLNR
	α_{s1}: 106–119	1.00	VPQLEIVPNSAEER
	α_{s1}: 105–119[c]	0.25	KVPQLEIVPNSAEER
	α_{s1}: 104–119[c]	0.10	YKVPQLEIVPNSAEER
	β: 192–202[c]	0.35	LYQEPVLGPVR
	α_{s1}: 174–193	0.50	TDAPSFSDIPNPIGSENSEK
	β: 33–52	0.50	FQSEEQQQTEDELQDKIHPF
	α_{s1}: 170–193[c]	0.20	GTQYTDAPSFSDIPNPIGSENSEK
	α_{s1}: 25–34[c]	0.40	VAPFPQVFGK
5	β: 98–99	0.25	VK
	α_{s2}: 204–205[c]	0.40	VR
	α_{s2}: 171–173[c]	0.50	YQK
	β: 94–97	0.30	GVSK
	α_{s2}: 42–45[c]	0.90	EVVR
	β: 164–169[c]	1.30	SLSQSK
	α_{s2}: 194–197[c]	0.50	IQPK
	β: 100–105	0.30	EAMAPK
	α_{s1}: 37–42	0.20	VNELSK
	β: 89–97	0.50	QPEVMGVSK
	β: 177–183	0.60	AVPYPQR
	β: 49–52	0.30	IHPF
	β: 108–113	1.00	EMPFPK
	α_{s1}: 146–154	0.40	YPELFRQFY
	α_{s1}: 106–119	0.30	VPELEIVPNSAEER
	α_{s2}: 100–109	0.20	YQGPIVLNPW
	β: 134–139	0.15	HLPLPL
	α_{s1}: 25–34	0.20	VAPFPEVFGK
6	α_{s2}: 171–173	0.17	YQK
	β: 126–127	0.40	TL

(*Continued on p. 310*)

TABLE II (*continued*)

Fraction No. from HPSEC[a]	Corresponding casein fragment	Molar composition (nmol per 10 μl)[b]	Sequence
	α_{s2}: 183–184[c]	0.40	VY
	α_{s1}: 91–92	0.60	YL
	κ: 17–21	0.23	FFSDK
	β: 193–198[c]	0.20	YQEPVL
	β: 177 183	0.10	AVPYPQR
	α_{s2}: 200 207	0.20	VIPYVRYL
	β: 191–193[c]	0.70	LLY
	β: 49–52[c]	0.92	IHPF
	κ: 44–50	0.13	QQKPVAL
	α_{s1}: 146–150[c]	0.15	YPELF
	α_{s2}: 142–147[c]	0.30	ESTEVF
	α_{s2}: 100–106[c]	0.30	YQGPIVL
	β: 184–190	0.40	DMPIQAF
	α_{s1}: 166–173[c]	1.50	YVPLGTQY
	β: 114–119	1.00	YPVEPF
	β: 184–190	1.00	DMPIQAF
	α_{s1}: 133–144	0.30	EPMIGVNQELAY
	α_{s1}: 152–164	0.20	QFYQLDAYPSGAW
	α_{s2}: 97–109	0.20	QYLYQGPIVLNPW
	α_{s1}: 25–32	0.25	VAPFPQVF
	β: 203–209	0.50	GPFPIIV
7			F
			Y
	α_{s2}: 206–207	0.50	YL
	α_{s2}: 99–100	0.60	LY
	α_{s1}: 152–154[c]	0.40	QFY
	β: 184–190	0.30	DMPIQAF
	β: 114–119[c]	0.80	YPVEPF
	α_{s1}: 166–173[c]	0.20	YVPLGTQY
	κ: 26–30[c]	0.16	IPIQY
	β: 184–190	0.90	DMPIQAF
	α_{s2}: 92–96[c]	0.40	FPQYL
	α_{s1}: 145–150	0.15	FYPELF
8			N
	α_{s1}: 23–24	0.15	FF
	α_{s1}: 152 154	0.30	LFY

[a] Fig. 1B.
[b] Peptide molar composition determined from an injection volume of 10 μl.
[c] Peptides identified from the second RP system.

action of chymotrypsin and the calculation was then based on the values obtained for aspartic acid, glutamic acid, proline and alanine residues.

According to the peptide molar composition values we found that some of the peptides were contaminated (data not shown). This contamination could be explained by the so-called "memory effect" [24], in which remaining hydrophobic peptides bound to the gel could elute after successive runs.

DISCUSSION

We were able to isolate and identify 213 and 187 peptides from less than 2 mg of CHDS and CHPS, respectively. Although the elution profiles of casein digests from HPSEC were almost the same, most of the identified peptides came from fraction 4 (550–840 daltons) of CHPS and fraction 7–8 (210–410 daltons) of CHDS and had comparable elution times in both hydrolysates. The identified peptides were found to originate from the four phosphoproteins, α_{s1}-, α_{s2}-, β- and κ-caseins, in the proportions 35, 21, 34 and 9%, respectively.

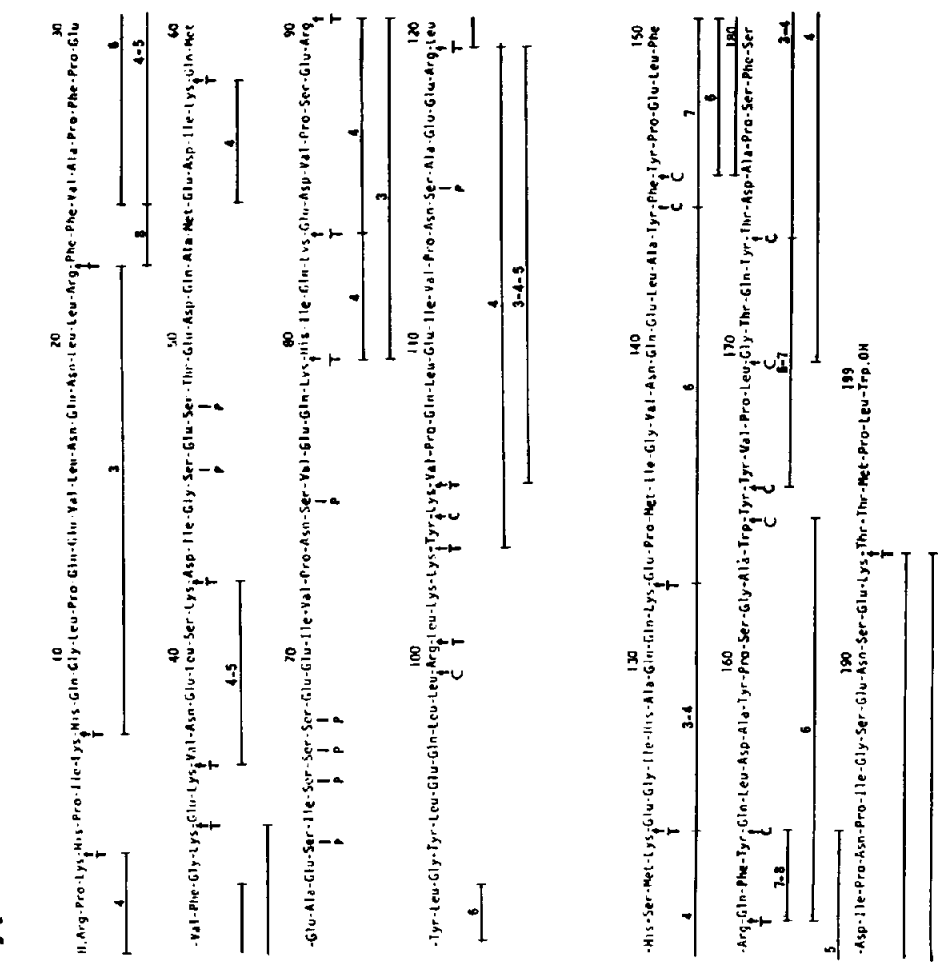
The peptides obtained from CHDS and CHPS had lysine, arginine, tyrosine, phenylalanine and valine as the C- and N-terminal amino acids as the enzymes used were endopeptidases, trypsin and chymotrypsin. Trypsin is highly specific for peptide bonds linking the carboxyl groups of two basic amino acids, lysine and arginine. However, chymotrypsin, although less specific than trypsin, has a preference for peptide bonds linking the carboxyl groups of the aromatic amino acids, phenylalanine, tryptophan and tyrosine. Keil [25] observed that chymotrypsin also cleaves proteins at the carboxylic side of other amino acids such as leucine, methionine and histidine at a slower rate. The cleavage sites of chymotrypsin and trypsin which are shown in Figs. 5 and 6 are those reported by Pelissier [26]. Although it is possible to have a number of cleavage sites for chymotrypsin [26], we observed only a few of them.

The elucidation of primary amino acid sequences of caseins was performed previously by conventional methods [9–12]. However, Stewart and co-workers [21,22] recently characterized bovine caseins at mRNA levels by cloning cDNAs. As this method provides accurate primary amino acid sequences of caseins, we used these sequences [21–23] to identify and locate the peptides isolated from CHDS and CHPS.

Peaks obtained from HPSEC using a TSK G2000SW column are not sharp and well separated (Fig. 1), as the resolution of the column is low. Hence identical peptides were eluted in two or more different fractions as given in Tables I and II. When fractions obtained from HPSEC were injected onto the reversed-phase column, these identical peptides had the same retention time (data not shown) but differed in concentration. If the HPSEC column had a better resolution, the fraction which contained the highest amount of the peptide would have had less contamination due to other peptides.

Peptide β: 184–190 was identified at two different elution times in the first RP-HPLC system; this could be explained by the oxidation of the methionine residue to a more polar sulphoxide or sulphone [27]; this was confirmed by performing PTC-amino acid analysis: methionine sulphoxide was eluted between arginine and threonine and methionine sulphone was eluted after proline.

Peptides with one (α_{s1}: 106–119; α_{s2}: 138–150; β: 33–48) or two serine residues (α_{s2}: 126–137) which were phosphorylated or dephosphorylated were found to have identical elution times in both casein digests. This finding does not agree with previous results obtained by Grego *et al.* [28], who observed longer retention times for corresponding dephosphorylated peptides. This result could be explained by the possible contamination of CHPS by dephosphorylated peptides [29]. The elution times of peptides with one or two dephosphorylated serines were lower than that of the peptide with four dephosphorylated serines (α_{s2}: 7–20) and with an intermediate hydrophobicity of 12.67 kcal/mol [30]. Thus, the elution of the peptide α_{s2}: 7–20 from


A

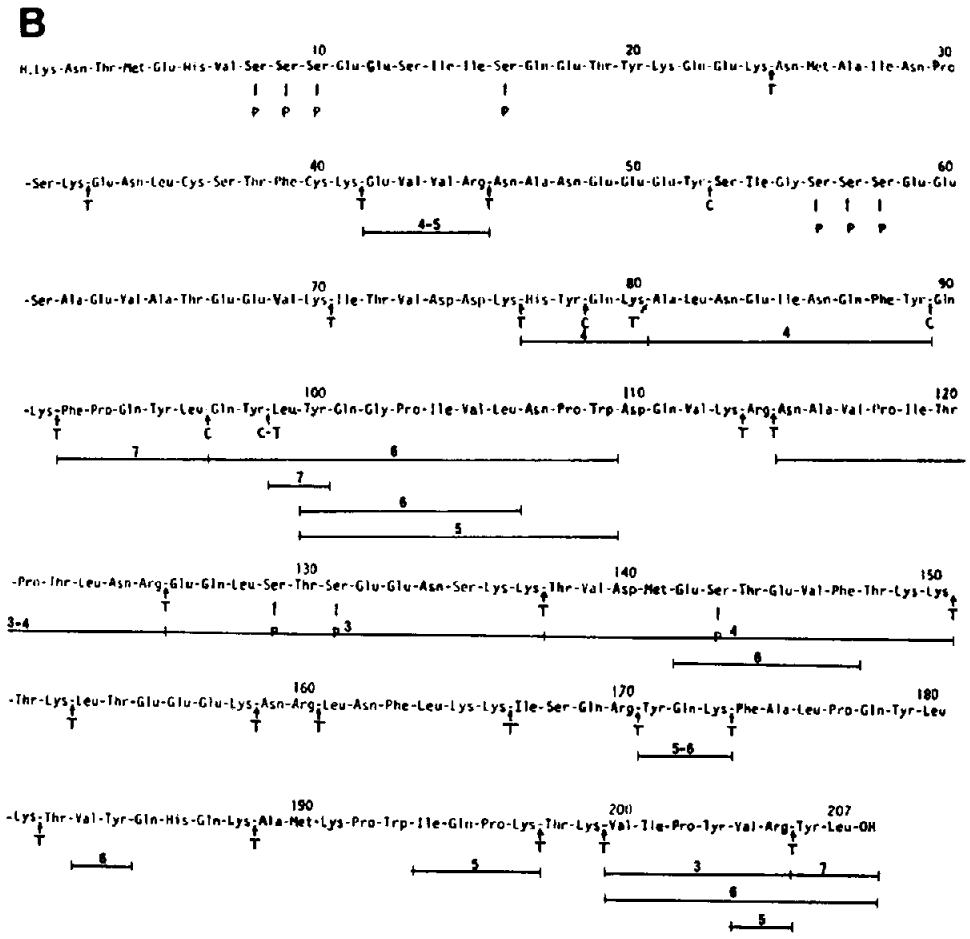
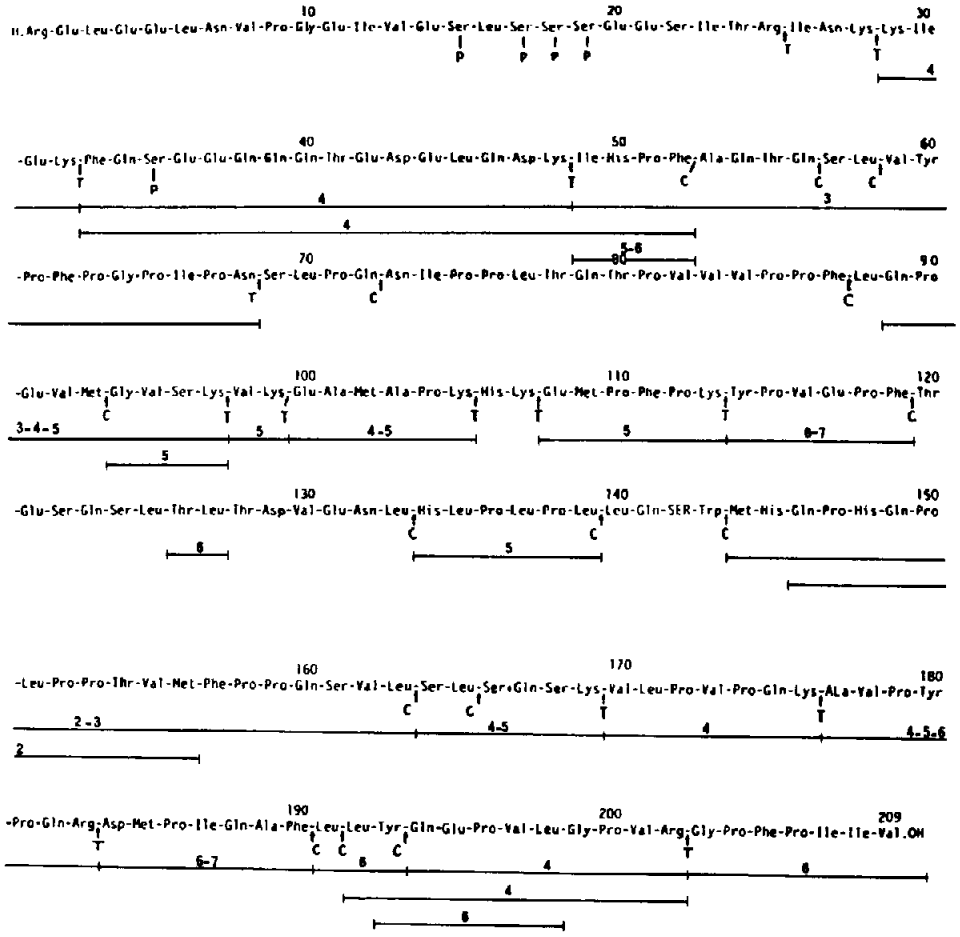


Fig. 5.

(*Continued on p. 314*)

C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20
30
H.Arg-Glu-Leu-Glu-Glu-Leu-Asn-Val-Pro-Gly-Glu-Ile-Val-Glu-Ser-Leu-Ser-Ser-Ser-Glu-Glu-Ser-Ile-Thr-Arg-Ile-Asn-Lys-Lys-Ile
P P P P T T
4
40
50
60
-Glu-Lys-Phe-Gln-Ser-Glu-Glu-Gln-Gln-Gln-Thr-Glu-Asp-Glu-Leu-Gln-Asp-Lys-Ile-His-Pro-Phe-Ala-Gln-Thr-Gln-Ser-Leu-Val-Tyr
T P T C C C
4
3
4
5-6
6
70
80
90
-Pro-Phe-Pro-Gly-Pro-Ile-Pro-Asn-Ser-Leu-Pro-Gln-Asn-Ile-Pro-Pro-Leu-Thr-Gln-Thr-Pro-Val-Val-Val-Pro-Pro-Phe-Leu-Gln-Pro
T C C
100
110
120
-Glu-Val-Met-Gly-Val-Ser-Lys-Val-Lys-Glu-Ala-Met-Ala-Pro-Lys-His-Lys-Glu-Met-Pro-Phe-Pro-Lys-Tyr-Pro-Val-Glu-Pro-Phe-Thr
C T T T T T C
3-4-5
5
4-5
5
8-7
5
130
140
150
-Glu-Ser-Gln-Ser-Leu-Thr-Leu-Thr-Asp-Val-Glu-Asn-Leu-His-Leu-Pro-Leu-Pro-Leu-Leu-Gln-SER-Trp-Met-His-Gln-Pro-His-Gln-Pro
C C C
6
5
160
170
180
-Leu-Pro-Pro-Thr-Val-Met-Phe-Pro-Pro-Gln-Ser-Val-Leu-Ser-Leu-Ser-Gln-Ser-Lys-Val-Leu-Pro-Val-Pro-Gln-Lys-ALa-Val-Pro-Tyr
C C T T
2-3
4-5
4
4-5-6
2
190
200
209
-Pro-Gln-Arg-Asp-Met-Pro-Ile-Gln-Ala-Phe-Leu-Leu-Tyr-Gln-Glu-Pro-Val-Leu-Gly-Pro-Val-Arg-Gly-Pro-Phe-Pro-Ile-Ile-Val.OH
T C C C T
6-7
6
4
6
4
6

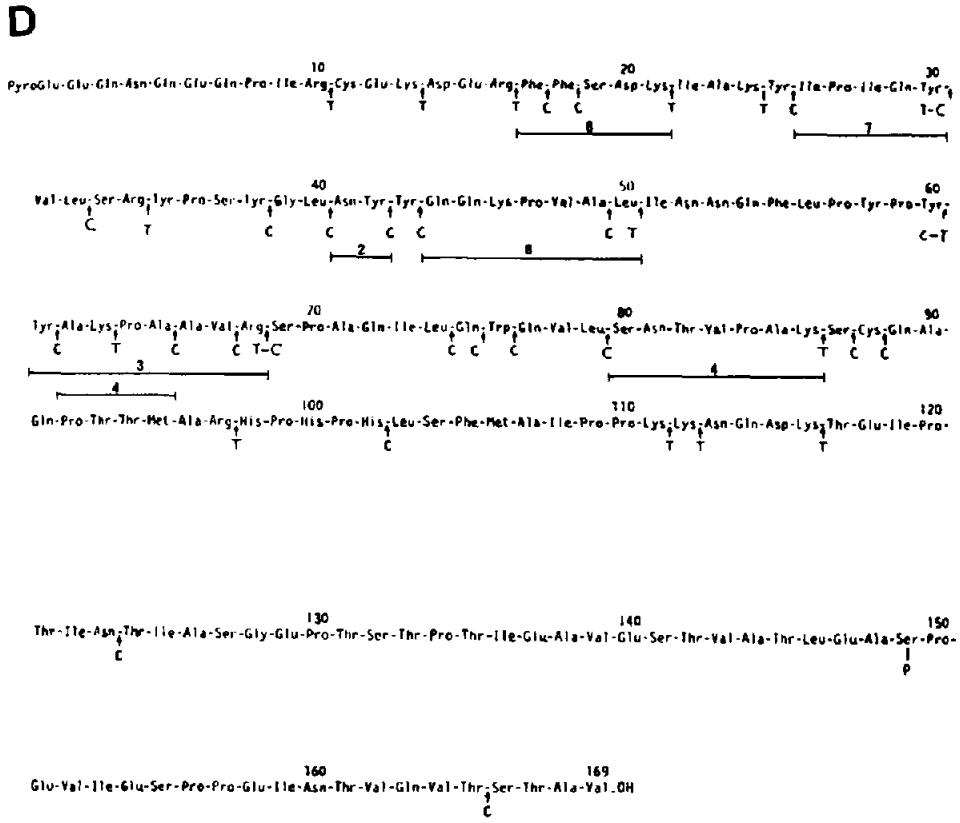
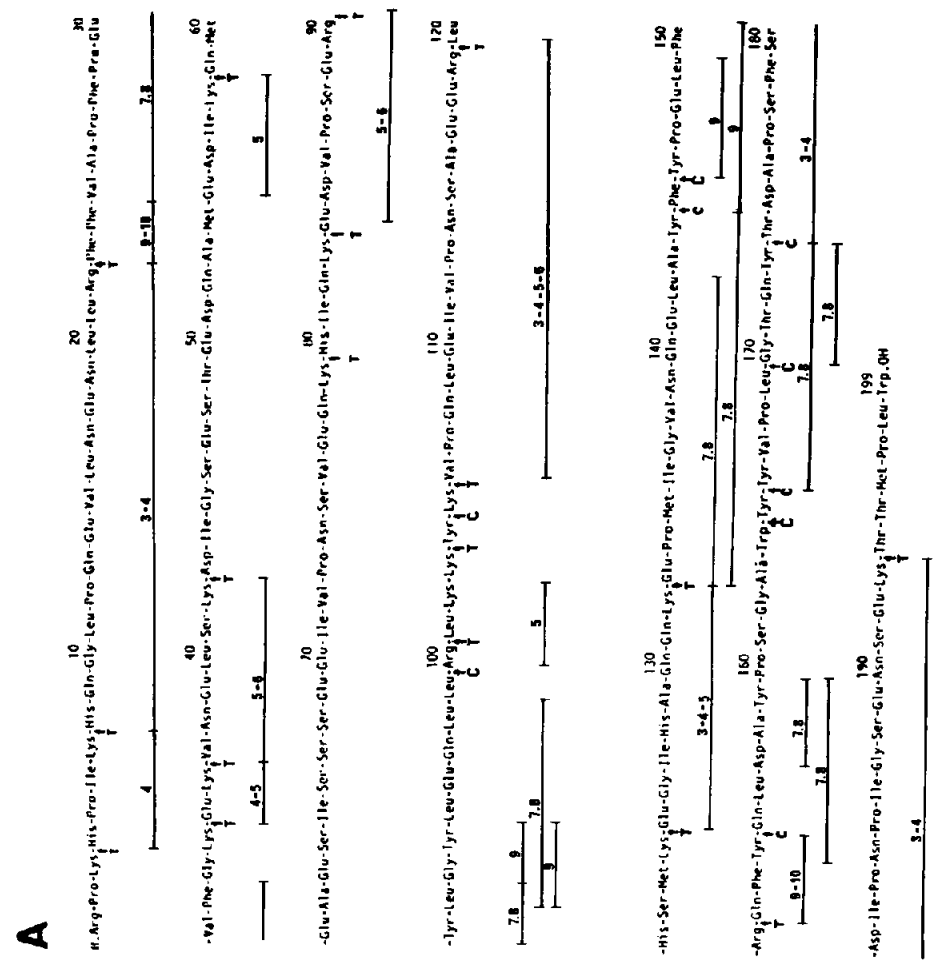


Fig. 5. (A) Underlined α_{s1}-casein peptides identified from a casein hydrolysate phosphorylated (CHPS). (B) Underlined α_{s2}-casein peptides identified from a casein hydrolysate phosphorylated (CHPS). (C) Underlined β-casein peptides identified from a casein hydrolysate phosphorylated (CHPS). (D) Underlined κ-casein peptides identified from a casein hydrolysate phosphorylated (CHPS). C and T: chymotrypsin and trypsin identified cleavage sites.


A
10
H.Arg-Pro-Lys-His-Pro-Ile-Lys-His-Gln-Gly-Leu-Pro-Gln-Glu-Val-Leu-Asn-Glu-Asn-Leu-Leu-Arg-Phe-Phe-Val-Ala-Pro-Phe-Pro-Glu
20
30
4
3-4
8-10
7,8
40
50
60
-Val-Phe-Gly-Lys-Glu-Lys-Val-Asn-Glu-Leu-Ser-Lys-Asp-Ile-Gly-Ser-Glu-Ser-Thr-Glu-Asp-Gln-Ala-Met-Glu-Asp-Ile-Lys-Gln-Met
4-5
5-6
5
70
80
90
-Glu-Ala-Glu-Ser-Ile-Ser-Ser-Ser-Glu-Glu-Ile-Val-Pro-Asn-Ser-Val-Glu-Gln-Lys-His-Ile-Gln-Lys-Glu-Asp-Val-Pro-Ser-Glu-Arg
5-6
100
110
120
-Tyr-Leu-Gly-Tyr-Leu-Glu-Gln-Leu-Leu-Arg-Leu-Lys-Lys-Tyr-Lys-Val-Pro-Gln-Leu-Glu-Ile-Val-Pro-Asn-Ser-Ala-Glu-Glu-Arg-Leu
7,8
9
7,8
9
5
3-4-5-6
130
140
150
-His-Ser-Met-Lys-Glu-Gly-Ile-His-Ala-Gln-Gln-Lys-Glu-Pro-Met-Ile-Gly-Val-Asn-Gln-Glu-Leu-Ala-Tyr-Phe-Tyr-Pro-Glu-Leu-Phe
3-4-5
7,8
9
9
160
170
180
-Arg-Gln-Phe-Tyr-Gln-Leu-Asp-Ala-Tyr-Pro-Ser-Gly-Ala-Trp-Tyr-Tyr-Val-Pro-Leu-Gly-Thr-Gln-Tyr-Thr-Asp-Ala-Pro-Ser-Phe-Ser
8-10
7,8
7,8
7,8
7,8
3-4
190
199
-Asp-Ile-Pro-Asn-Pro-Ile-Gly-Ser-Glu-Asn-Ser-Glu-Lys-Thr-Thr-Met-Pro-Leu-Trp.OH
3-4

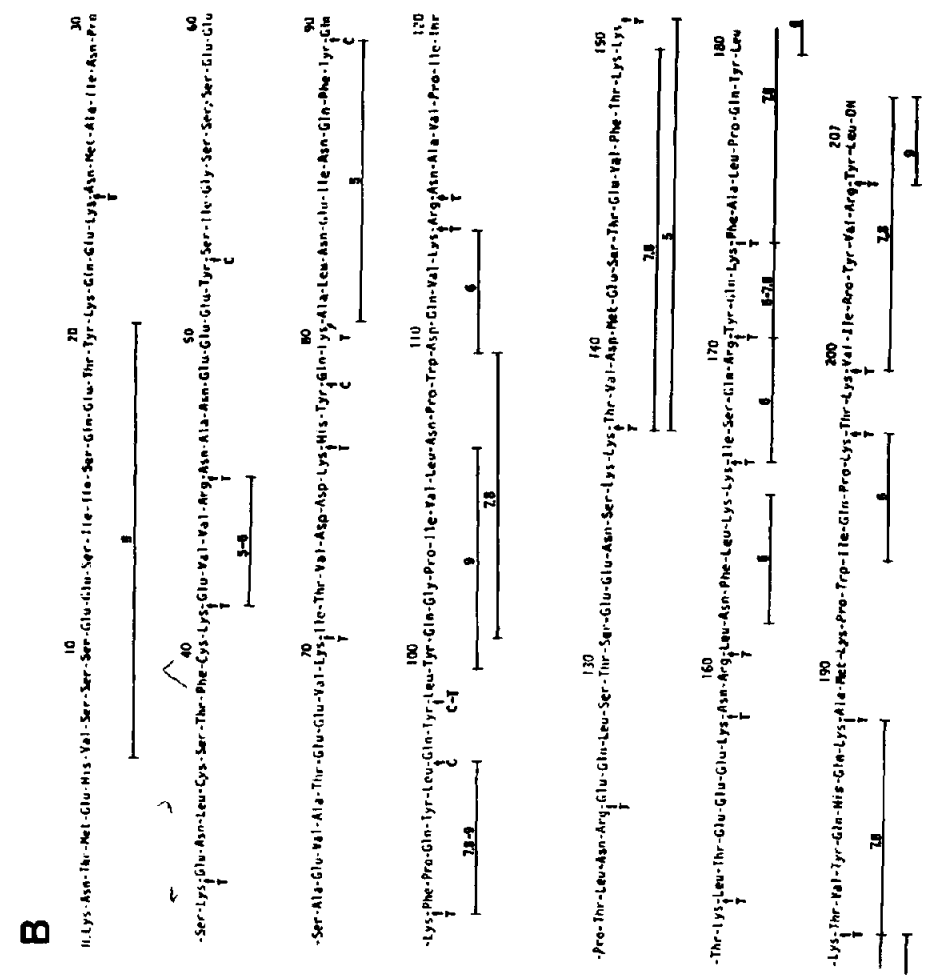


Fig. 6.

(*Continued on p. 318*)

C

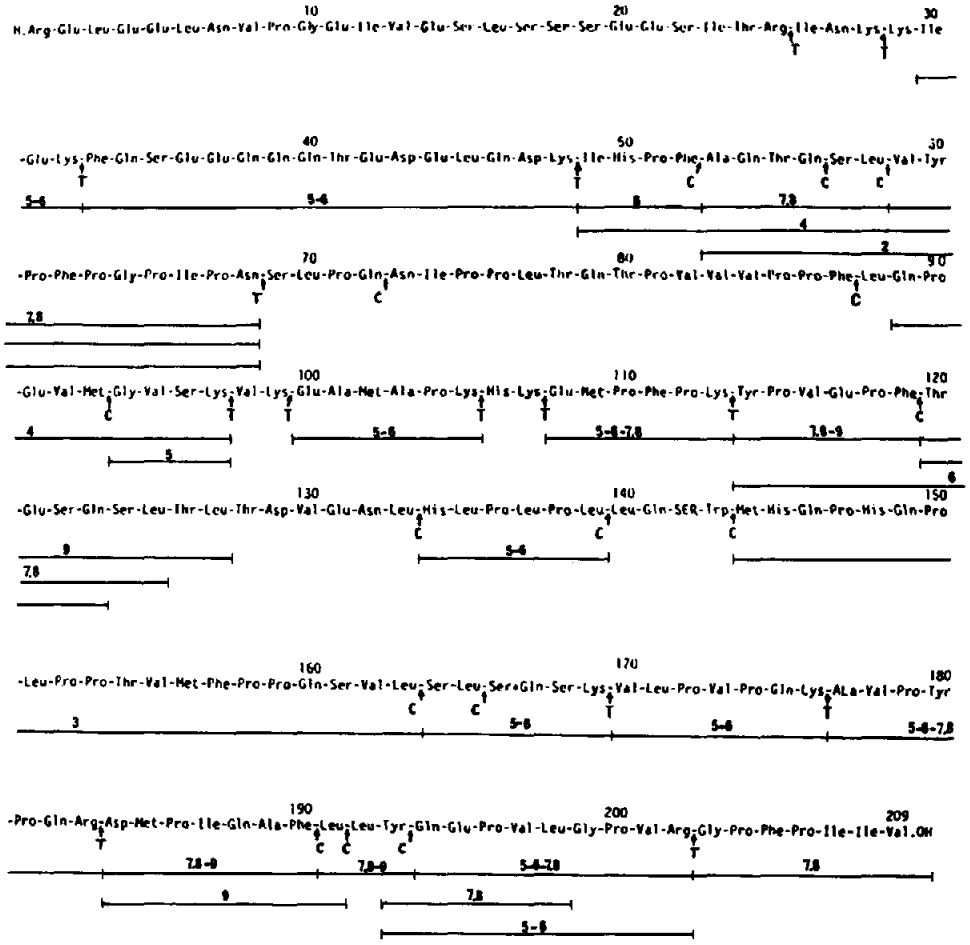

10
20
30
H.Arg-Glu-Leu-Glu-Glu-Leu-Asn-Val-Pro-Gly-Glu-Ile-Val-Glu-Ser-Leu-Ser-Ser-Ser-Glu-Glu-Ser-Ile-Thr-Arg-Ile-Asn-Lys-Lys-Ile
40
50
60
-Glu-Lys-Phe-Gln-Ser-Glu-Glu-Gln-Gln-Thr-Glu-Asp-Glu-Leu-Gln-Asp-Lys-Ile-His-Pro-Phe-Ala-Gln-Thr-Gln-Ser-Leu-Val-Tyr
5-6
5-6
8
7,8
4
2
70
80
90
-Pro-Phe-Pro-Gly-Pro-Ile-Pro-Asn-Ser-Leu-Pro-Gln-Asn-Ile-Pro-Pro-Leu-Thr-Gln-Thr-Pro-Val-Val-Val-Pro-Pro-Phe-Leu-Gln-Pro
7,8
100
110
120
-Glu-Val-Met-Gly-Val-Ser-Lys-Val-Lys-Glu-Ala-Met-Ala-Pro-Lys-His-Lys-Glu-Met-Pro-Phe-Pro-Lys-Tyr-Pro-Val-Glu-Pro-Phe-Thr
4
5-6
5-6-7,8
7,8-9
5
6
130
140
150
-Glu-Ser-Gln-Ser-Leu-Thr-Leu-Thr-Asp-Val-Glu-Asn-Leu-His-Leu-Pro-Leu-Pro-Leu-Leu-Gln-SER-Trp-Met-His-Gln-Pro-His-Gln-Pro
9
5-6
7,8
160
170
180
-Leu-Pro-Pro-Thr-Val-Met-Phe-Pro-Pro-Gln-Ser-Val-Leu-Ser-Leu-SerGln-Ser-Lys-Val-Leu-Pro-Val-Pro-Gln-Lys-ALa-Val-Pro-Tyr
3
5-6
5-6
5-6-7,8
190
200
209
-Pro-Gln-Arg-Asp-Met-Pro-Ile-Gln-Ala-Phe-Leu-Leu-Tyr-Gln-Glu-Pro-Val-Leu-Gly-Pro-Val-Arg-Gly-Pro-Phe-Pro-Ile-Ile-Val.OH
7,8-9
7,8-9
5-6-7,8
7,8
9
7,8
5-6

Fig. 6. (A) Underlined α_{s1}-casein peptides identified from a casein hydrolysate dephosphorylated (CHDS). (B) Underlined α_{s2}-casein peptides identified from a casein hydrolysate dephosphorylated (CHDS). (C) Underlined β-casein peptides identified from a casein hydrolysate dephosphorylated (CHDS). (D) Underlined κ-casein peptides identified from a casein hydrolysate dephosphorylated (CHDS). C and T: chymotrypsin and trypsin identified cleavage sites.

the reversed-phase column seems to be controlled not only by its hydrophobicity but also, as suggested by Juhl and Soderling [31], by its molecular weight.

The peptide, β: 203–209, isolated from CHDS and CHPS, was similar to the peptide found in bitter cheese. Pelissier *et al.* [32] have reported a number of bitter peptides originating from α_{s1}- and β-casein hydrolysates. However, we did not identify many of these bitter peptides in CHDS and CHPS, probably because of the conditions of the proteolysis.

Peptides such as β: 49–68 and β: 59 68 isolated from CHDS and CHPS may possess an immunostimulant property, since Maubois and Léonil [8] found that the peptide β: 63 68 was an immunomodulator. Other peptides have been associated with various biological functions [8] such as bioavailability of oligo-elements (β: 1–25); opioids (β: 60–66, β-casomorphin 7; α_{s1}: 90–96, α-casein exorphin), antithrombosis (κ: 106 116; κ: 106–112; κ: 113–116) and antihypertension (β: 177–183 or CEI B_7; α_{s1}: 23–34 or CEI_{12}). Among the peptides known to inhibit the angiotensin-converting enzyme, ACE (E.C. 3.14.15.1), CEI B_7 was found in both casein digests whereas CEI_{12} was identified only in CHPS. Further, peptide α_{s1}: 106–119 isolated from CHPS has previously been found to act as a mineral carrier [33] and peptide β: 191–193 from CHPS and CHDS to exert a stimulating function on the immune system [34].

Figs. 5 and 6 show that most of the peptides in the casein were identified. However, there are some missing links in the sequences. It is possible that some of the peptides produced in the enzymatic–ultrafiltration process may have been lost in the ultrafiltration step owing to some steric effects or electrostatic interactions [35]. In addition, lack of material, significant loss of polypeptides at low concentrations and adsorption on plastic surfaces or glassware may be responsible for the missing links in the sequences. Further, irreversible bonding of peptides at low concentrations can occur on the stationary phase, as reported for phosphopeptides on a C_{18} column [36].

CONCLUSION

A combination of size-exclusion and reversed-phase HPLC methodologies was efficient for the separation of protein hydrolysates and identification by Pico-Tag amino acid analysis. These methods also led to the isolation of some biologically active casein fragments. Work is in progress to evaluate the accuracy of HPSEC for the separation of peptides from protein hydrolysates according to their molecular weight.

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