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Pancreatic hydrolysis of bovine casein: Identification and release kinetics of phosphopeptides

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Abstract

Bovine casein was digested with pancreatin at pH 8.0 in a batched stirred tank reactor. In total, 52 casein phosphopeptides (CPPs) in the time-course samples were separated and identified using liquid chromatography-tandem mass spectrometry by means of neutral loss scanning and database searching. Of these peptides, 23, 7, 8, 10 and 4 are singly, doubly, triply, quadruply and quintuply phosphorylated, respectively. Furthermore, 14 peptides contained the cluster sequence, S(P)S(P)S(P)EE, providing the mineral binding sites; these peptides were all formed after 10 min of hydrolysis, but none of them survived after 6 h of pancreatic digestion. A study of the release kinetics of CPPs allowed determination of the degrees of hydrolysis for the preparation of target peptides with high yields. The discrimination of pancreatic attack on different phosphorylated regions of bovine casein was also analyzed in terms of the release of CPPs.

Keywords: Casein phosphopeptides; Pancreatin; Tandem mass spectrometry; Neutral loss scanning; Release kinetics

1. Introduction

Caseins, which account for approximately 80% of the total proteins in mammalian milk, exist in a micelle form aggregated by four phosphorylated proteins, namely α_{S1}-, α_{S2}-, β- and κ-caseins (α_{S1}-, α_{S2}-, β- and κ-CN). After the milk is fed to the suckling offspring, the casein micelles serve as the carrier of calcium phosphate, providing the newborn with minerals for their bone and teeth formation (Horne, 2002). Although caseins will undergo gastrointestinal digestion *in vivo*, their mineral binding ability (Philippe, Le Graet, & Gaucheron, 2005) can be retained and even strengthened by their hydrolysates, i.e. casein phosphopeptides (CPPs) (Meisel & FitzGerald, 2003). These peptides have been proven to play an essential role in the nutritional system, with many biological functions, such as antioxidant (Kitts, 2005), anticariogenic (Andrews et al., 2006), immunomodulating (Pizzano et al., 2000), and binding of many important minerals, including calcium and iron (Meisel & FitzGerald, 2003).

It is now accepted that the CPPs are latent in an inactive state within the amino acid sequence of caseins, and can be liberated by the proteolytic enzymes during food processing or gastrointestinal digestion. To date, the actions of trypsin (Wei, Zhimin, & Deqing, 2003), of chymotrypsin (McDonagh & FitzGerald, 1998), of plamin (Andrews et al., 2006), of a *Lactobacillus* proteinase (Corsetti, Massitti, Minervini, Fox, & Gobbetti, 2003), of pancreatin (Adamson & Reynolds), and of simulated gastrointestinal proteases (Miquel et al., 2006) on bovine caseins, Na-caseinates or milk have been typically studied for the CPPs production. However, most of these studies were concentrated on the identification of phosphopeptides in the hydrolysates after extensive digestion, but less on their release behaviours. In order to prepare the CPPs with high yield *in vitro* or to investigate the biological phenomenon after casein feeding *in vivo*, it is necessary to identify the peptide components and to determine their contents over the entire hydrolysis process. Therefore, insights into how this process proceeds and how it can be manipulated will be important.

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In recent years, the identification of phosphorylated peptides has been most robustly accomplished by the use of tandem mass spectrometry (MS/MS), with which the phosphopeptides are fragmented to determine their sequence and phosphorylated sites (Mann et al., 2002). In addition, fragmentation of phosphopeptides by MS/MS commonly resulted in several dominant peaks, corresponding to neutral loss of phosphoric acids, which has provided a key approach, namely neutral loss scanning, to increase the efficiency of phosphopeptide identification (Hoffert, Pisitkun, Wang, Shen, & Knepper, 2006).

The objective of this study, therefore, was to (1) identify the CPPs, in an exhaustive manner, using LC-ESI-MS/MS by means of neutral loss scanning and database searching, (2) follow the time-dependant release of the CPPs during pancreatic hydrolysis of bovine casein, and (3) analyze the cleavage behaviour of pancreatin on the phosphorylated regions of bovine casein in terms of the release of CPPs.

2. Materials and methods

2.1. Materials

Bovine casein (Product Code C7078), of technical grade, and porcine pancreatin (P7545) were purchased from Sigma Co. (USA). Acetonitrile (ACN) and trifluoroacetic acid (TFA), of HPLC grade, were purchased from Merck Co. (Darmstadt, Germany) and Dikma Co. (Beijing, China), respectively. Ultrapure water was obtained from an ELGA water purification unit (ELGA Ltd., Bucks, England). All other common reagents and solvents were of analytical grade from commercial sources.

2.2. Pancreatic hydrolysis of bovine casein

Bovine casein at 10 mg ml^{-1} was digested with pancreatin (0.1 mg ml^{-1}) at 37 °C in a batch stirred tank reactor. The pH was kept at a stable value of 8.0 by adding 0.1 M NaOH solution, by a pH-stat method. During the reaction, aliquots of bovine casein and pancreatin mixture were taken out at various times. Each aliquot was heated in boiling water for 10 min to inactivate the pancreatin and stop the reaction. The samples were lyophilized, and then stored at −20 °C.

2.3. Determination of the degree of hydrolysis

The degree of hydrolysis (DH) is defined as the percentage of peptide bond cleaved during the enzymatic reaction. The DH value can be estimated by the following equation (Bressollier, Petit, & Julien, 1988):

$$\mathrm{DH}(\%) = \frac{h \times 100}{h_{\mathrm{tot}}} = \frac{BN_{\mathrm{B}}}{\alpha h_{\mathrm{tot}} MP} \times 100$$

where h is the number of peptide bonds broken, called the hydrolysis equivalence and expressed as the equivalents per kg protein; h_{tot} the total number of peptide bonds in the bovine casein protein substrate; B the base consumption in ml; N_{B} is the normality of the base; α the average degree of dissociation of α-NH_2; and MP is the mass of protein in grammes.

2.4. RPLC-ESI-MS and tandem MS

All samples were analyzed by an on-line liquid chromatography-tandem mass spectrometry (LC-MS/MS) setup, consisting of a Surveyor LC pump and a Surveyor auto sampler fitted to a Surveyor PDA detector and a LCQ ion trap mass spectrometer (Thermo Finnigan, San Jose, CA).

For analytical runs, 10 μl of the digestion mixture was loaded onto a 4.5 × 250 mm column (5 μm, 300 Å, 214TP54 C4, Vydac). The peptides were eluted by buffer A (water containing 0.1% TFA and 2% ACN) and buffer B (ACN containing 0.085% TFA and 5% water) at a flow rate of 1 ml min^{-1} over the column. The programme started with isocratic elution 100% A for 5 min, followed by linear gradient elution 100–50% A for 50 min, and then maintaining of 50% A for 10 min.

The MS and tandem MS experiments were performed with an electrospray interface operated in the positive ion mode and in a data-dependent scan mode to automatically switch between MS and MS/MS acquisition, controlled by the Xcalibur software. Full scans were performed between m/z 300 and 2000. A full scan MS spectrum was acquired, followed by tandem mass spectra using collision-induced dissociation (CID) of the three most intense precursor ions present in the MS scan. Electrospray conditions were as follows: capillary temperature, 300 °C; ESI voltage, +5.0 kV; capillary voltage, 2.1 kV.

2.5. Database analysis

All the MS/MS spectra were initially analyzed using the SALSA algorithm (Thermo Finnigan, Bioworks 3.1 version), a tool for identifying MS/MS spectra by user-defined parameters (Liebler, Hansen, Davey, Tiscareno, & Mason, 2002). Subsequently, they were searched against a bovine database for peptide sequence and identification of site(s) of phosphorylation by the SEQUEST algorithm (Thermo Finnigan, Bioworks 3.1 version) (Eng, Mccormack, & Yates, 1994). Search parameters included a differential mass modification of +80 Da (presence or absence of phosphate) on serine and threonine due to their possible phosphorylation.

3. Results and discussion

3.1. Pancreatic hydrolysis of bovine casein

To follow the release kinetics of CPPs, bovine casein pancreatic hydrolysates were obtained at different degrees of hydrolysis (DH) (1.5%, 3%, 6%, 12%, 16%, 20%, 25%,

28% and 28.5%), corresponding to 2.5, 5, 10 and 30 min, 1, 2, 6, 12 and 24 h, respectively. RP-HPLC profiles of enzymatic digestion (Fig. 1) show that all the individual caseins were rapidly cleaved into different peptides during the course of hydrolysis. Furthermore, the components and contents of hydrophobic peptides with relatively higher retention times (45–55 min) continued to decrease after 5 min of hydrolysis, whereas those of hydrophilic peptides with low elution times (<30 min) increased as the reaction proceeded. Therefore, pancreatic hydrolysis of bovine casein for a short reaction time (~5 min) was proposed, to yield hydrolysates of a more hydrophobic nature, whereas an extensive reaction (>6 h) was suggested to prepare the hydrophilic digestions.

3.2. Identification of casein phosphopeptides by LC-MS/MS

After pancreatic digestion of bovine casein, the released peptides were separated by RP-HPLC and were subsequently introduced to the mass spectrometer. The SALSA algorithm was initially applied to search MS/MS spectra for fragment ions formed by neutral loss of phosphoric acid, namely 24.5, 32.7, 49 and 98 Da from $(M + 4H)^{4+}$, $(M + 3H)^{3+}$, $(M + 2H)^{2+}$ and $(M + H)^{+}$ ions, respectively. It is well known that phosphoserine and phosphothreonine commonly undergo a β-elimination reaction and lose phosphoric acid readily during the CID process in the ion-trap mass spectrometer (Mann et al., 2002). Therefore, appearance of significant neutral loss of phosphoric acid(s) in the MS/MS spectra strongly indicates that the peptide is a phosphopeptide containing at least one phosphoserine or phosphothreonine residue.

Fig. 2a displays the RPLC-TIC chromatogram of bovine casein pancreatic digestion obtained after 2 h of hydrolysis. The mass spectrum of one selected peak at the retention time of 31.84 min is shown in Fig. 2b. The precursor ion m/z 976.6 was a doubly charged ion, indicating that the relative molecular mass of the peptide was approximately 1951 Da. Its fragment ion spectrum in Fig. 2c showed a significant signal at $m/z = 927.5$, corresponding to neutral loss of 49 Da, and thus provided an immediate indication of a phosphoserine- or phosphothreonine-containing peptide. Database searching of this CID spectrum matched with α-CN f(104–119), a singly phosphorylated peptide with sequence YKVPQLEIVPNS* AEER (asterisk * denotes a phosphate group). In the MS/MS spectrum, with the exception of m/z 927.5, fragment ions at m/z 882.3 and 1070.6, corresponding to $y7$ and $b9$, respectively, were the most noticeable. This fragment phenomenon was agreement with the fact that N-terminal cleavage to Pro would readily produce relatively abundant $y$and b ions (Breci, Tabb, Yates, & Wysocki, 2003), and this increased the efficiency of the peptide's identification.

We next evaluated this procedure for the identification of multiply phosphorylated peptides. Fig. 3 shows the fragmentation profiles of the 2–4 charged precursor ions, attributed to four peptides that are doubly, triply, quadruply and quintuply phosphorylated, respectively. As can be seen, these phosphopeptides underwent multiply sequential losses of 49 Da, 32.7 Da or 24.5 Da, depending on their charge states, which indicated the universality of neutral loss of phosphoric acid during CID. Furthermore, the ions corresponding to the losses of H_3PO_4 were remarkably over-represented in the tandem mass spectra, and this effect would be more significant if more phosphoserine sites were in a single peptide. In addition, the charge states, molecular masses and number of phosphorylation sites of peptides could be determined on the basis of these cluster ions. As an example, the MS/MS spectrum of the quintuply phosphorylated peptide β-CN 1-52 (5P) exhibited a series of five peaks (Fig. 3d), which were separated by 24.5 Da, indicating that the peptide was quadruply charged during the MS scan. The molecular mass of peptide could thus be determined to be around 6511 Da ($1628.8 \times 4 - 4$), and the number of the consecutive 24.5 Da losses was observed to equal the number of phosphorylated residues. This phenomenon was also found in the case of other phosphopeptides, as shown in Fig. 3a–c.

In total, 52 CPPs were identified by neutral loss scanning and database searching during the course of pancreatic digestion, as shown in Table 1 and Fig. 1. Of these, 23 (44.2%) are singly phosphorylated, 7 (13.5%) are doubly phosphorylated, 8 (15.4%) are triply phosphorylated, 10 (19.2%) are quadruply phosphorylated, and 4 (7.7%) are quintuply phosphorylated. Moreover, 14 (27%) of these peptides contained the cluster sequence S(P)S(P)S(P)EE which is the binding site for di- and tri-valent minerals (Meisel & FitzGerald, 2003).

3.3. Characterization of casein phosphopeptides from pancreatic hydrolysates

Data in Table 1 represent a relatively large percentage of singly phosphorylated peptides in our experiment, in contrast to the case of only two identified monophosphopeptides after the simulated gastrointestinal digestion of casein (Miquel et al., 2006). It may be concluded that the selective precipitation of CPPs by Miquel et al. (2006) could be applied to obtain multiply phosphorylated peptides, but not to recover all the CPPs, especially the monophosphopeptides. Therefore, direct analysis of unseparated digestion by LC-MS/MS in our experiment may provide a more favourable approach to completely study the release kinetics of CPPs during enzymatic hydrolysis.

Fig. 4 displays the identified CPPs in the amino acid sequence of bovine casein over the entire process of hydrolysis. Among these CPPs, the identity of 4 di-phosphopeptides (peptides 1, 5, 7 and 8 in Table 1) and 4 tri-phosphopeptides (peptides 2, 3, 4 and 6 in Table 1) indicated that S_{41} in α_{S1}-CN was partially phosphorylated by Golgi apparatus casein kinase, G-CK (Lasa-Benito, Marin, Meggio, & Pinna, 1996). According to the results of Lasa-Benito et al. (1996), the potentiality for phosphorylation of the amino acid triplets by G-CK showed a downtrend order with

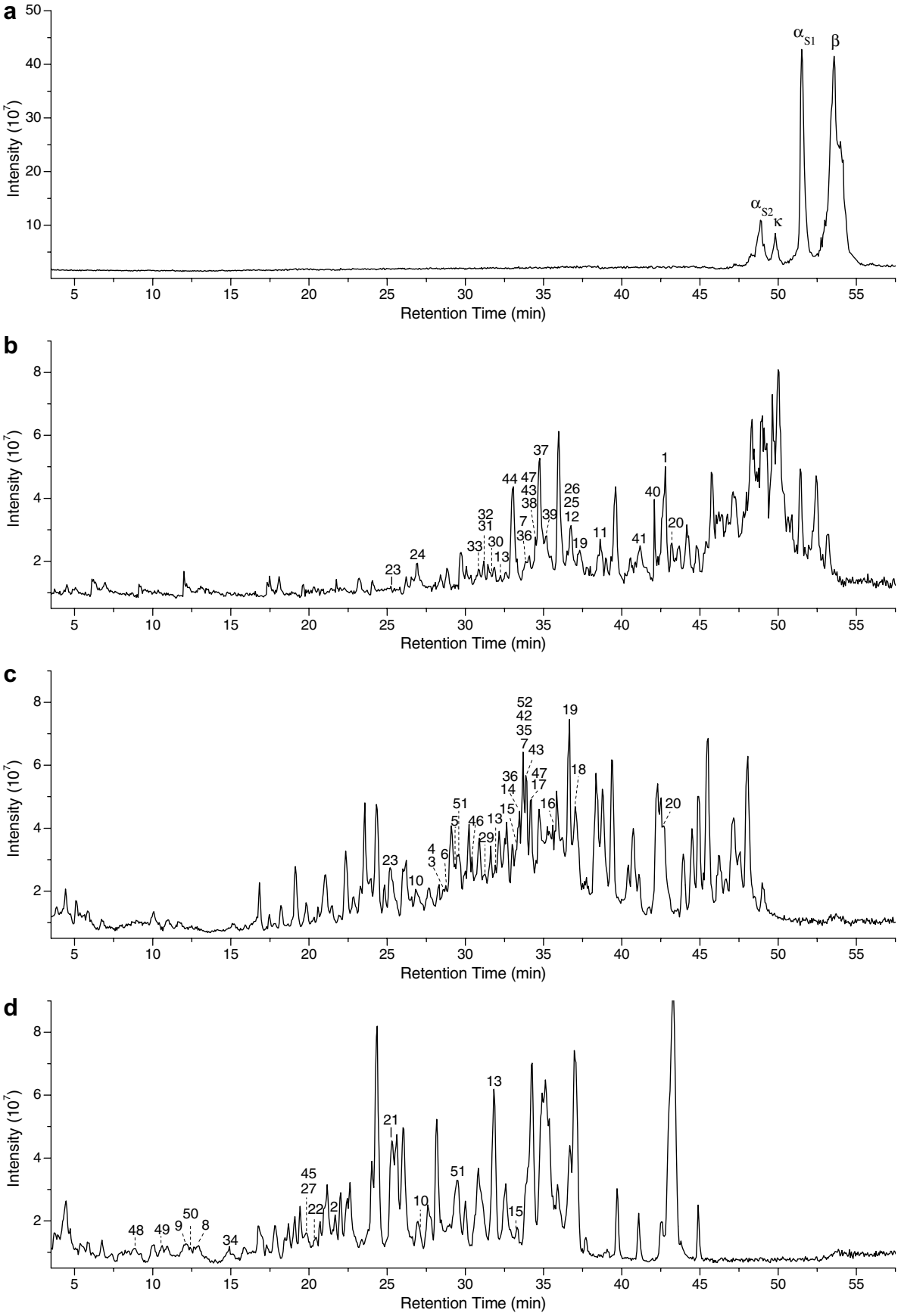

Fig. 1. RPLC-TIC chromatograms of bovine casein undergoing hydrolysis with pancreatin for 0 (a, control), 5 (b), 30 (c), 360 (d) min at pH 8.0 and 37 °C, using the ratio of enzyme to substrate of 1:100 (w/w). Mass spectrometry identification of the numbered peptides is presented in Table 1.

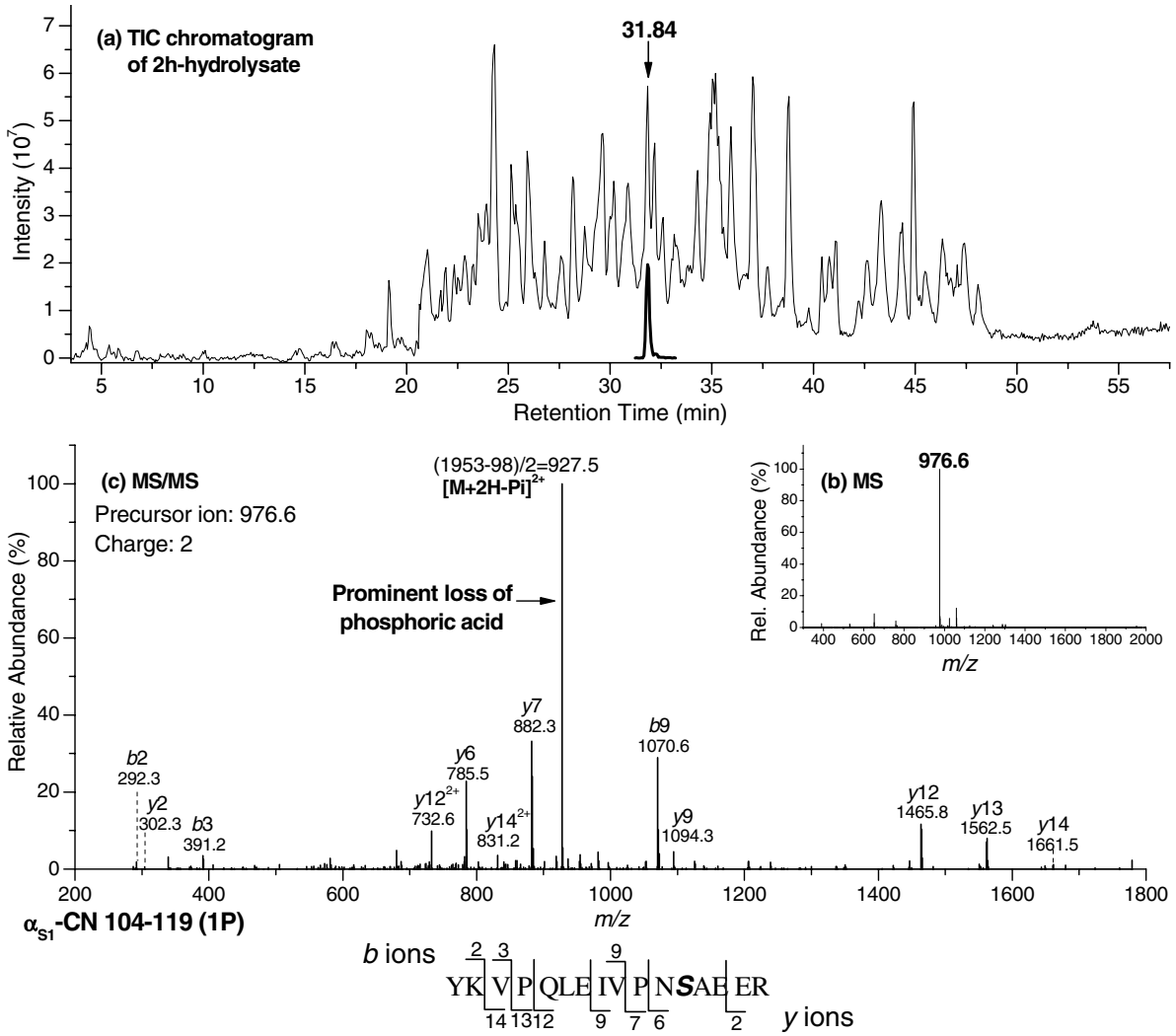

Fig. 2. (a) RPLC-TIC chromatogram of bovine casein pancreatic digestion after a 2 h hydrolysis. Shown in bold is the trace for the intensity of the selected ions (m/z 976.6 ± 0.5), i.e. extracted ion current; (b) Mass spectrum of the selected peak in (a); (c) Tandem mass spectrum of doubly charged ion m/z 976.6, showing a typical extensive neutral loss of phosphoric acid. Based on sequence interpretation and database searching, the tandem mass spectrum was matched to α_{S1}-CN 104–119 (1P). The sequence of the peptide is displayed with the fragment ions observed in the spectrum. The phosphorylated Ser residue is shown in bold and italic, i.e. ***S***. For clarity, only the *b* and the *y* fragments ions are labelled.

–S–X–E– > –T–X–E– > –S–X–D– > –T–X–D–. As a result, the lack of efficient phosphorylation of S_{41} might be due to the presence of D_{43} residue that would strongly reduce G-CK activity. A similar case was observed for the S_{31}in α_{S2}-CN, since experimental masses 5340.0 Da ($1336.0 \times 4 - 4$) and 5420.1 Da ($1807.7 \times 3 - 3$) were respectively attributed to α_{S2}-CN 1-44 (4P) and α_{S2}-CN 1-44 (5P) (peptides 25 and 26 in Table 1) in the early stage of hydrolysis. Therefore, the S_{31} residue was also partially phosphorylated, despite the presence of a favourable residue E_{33} (Lasa-Benito et al., 1996). This phenomenon was also found in the case of bovine α_{S2}-CN hydrolyzed with trypsin (Tauzin, Miclo, Roth, Mollé, & Gaillard, 2003).

As shown in Table 1 and Fig. 4, more than 50% of CPPs in our experiment were released due to C-terminal cleavages to R and K residues, which were consistent with the tryptic activity in pancreatin (Antal et al., 2001). Among the other cleavages, C-terminal to F and L residues resulted from the chymotryptic activity (Antal et al., 2001), and C-terminal to A and V residues was due to the elastase in pancreatin (Narayana & Anwar, 1969). The CPPs with their C-terminal residues of Q, M, S, T and P were also released after pancreatic digestion. This phenomenon was agreement with the results of Adamson and Reynolds (1995) that pancreatin exhibits broad specificity in cleaving peptide bonds on the C-terminus of R, K, Y, L, M, Q, S, T, E, V and P.

3.4. Time-dependent release of casein phosphopeptides

Table 1 also represents the formation process of the CPPs during pancreatic hydrolysis of bovine casein. As can be seen, the population of larger peptides at the early stage of hydrolysis (2.5 min) was much higher than that after 24 h. None of the phosphopeptides identified in the 2.5 min digestion had a molecular mass <3 kDa, whereas only one peptide (α_{S1}-CN 35–52 (3P)), with a molecular

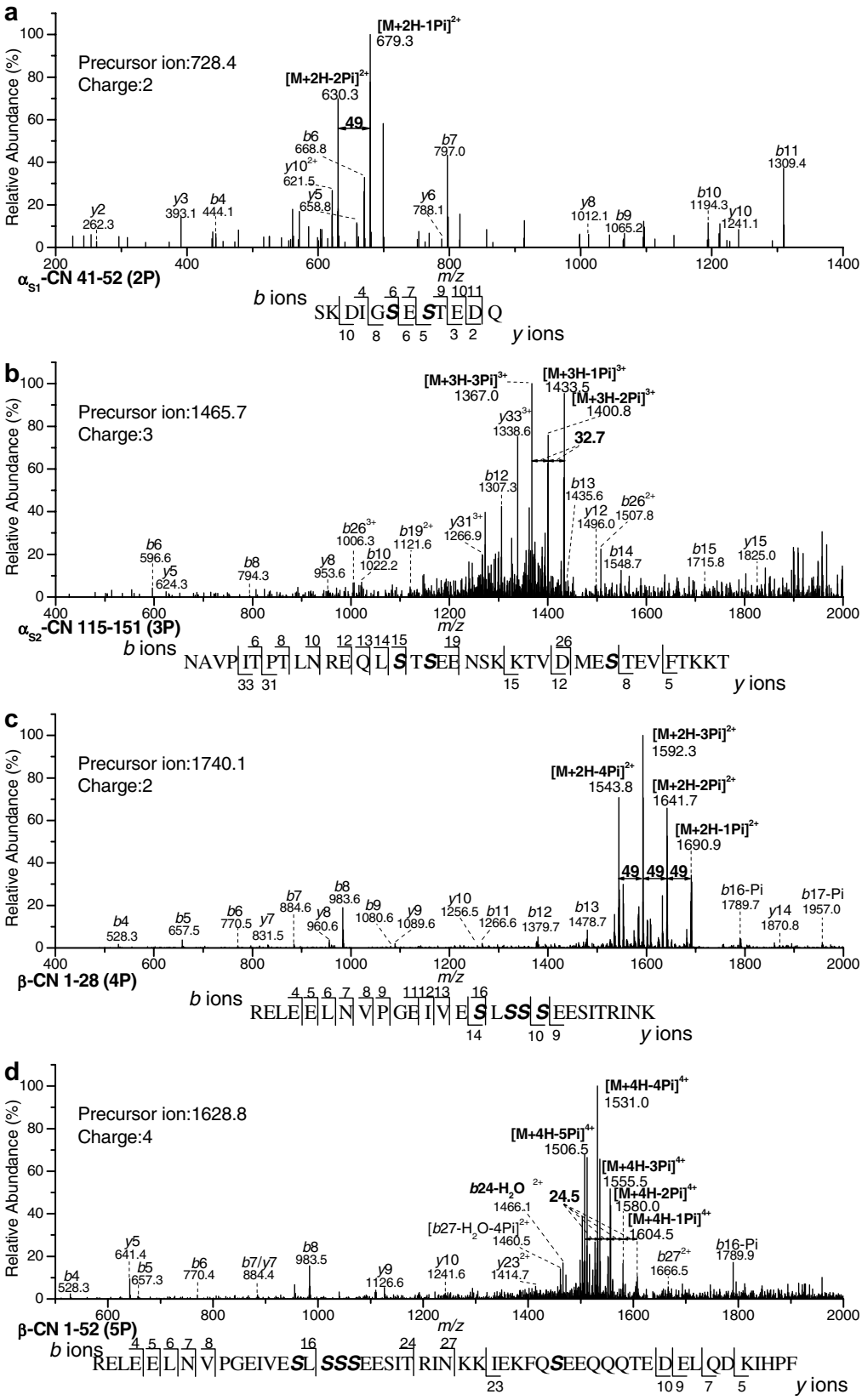

Fig. 3. Tandem mass spectra of ions (a) m/z 728.4, z 2; (b) m/z 1465.7, z 3; (c) m/z 1740.1, z 2; and (d) m/z 1628.8, z 4. Multiply phosphorylated peptides underwent multiple sequential losses of 49, 32.7 and 24.5 Da while the charges of precursor ions were 2, 3 and 4, respectively. Based on sequence interpretation and database searching, the tandem mass spectra were matched to (a) α_{S1}-CN 41–52 (2P); (b) α_{S2}-CN 115–151 (3P); (c) β-CN 1–28 (4P) and (d) β-CN 1–52 (5P), respectively.

Table 1
Identification of CPPs released from pancreatic hydrolysis of bovine casein

No[a]	Casein fragment	RT[b] (min)	Sequence identified[c]	Mass[d] (m/z)	Ion selected for MS^2 (Charge)	Peptide formation[e]
1	α_{S1} 23–60(2P)	42.51	R⟨FFVAPFPEVFGKEKVNELSKDIGS*ES*TEDQAMEDIKQM⟩E	4480.0	1495.0 (3)	1,2,3
2	α_{S1} 35–52(3P)	21.62	K⟨EKVNELS*KDIGS*ES*TEDQ⟩A	2247.8	1124.1 (2)	5,6,7,8,9
3	α_{S1} 35–58(3P)	28.52	K⟨EKVNELS*KDIGS*ES*TEDQAMEDIK⟩Q	2935.2	979.8 (3)	4,5,6
4	α_{S1} 35–59(3P)	28.52	K⟨EKVNELS*KDIGS*ES*TEDQAMEDIKQ⟩M	3063.2	1533.4 (2)	4,5,6
5	α_{S1} 37–58(2P)	29.31	K⟨VNELSKDIGS*ES*TEDQAMEDIK⟩Q	2598.1	1300.8 (2)	4,5
6	α_{S1} 37–59(3P)	28.62	K⟨VNELS*KDIGS*ES*TEDQAMEDIKQ⟩M	2806.1	1403.9 (2)	3,4,5
7	α_{S1} 37–60(2P)	33.69	K⟨VNELSKDIGS*ES*TEDQAMEDIKQM⟩E	2857.2	953.5 (3)	2,3,4,5
8	α_{S1} 41–52(2P)	13.07	L⟨SKDIGS*ES*TEDQ⟩A	1455.5	728.5 (2)	7,8,9
9	α_{S1} 43–52(2P)	12.00	K⟨DIGS*ES*TEDQ⟩A	1240.4	1241.3 (1)	6,7,8,9
10	α_{S1} 43–58(2P)	26.83	K⟨DIGS*ES*TEDQAMEDIK⟩Q	1927.7	965.6 (2)	4,5,6,7
11	α_{S1} 60–98(5P)	38.50	Q⟨MEAES*IS*S*S*EEIVPNS*VEQKHIQKEDVPSERYLGYLEQL⟩L	4891.0	1631.2 (3)	1,2,3
12	α_{S1} 101–143(1P)	36.11	R⟨LKKYKVPQLEIVPNS*AEERLHSMKEGIHAQQKEPMIGVNQELA⟩Y	4990.6	1665.2 (3)	2,3
13	α_{S1} 104–119(1P)	31.84	K⟨YKVPQLEIVPNS*AEER⟩L	1952.0	976.6 (2)	2,3,4,5,6,7,8
14	α_{S1} 104–121(1P)	33.30	K⟨YKVPQLEIVPNS*AEERLH⟩S	2202.1	735.2 (2)	4,5,6
15	α_{S1} 104–122(1P)	33.20	K⟨YKVPQLEIVPNS*AEERLHS⟩M	2289.1	764.1 (3)	3,4,5,6,7
16	α_{S1} 104–123(1P)	35.59	K⟨YKVPQLEIVPNS*AEERLHSM⟩K	2420.2	1211.0 (2)	3,4,5
17	α_{S1} 104–124(1P)	34.28	K⟨YKVPQLEIVPNS*AEERLHSMK⟩E	2548.3	850.7 (3)	3,4,5
18	α_{S1} 104–142(1P)	36.87	K⟨YKVPQLEIVPNS*AEERLHSMKEGIHAQQKEPMIGVNQEL⟩A	4550.3	1518.3 (3)	3,4,5
19	α_{S1} 104–143(1P)	36.64	K⟨YKVPQLEIVPNS*AEERLHSMKEGIHAQQKEPMIGVNQELA⟩Y	4621.3	1541.7 (3)	2,3,4,5
20	α_{S1} 104–149(1P)	42.79	K⟨YKVPQLEIVPNS*AEERLHSMKEGIHAQQKEPMIGVNQELAYFYPEL⟩F	5433.7	1812.6 (3)	1,2,3,4
21	α_{S1} 109–119(1P)	25.88	Q⟨LEIVPNS*AEER⟩L	1336.6	669.4 (2)	6,7,8,9
22	α_{S1} 110–119(1P)	20.31	L⟨EIVPNS*AEER⟩L	1223.5	612.4 (2)	7,8,9
23	α_{S2} 1–19(4P)	25.63	–⟨KNTMEHVS*S*S*EESIIS*QET⟩Y	2455.8	1229.5 (2)	2,3,4,5,6
24	α_{S2} 1–24(4P)	26.66	–⟨KNTMEHVS*S*S*EESIIS*QETYKQEK⟩N	3132.2	1567.3 (2)	1,2,3,4
25	α_{S2} 1–44(4P)	36.65	–⟨KNTMEHVS*S*S*EESIIS*QETYKQEKNMAINPSKENLCSTFCKEVV⟩R	5340.2	1336.0 (4)	1,2,3
26	α_{S2} 1–44(5P)	36.65	–⟨KNTMEHVS*S*S*EESIIS*QETYKQEKNMAINPS*KENLCSTFCKEVV⟩R	5420.2	1807.7 (3)	1,2,3
27	α_{S2} 25–34(1P)	19.78	K⟨NMAINPS*KEN⟩L	1197.5	599.6 (2)	7,8,9
28	α_{S2} 44–81(4P)	42.43	V⟨VRNANEEEYSIGS*S*S*EES*AEVATEEVKITVDDKHYQKA⟩L	4561.9	1521.9 (3)	1,2,3
29	α_{S2} 46–80(4P)	31.28	R⟨NANEEEYSIGS*S*S*EES*AEVATEEVKITVDDKHYQK⟩A	4235.7	1413.3 (3)	3,4
30	α_{S2} 115–149(3P)	31.55	R⟨NAVPITPTLNREQLS*TS*EENSKKTVDMES*TEVFTK⟩K	4163.9	1389.1 (3)	1,2,3,4
31	α_{S2} 115–150(3P)	31.19	R⟨NAVPITPTLNREQLS*TS*EENSKKTVDMES*TEVFTKK⟩T	4292.0	1432.1 (3)	1,2,3,4
32	α_{S2} 115–151(3P)	31.19	R⟨NAVPITPTLNREQLS*TS*EENSKKTVDMES*TEVFTKKT⟩K	4393.0	1465.7 (3)	1,2,3
33	α_{S2} 115–152(3P)	30.43	R⟨NAVPITPTLNREQLS*TS*EENSKKTVDMES*TEVFTKKTK⟩L	4521.1	1508.1 (3)	1,2,3
34	α_{S2} 126–136(2P)	14.43	R⟨EQLS*TS*EENSK⟩K	1411.5	706.3 (2)	6,7,8,9
35	β 1–24(4P)	33.73	–⟨RELEELNVPGEIVES*LS*S*S*EESIT⟩R	2966.2	1483.8 (2)	3,4,5
36	β 1–25(4P)	33.27	–⟨RELEELNVPGEIVES*LS*S*S*EESITR⟩I	3122.3	1562.0 (2)	1,2,3,4,5
37	β 1–28(4P)	34.30	–⟨RELEELNVPGEIVES*LS*S*S*EESITRINK⟩K	3477.5	1739.8 (2)	1,2,3,4
38	β 1–29(4P)	34.38	–⟨RELEELNVPGEIVES*LS*S*S*EESITRINKK⟩I	3605.6	1203.4 (3)	1,2,3
39	β 1–32(4P)	35.16	–⟨RELEELNVPGEIVES*LS*S*S*EESITRINKKIEK⟩F	3975.8	1326.4 (3)	1,2,3,4
40	β 1–52(5P)	41.95	–⟨RELEELNVPGEIVES*LS*S*S*EESITRINKKIEKFQS*EEQQQTEDELQDKIHPF⟩A	6432.9	1629.7 (4)	1,2,3
41	β 1–56(5P)	40.77	–⟨RELEELNVPGEIVES*LS*S*S*EESITRINKKIEKFQS*EEQQQTEDELQDKIHPFAQTQ⟩S	6959.1	1737.0 (4)	1,2
42	β 26–56(1P)	34.20	R⟨INKKIEKFQS*EEQQQTEDELQDKIHPFAQTQ⟩S	3837.8	1281.1 (3)	1,2,3,4,5
43	β 29–52(1P)	34.50	K⟨KIEKFQS*EEQQQTEDELQDKIHPF⟩A	3054.4	1528.6 (2)	1,2,3,4
44	β 29–56(1P)	32.73	K⟨KIEKFQS*EEQQQTEDELQDKIHPFAQTQ⟩S	3482.6	1162.5 (3)	1,2,3,4,5,6
45	β 30–40(1P)	19.81	K⟨IEKFQS*EEQQQ⟩T	1473.6	737.3 (2)	6,7

46	β 30–51(1P)	30.51	K⟨IEKFQS*EEQQQTEDELQDKIHP⟩F	2779.3	927.6 (3)	3,4,5,6
47	β 30–52(1P)	34.28	K⟨IEKFQS*EEQQQTEDELQDKIHPF⟩A	2926.3	976.5 (3)	2,3,4,5
48	β 33–38(1P)	8.88	K⟨FQS*EEQ⟩Q	847.3	847.3 (1)	7,8,9
49	β 33–39(1P)	10.61	K⟨FQS*EEQQ⟩Q	975.4	975.5 (1)	7,8,9
50	β 33–40(1P)	12.47	K⟨FQS*EEQQQ⟩T	1103.4	1103.8 (1)	6,7
51	β 33–51(1P)	29.52	K⟨FQS*EEQQQTEDELQDKIHP⟩F	2409.0	1205.8 (2)	3,4,5,6,7
52	β 33–52(1P)	33.73	K⟨FQS*EEQQQTEDELQDKIHPF⟩A	2556.1	853.6 (3)	3,4,5,6

[a] Numbers refer to HPLC peaks reported in Fig. 1.
[b] Average retention time of each peptide during RPLC.
[c] The sequences of peptides were identified in the bracket with adjacent amino acids. Phosphoserine in the peptides is indicated by an asterisk, i.e. S*.
[d] Calculated monoisotopic mass.
[e] 1, 2, 3, 4, 5, 6, 7, 8 and 9 mean, respectively, the digestion of bovine casein with pancreatin for 2.5, 5, 10, 30 min and 1, 2, 6, 12 and 24 h.

mass >2 kDa, survived after 24 h of hydrolysis. It may be concluded that the larger phosphopeptides released after a short time of hydrolysis were all digested further into smaller ones. As an example, the large peptide α_{S1}-CN 23–60 (2P) was quickly formed after 2.5 min hydrolysis, and then it was converted into several intermediate peptides, including α_{S1}-CN 37–60 (2P) and α_{S1}-CN 37–58 (2P). After a further hydrolysis, the latter peptides continued to be degraded slowly into smaller ones, namely α_{S1}-CN 41–52 (2P), α_{S1}-CN 43–52 (2P) and α_{S1}-CN 43-58 (2P).

The middle region (101-149) of α_{S1}-CN appeared to be an easy susceptible area for pancreatin (Fig. 4), since most of the peptides had been formed after 10 min of hydrolysis (Table 1). The scissile bond $K_{103} - Y_{104}$ in α_{S1}-CN was quite favourable for trypsin, involving the release of eight phosphopeptides (peptides No. 13–20 in Table 1). This may have resulted from the presence of favourable adjacent residues, i.e. Y_{104} and K_{105} in P'_1 and P'_2 positions, in which aliphatic, aromatic and basic residues are preferred for trypsin according to the results of Schellenberger, Turck, Hedstrom, and Rutter (1993).

To follow the release kinetics of CPPs, we are interested, not only in their identity, but also in their quantity. Unfortunately, due to the complexity of pancreatic hydrolysates, a lot of peaks in the RPLC-TIC chromatograms may include several peptides with similar hydrophobic natures during their separation. In addition, the peptides may not be equimolar, even if their intensities in mass spectrum are equal, because their ionization efficiencies are different. Fortunately, this efficiency for the same peptide is reproducible in each MS scan. Therefore, the intensity and area of the chromatographic peaks for each peptide (extracted ion current; Fig. 2a) can be determined separately, and also could be a good indicator of its relative amount (Steen & Mann, 2004).

Fig. 5 shows the time-dependent release of selected peptides containing the cluster sequence S(P)S(P)S(P)EE. It should be noted that these peptides were all formed after 10 min of hydrolysis, but none of them survived after a 6 h digestion. The pentaphosphorylated peptide, α_{S1}-CN 60–98 (5P), was formed at the early stage of hydrolysis; it reached the maximum at a low DH of 3%, and disappeared rapidly at DH 12%. This CPP shares a common sequence, including S(P)S(P)S(P)EE in 66–70 positions, with the tryptic peptide α_{S1}-CN 59–79 (5P), which has a calcium binding constant of 0.51–0.58 mM^{-1} at pH 7.0 (Meisel & Olieman, 1998) and can promote calcium uptake in human intestinal tumor cells (Ferraretto, Gravaghi, Fiorilli, & Tettamanti, 2003). The CPPs might be expected to have similar mineral binding activities, if they share common sequences and the same phosphorylation sites (Miquel et al., 2006).

N-terminal digestion of α_{S2}-CN with pancreatin resulted in the release of four multiply phosphorylated peptides, including α_{S2}-CN 1–19 (4P), α_{S2}-CN 1–24 (4P), α_{S2}-CN 1–44 (4P) and α_{S2}-CN 1–44 (5P). The peptide α_{S2}-CN 1–19 (4P) was late detected at a DH of 3%, while the other

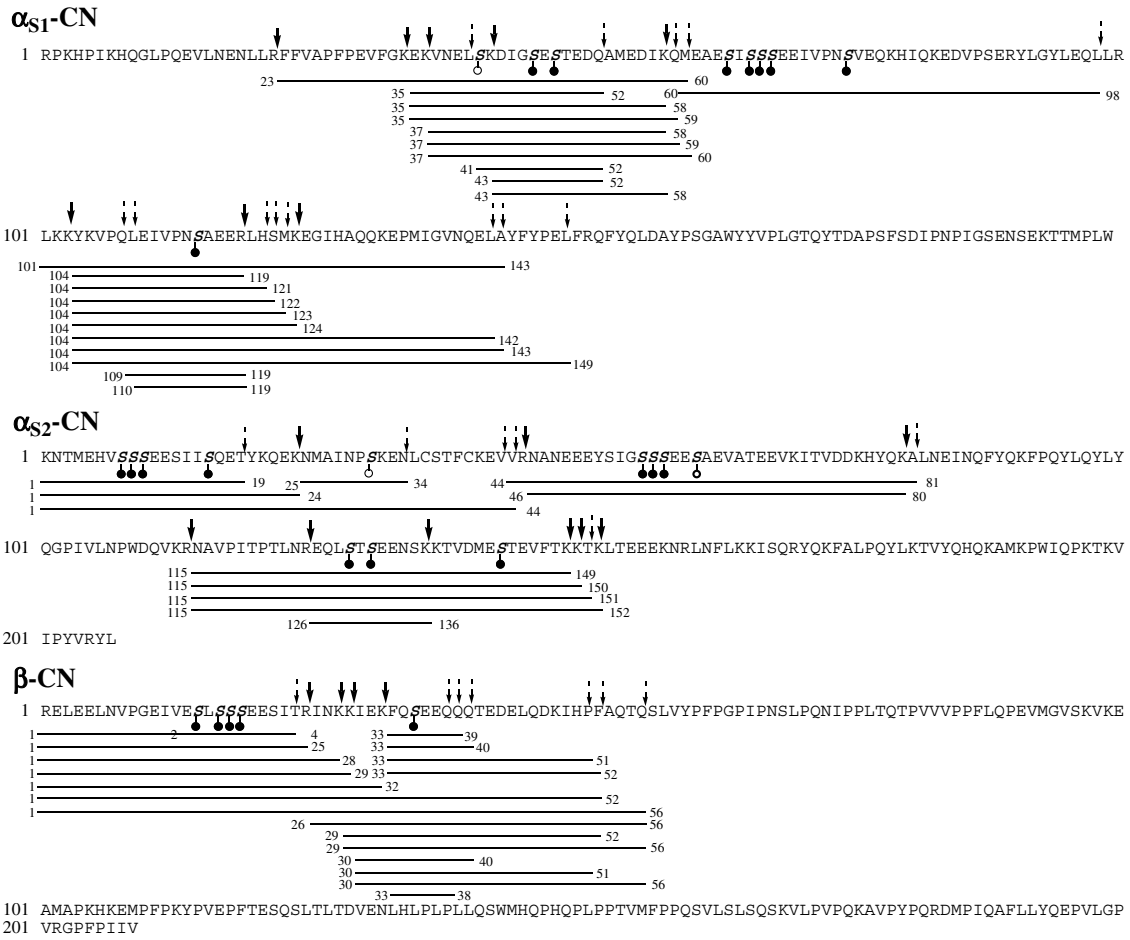

Fig. 4. Illustration of all unambiguously identified CPPs labelled with lines from α_{S1}-CN, α_{S2}-CN and β-CN. The phosphoserine residues were identified in this study (always and partially phosphorylated were shown with • and ○, respectively). Cleavage by trypsin and other proteinase were, respectively, displayed with solid and dashed arrows.

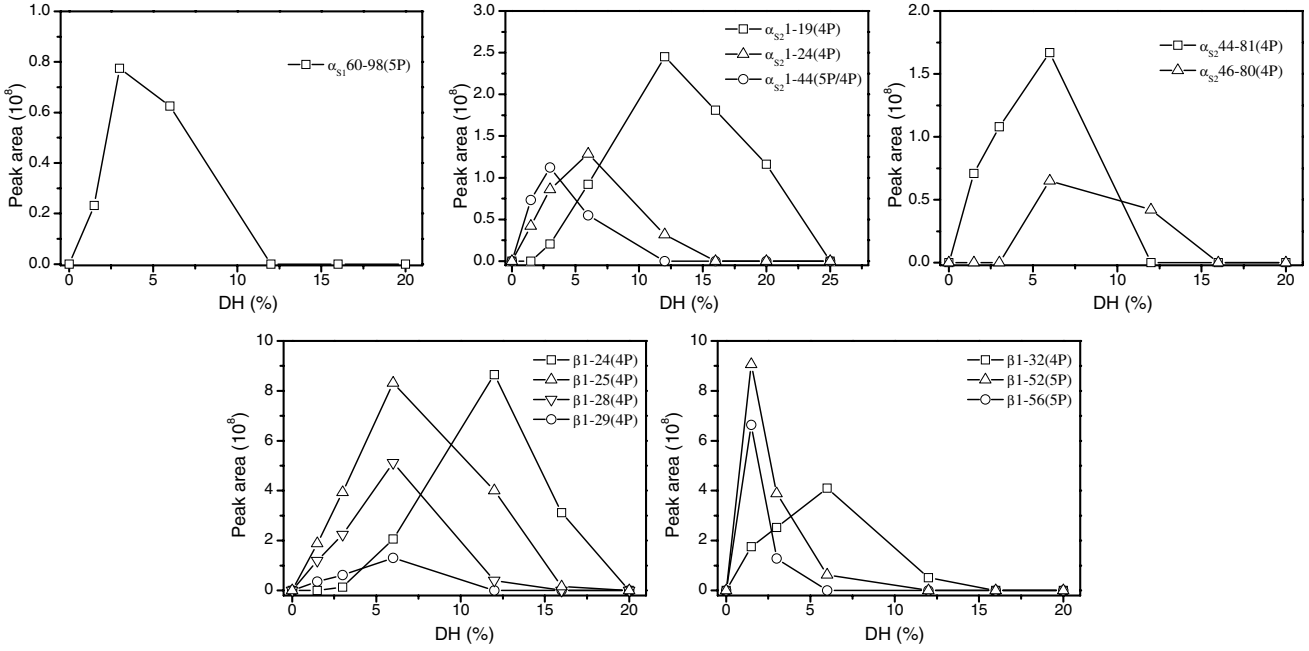

Fig. 5. Time-dependent release of selected CPPs, which contain the cluster sequence S(P)S(P)S(P)EE, derived from pancreatic hydrolysis of bovine casein. Peptides were separated and identified by LC-ESI-MS/MS, and their relative abundance was determined by peak area integration of their extracted ion current chromatogram (refer to the bold trace in Fig. 2a). Note the changes in scale.

peptides were all formed at the initial stage of hydrolysis. As shown in Fig. 5, a DH value of 3% was proposed to prepare α_{S2}-CN 1–44 (4P) and α_{S2}-CN 1–44 (5P) with maximum yields, whereas the DH values at 6% and 12% were suggested to prepare α_{S2}-CN 1–24 (4P) and α_{S2}-CN 1–19 (4P), respectively. Two other tetraphosphopeptides (α_{S2}-CN 44–81 (4P) and α_{S2}-CN 46–80 (4P)) were released from the other highly phosphorylated region of α_{S2}-CN. Although α_{S2}-CN 44–81 (4P) was released and disappeared, both at an earlier stage of hydrolysis than α_{S2}-CN 46–80 (4P), they both reached a maximum at the DH value of 6%.

The N-terminal region of β-CN seemed to be the most accessible to the pancreatic action, since the formation of nine phosphopeptides was observed after a short time of hydrolysis. Two pentaphosphorylated peptides, β-CN 1–56 (5P) and β-CN 1–52 (5P), were rapidly released and reached a maximum at a DH of 1.5%, then disappeared at the DH values of 3% and 6%, respectively. In the sequences of these peptides, they contain two tryptic peptides, β-CN 1–25 (4P) and β-CN 33–48 (1P), with calcium binding constants of 0.35–0.39 and 0.30–0.33 mM^{-1} at pH 7.0 (Meisel & Olieman, 1998). The tetraphosphopeptides from β-CN (four peptides No. 36–39 in Table 1) could be obtained productively at the DH value of 6% with the exception of β-CN 1–24 (4P), whose maximum content appeared at a DH of 12%.

4. Conclusions

The time-dependent release of CPPs from pancreatic digestion of bovine casein at pH 8.0 was investigated, in an extensive way, by using RPLC-ESI-MS/MS. The main conclusions drawn were as follows:

1. The combination of neutral loss scanning with database searching by Sequest algorithm offered a powerful approach to completely identify the CPPs. Fragmentations of the CPPs in our study all result in the production of several cluster peaks, corresponding to neutral loss(es) of phosphoric acid. The charge states, molecular masses and the number of phosphorylation sites of peptides could be quickly determined by the cluster peaks.
2. A total of 52 CPPs were identified in the time-course samples from bovine casein pancreatic digestion. Of these, 23, 7, 8, 10 and 4 were singly, doubly, triply, quadruply and quintuply phosphorylated, respectively. Fourteen peptides had the cluster sequence S(P)S(P)S(P)EE, which provides the mineral binding sites.
3. A study on the time-dependent release of CPPs allowed determination of the degrees of hydrolysis for the preparation of target peptides with the highest yields. The CPPs with the cluster sequence S(P)S(P)S(P)EE were all formed after 10 min of hydrolysis, but none of them survived after a 6 h pancreatic digestion. The discrimination of pancreatic attack on different phosphorylated regions of bovine casein was also analyzed in terms of the release kinetics of CPPs.

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