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Selective determination of phosphopeptide β-CN(1–25) in a β-casein digest by adding iron: characterization by liquid chromatography with on-line electrospray-ionization mass spectrometric detection

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Abstract

A β-casein tryptic digest has been analysed by reversed-phase high-performance liquid chromatography (RP-HPLC) with on-line electrospray-ionization mass spectrometry (ESI-MS). Analyses of peptides were carried out before and after addition of iron(II) to the peptides in solution. In both cases, the majority of peptides were identified by the determination of molecular masses by ESI-MS and by prior knowledge of the amino acid sequence of β-casein, and thus of its corresponding tryptic peptides. In the presence of iron(II), only phosphopeptide β-CN(1–25) was able to bind iron to form different complexes that have increased retention times on the RP-HPLC column and that also absorbed at 280 nm. The method presented here appears to be selective for peptides containing phosphoseryl cluster(s).

1. Introduction

α_{s1}-, α_{s2}-, and β-Casein are milk proteins that contain phosphoseryl residues grouped within a short length of the polypeptide chain. In milk, these phophorylated sequences or caseinophosphopeptides (CPPs) play an important role because they bind micellar calcium and contribute to the structure of casein micelles [1]. Moreover, CPPs might play a physiological role because they enhance both calcium intraluminal solubilization and its absorption [2]. Soluble salts of the CPPs formed from macroelements such as calcium and magnesium and/or from trace elements such as iron, zinc and copper could have dietetic and pharmacological applications [3]. For example, casein tryptic peptides containing multiple phosphoseryl residues associated with calcium phosphate can be used as toothpaste, mouthwash, and food additives for the prevention of dental caries [4]. Because of these properties, a method to identify specifically phosphopeptide(s) would be valuable. CPPs obtained after proteolytic digestion of caseinates [3] or proteolytic digestion of individual caseins [5,6] can be identified and isolated by means of several techniques: RP [5,7,8] and immobilised Fe^{3+} affinity [9] chromatography, capillary zone

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electrophoresis [10] and with peptides radiolabelled with ^{32}P adenosine triphosphate. The conversion of phosphoserine to S-ethylcysteine has been exploited to develop a procedure for the selective determination of phosphopeptides [11]. Recently, Nuwaysir and Stults [12] have determined phosphopeptides by use of immobilized metal-ion affinity chromatography coupled with electrospray-ionization mass spectrometry (ESI-MS).

ESI-MS is a powerful analytical technique for the identification of proteins and peptides by their molecular masses (M_r). Current applications are also the characterization of post-translational modifications (glycosylation, phosphorylation, oxidation, etc) and protein conformational changes in solution [13–17]. In addition, more information can be obtained by digesting proteins with a specific enzyme to generate a family of peptides that again may be characterized by ESI-MS after chromatographic separation [18].

This paper reports a simple and efficient reversed-phase high-performance liquid chromatographic (RP-HPLC) procedure for the determination of phosphopeptide β-CN(1–25) before and after addition of Fe(II) to the tryptic digest of β-casein. The characterization of iron–phosphopeptide β-CN(1–25) complexes has been performed by RP-HPLC with on-line ESI-MS.

2. Experimental

2.1. Purification of β-casein A1

β-Casein A^1 was purified as described by Mercier et al. [19] from whole casein obtained from the milk of a cow, which was homozygous for the variant A^1. The β-casein fraction was dialysed against water, brought to pH 7, and freeze dried. The concentration of β-casein in solution was determined spectrophotometrically using the molar absorptivity coefficient ϵ(1 mg/ml) = 0.46 at 278 nm [20]. It was found to be 90% homogeneous by HPLC analysis [21].

2.2. Hydrolysis of β-casein by the TPCK-treated trypsin

Tryptic digestion at 20°C for 2 h was performed on a solution of β-casein ($4.3 \cdot 10^{-5}$ *M*) in water at an enzyme/substrate ratio of 1:500 (w/w). TPCK-treated trypsin (EC 3.4.2.1.4) was purchased from Serva (Heidelberg, Germany). During hydrolysis, the pH was maintained at 7.5 by addition of 0.5 *M* NaOH using a pH Stat (Model 686, Metrohm, Herisau, Switzerland).

2.3. RP-HPLC–ESI-MS analyses of β-casein tryptic digest

Analyses of the β-casein tryptic digest were performed by RP-HPLC coupled with ESI-MS before and after the addition of Fe(II) to the tryptic digest solution. $FeCl_2 \cdot 4H_2O$ was obtained from Merck (Darmstadt, Germany).

The Fe(II) solution was freshly prepared in water directly prior to its addition to the peptides. The final concentration of Fe(II) in the peptide digest solution was $2.6 \cdot 10^{-4}$ *M* and the molar ratio Fe/phosphopeptide β-CN(1–25) was about 6. Addition was carried out with rapid mixing.

Peptides were separated on a Zorbax 300 SB RP-8 column (150 × 2.1 mm I.D., 5 μm particle size) (Rockland Technologies, Newport, RI, USA) by gradient elution with acetonitrile as organic modifier at 40°C and at a flow-rate of 0.2 ml/min. Solution A was 0.1% TFA dissolved in double-distilled water (v/v) and solution B was 0.1% TFA dissolved in acetonitrile–double-distilled water (80:20, v/v). After equilibration of the column with 10% of solution B, peptide digest solutions (50 μl) were applied to the C_8 column and eluted by increasing the concentration of solution B as follows: 0–10 min, 10–20%; 10–20 min, 20–50%; and 20–30 min, 50–60%. Eluted peaks were detected by absorbance at 214 and 280 nm, and analyzed by ESI-MS.

The mass spectrometer API I Sciex (Thornhill, Ont., Canada) was a single-quadrupole mass spectrometer equipped with an atmospheric-

pressure ionization ion source. Multiply-charged protein ions were generated by spraying the sample solution through a stainless steel capillary held at high potential. The voltage on the sprayer was usually set between 5 and 5.5 kV for positive ion production. A coaxial air-flow along the sprayer was provided to assist the liquid nebulization; the nebulizer pressure was usually adjusted in the range of 0.3–0.4 MPa. For the infusion experiment, the sample was delivered to the sprayer by a syringe infusion pump (Model 22, Harvard Apparatus, South Natick, MA, USA). The liquid flow-rate was usually set at 5–10 μl/min for sample introduction. For analyses by RP-HPLC coupled with ESI-MS, splitting of the liquid flow was achieved by a low-dead-volume connection, and the column effluent was diverted to the mass spectrometer (15% of the effluent) and to the UV detector (85% of the effluent). This arrangement permitted a straightforward correlation of the total-ion current (TIC) trace with the UV trace. The connection between the syringe infusion pump (or the HPLC system) and the ion source was a fused-silica capillary of 75 μm I.D. The interface between the sprayer and the mass analyser was made of a small conical orifice of 100 μm diameter. The potential on the orifice was 80 V. A gas curtain formed by a continuous flow (0.8–1.2 l/min) of nitrogen in the interface region served to break up any clusters. The instrument m/z scale was calibrated with poly-(propylene glycols). All protein and peptide mass spectra were obtained from the signal averaging of multiple scans. Each scan was acquired over the range of mass-to-charge (m/z) values from 350 to 2400 using a step size of 0.33 Da and a dwell-time of 0.5 ms. Molecular masses were determined from the measured m/z values for the protonated molecules. Data were acquired on an Apple Macintosh computer and were processed using the software package Mac Spec 3.2 Sciex. Assignment of individual sequences based on the molecular masses of peptides and the known sequence of bovine β-casein, was assisted by the use of Mac Bio Spec data base. The reconstructed molecular mass profile was obtained by using a deconvolution algorithm (Mac Spec 3.2 Sciex).

3. Results and discussion

3.1. ESI-MS analysis of β-casein A^1

Initially, the purity of β-casein variant A^1 was verified by ESI-MS. The ionspray mass spectrum of β-casein A^1 is shown in Fig. 1. A maximal charge-state of 33^+ was observed (Fig. 1). All positive ions in the charge distribution were due to the protonation of the basic amino acid residues (Arg, Lys, and His) and of the N-terminal group in the protein. Because β-casein A^1 contains 22 potential sites of protonation (4 Arg, 11 Lys, 6 His, and the N-terminal group) [22], other sites were also protonated, but they are not identified. Some glutamine (22 residues) and asparagine (5 residues) of β-casein might have been protonated under ESI conditions, as also reported for the ESI-MS analysis of lysozyme and Met-human growth hormone [23]. From the measured m/z values for the proton-

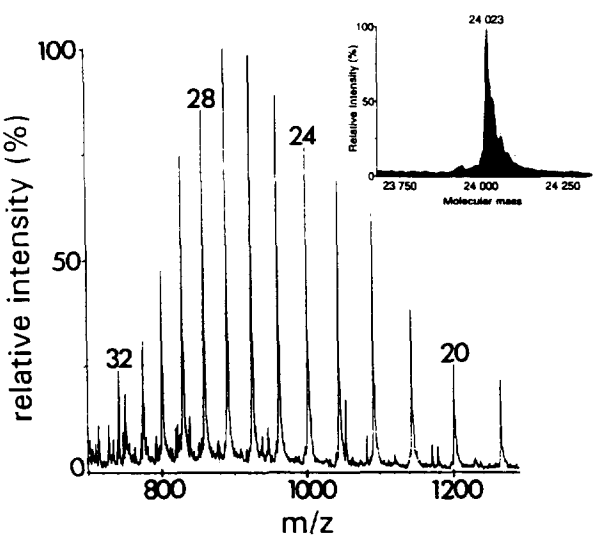

Fig. 1. ESI-MS spectrum and reconstructed molecular mass profile (insert) of purified β-casein A^1. The number of positive charges is indicated above some peaks of the ESI-MS spectrum.

ated molecules, a molecular mass of 24022.6 Da was determined. This value is the same as the molecular mass deduced from the amino acid sequence of β-casein A^1 [22]. The purity of β-casein A^1 is better revealed from the reconstructed molecular mass profile of the mass spectrum (insert of Fig. 1).

3.2. RP-HPLC–ESI-MS analyses of β-casein tryptic digest

The tryptic digest of β-casein was analysed, before and after adding Fe(II) to the tryptic digest, by on-line RP-HPLC and ESI-MS. Under these chromatographic conditions, satisfactory separations were performed.

Analysis in the absence of iron

Peptide profiles of the tryptic digest of β-casein detected by TIC or by UV absorbance were qualitatively similar (Fig 2A,B). The relative heights of some peaks were different when using both detection methods because the measurements were based on different physical principles. Sixteen peaks at 214 nm and four at 280 nm were detected after separation by RP-HPLC (Fig. 3A,B). Each peak, except peak 12, was identified by the determination of molecular masses by ESI-MS and by prior knowledge of the amino acid sequence of β-casein A^1, and thus its corresponding tryptic peptides [22] (Table 1). For example, Fig. 4 shows the mass spectrum of peak 9 (retention time on RP-HPLC of 15.6 min). Three signals at m/z values of 1042.0, 1562.8, and 2082.8, respectively, were observed. These signals corresponded to $[M+3H]^{3+}$, $[M+2H]^{2+}$, and $[2M+3H]^{3+}$ ions, respectively. From these values, a molecular mass of 3123.0 Da was determined and assigned to the phosphopeptide β-CN(1–25). The formation of the dimer species for peptides as well proteins has been previously described in the literature [24].

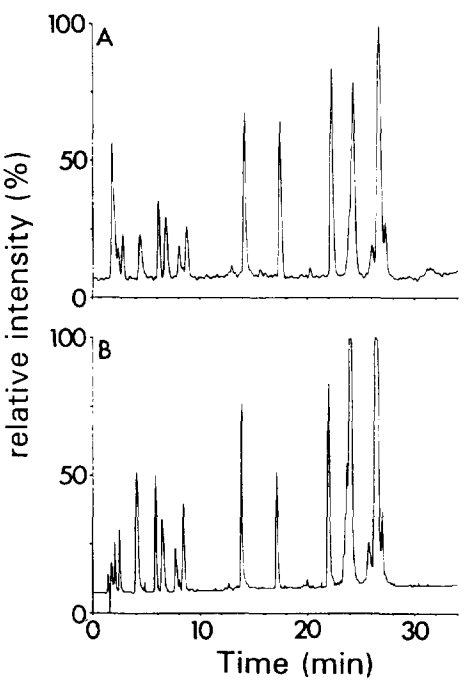

Fig. 2. Tryptic peptides from β-casein A^1 separated by RP-HPLC and detected by mass spectrometry (total-ion current) (A) or UV absorbance at 214 nm (B).

It is noteworthy that a peptide originally containing a Met residue (peak 15) exhibits an upward shift of 16 Da. Such a difference can be ascribed to the formation of the corresponding sulfoxide, leading to earlier elution of the peptide. The oxidation of Met residues is a problem that is frequently observed for peptides stocked in solution as well as in powder form. Presumably it is an oxidation by O_2.

Analysis in the presence of iron

The same experiment (RP-HPLC separation coupled with ESI-MS) was repeated with the addition of Fe(II) to the peptide digest solution. Sixteen peaks at 214 nm and five at 280 nm were detected after RP-HPLC separation (Fig. 3C,D). The peptide profile was qualitatively and quantitatively similar to that obtained in the absence of iron, except for peak 9. This peak (retention time = 15.6 min observed in Fig. 3A) disappeared, whereas a new and broad peak appeared (retention time = 18.1 min as shown in Fig. 3C). The mass spectrum corresponding to this peak is shown in Fig. 5. To know the iron binding stoichiometry, the molecular ion signals found in Fig. 5 were tentatively assigned to the following formula: $M_r = [2M_r(\beta\text{-CN}(1\text{–}25) + n\ M_r(\text{Fe}) -$

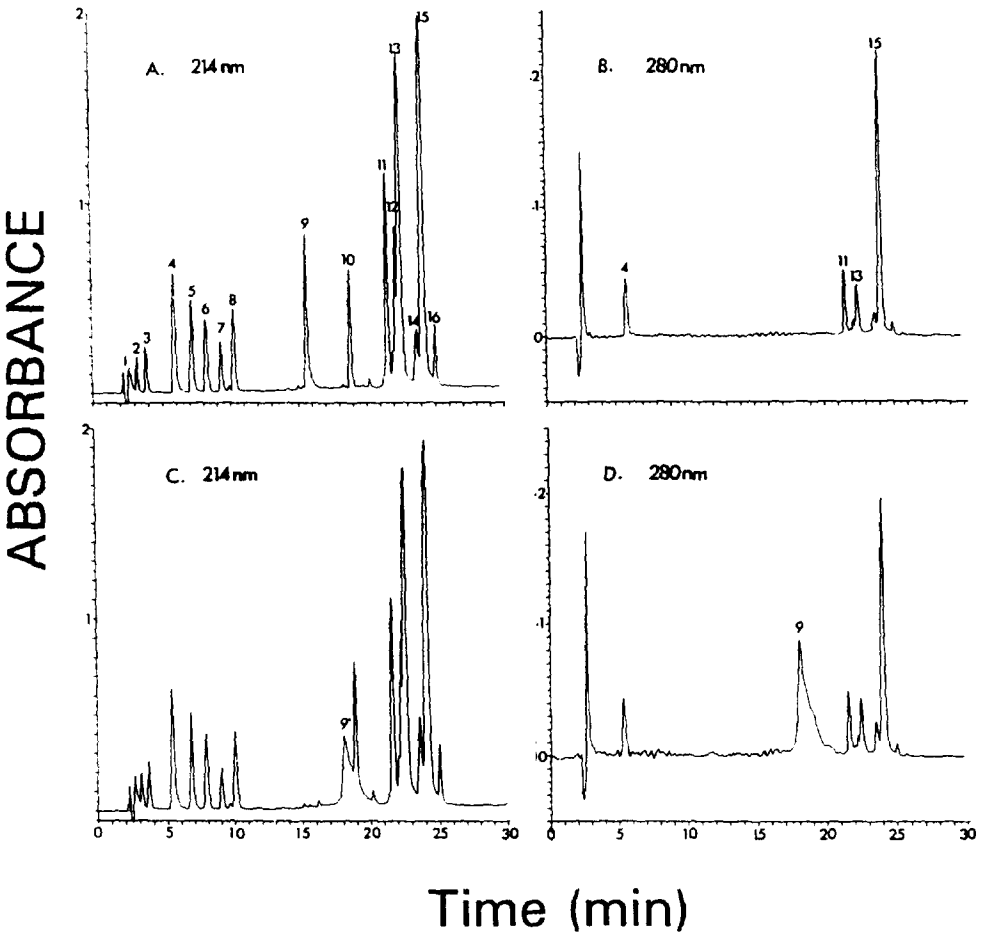

Fig. 3. Tryptic peptides from β-casein A[1] separated by RP-HPLC before (A, B) and after (C, D) addition of Fe(II) to the peptide solution. Eluted peaks were detected at 214 (A, C) and 280 nm (B, D).

$3\,nM_r(H)$] with values of n (number of bound iron atoms) varying from 4 to 6. The calculated and observed phosphopeptide–iron molecular masses are summarized in Table 2. Thus, three iron–phosphopeptide β-CN(1–25) complexes were detected. The width of this chromatographic peak was probably due to the presence of these multiple species. It can be hypothesized that phosphopeptide β-CN(1–25) binds iron via the oxygen of the phosphate group by coordination with Fe [25]. This binding will induce a release of three protons (Table 2) for one bound iron atom. This release of protons may correspond to an oxidation of iron from the ferrous to the ferric state [25]. Presumably Fe(II) adopts a tetragonal-bipyramidal structure in the complex. However this oxidation may be associated with a change in the complex structure compared to Fe(II). The three species differed only by the number of bound iron atoms (4 to 6). The major species contained 5 iron atoms. However, in these three species, we can not differentiate iron bridge(s) between two phosphopeptides β-CN(1–25), as suggested by Bouhallab et al. [26], and a dimer of phosphopeptide containing several iron atoms but with no iron bridge(s). Further investigations are in progress.

From the shift of chromatographic peak 9, it may be deduced that the hydrophobic character of the complexes is substantially higher than that

Table 1
Molecular masses (Da) and sequences of tryptic fragments of β-casein A[1] observed by RP-HPLC–ESI-MS

Peak No.	Molecular masses (Da)		Tryptic fragments
	Observed	Theoretical	
1	516.5	516.4	29–32
	501.3	501.6	26–29
	373.2	373.5	26–28
	388.4	388.5	30–32
2	645.5	645.8	100–105
3	872.5	873.1	98–105
4	829.3	830.0	177–183
5	2061.2	2062.0	33–48
6	779.5	780.0	170–176
7	1013.3	1013.2	106–113
8	747.5	747.9	108–113
9	3122.5	3123.0	1–25
10	741.5	741.9	203–209
11	2185.9	2186.6	184–202
12	5553.9	N.D.	N.D.
13	5358.4	5359.3	49–97
14	6378.4	6377.4	114–169*
15	6362.2	6361.4	114–169
16	5820.1	5817.7	114–164

Observed molecular masses are compared to theoretical molecular masses. The theoretical molecular masses were calculated using the sequence established by Ribadeau-Dumas et al. [22]. N.D. = not determined. *: This peptide contains an oxidized methionine residue.

of the "native" structure. Thus it seems that complexation, which takes place at phosphoseryl residues sites, changes the structure in such a manner that the more hydrophobic parts of the

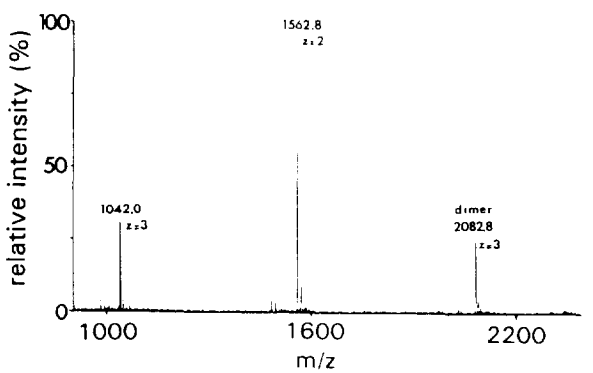

Fig. 4. ESI-MS spectrum of the phosphopeptide β-CN(1–25) after separation by RP-HPLC (chromatographic peak 9).

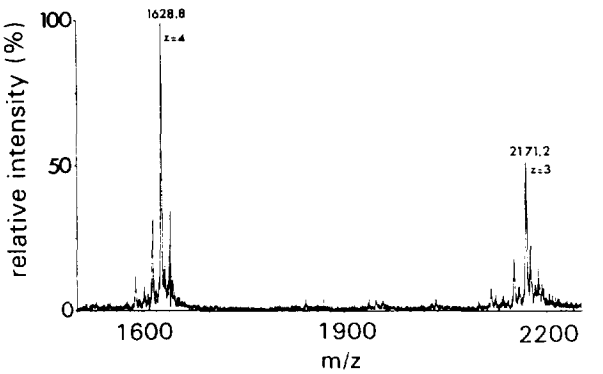

Fig. 5. ESI-MS spectrum of the phosphopeptide β-CN(1–25) after addition of Fe(II) to the peptides in solution and separation by RP-HPLC (chromatographic peak 9).

peptide are preferably exposed to the octylsilyl silica matrix.

In parallel, the chelation of this cation by phosphopeptide β-CN(1–25) has induced an absorption of peak 9 at 280 nm (Fig. 3D) even though phosphopeptide β-CN(1–25) does not contain any aromatic amino acids. Moreover, iron–phosphopeptide β-CN(1–25) complexes seem stable because they were detected after chromatography (presence of acetonitrile, pH 2 with 0.1% TFA) and after ionization by spraying across an electrostatic field gradient.

It is noteworthy that only phosphopeptide β-CN(1–25) was selectively complexed with iron, although another peptide that contains only one phosphoseryl residue [β-CN(33–48)] (peak 5) was also present in the tryptic digest solution. Mass spectra of phosphopeptide β-CN(33–48) were exactly the same in the presence or absence of iron. This result suggested that this peptide was not able to bind iron or that it bound iron insufficently. The stability of these complexes depends on the affinity of iron towards oxygen of phosphoseryl residues but also on the steric requirements. It might seem, therefore, that the high electronegativity of the phosphoseryl cluster containing four phosphoseryl residues (positions 15, 17, 18 and 19) in phosphopeptide β-CN(1–25) was not the only factor responsible for this higher affinity [27,28]. The presumable lack of enough sites in the 33–48 fragment for successful complexation in order to reach the optimum

Table 2
Determination by RP-HPLC and ESI-MS of molecular masses (Da) of iron–phosphopeptide β-CN(1–25) complexes

m/z	Charge	Observed molecular masses	Calculated molecular masses $[2M_r(\beta\text{-CN}(1\text{–}25)) + n\ M_r(\text{Fe}) - 3n\ M_r(\text{H})]$
1642.0	4	6564.0	6563.4 ($n = 6$)
2189.3	3	6564.7	
1628.8	4	6511.0	6510.5 ($n = 5$)
2171.3	3	6510.7	
1615.5	4	6458.0	6457.6 ($n = 4$)
2153.5	3	6457.5	

The m/z values derive from the experiment presented in Fig. 5. For calculation of theoretical molecular masses of iron–phosphopeptide β-CN(1–25) complexes, the molecular masses of 3123.0, 55.9, and 1.0 Da are used for phosphopeptide β-CN(1–25), iron, and proton molecular masses respectively; n represents the number of bound iron atoms.

steric configuration for efficient complexation may play a crucial role.

Mass spectra of peptides without phosphoseryl residues within their sequences were identical in the presence or absence of iron. This result indicated that these peptides were not able to bind iron.

4. Conclusions

Several methods have been developed for the determination of phosphopeptides [4,7–12]. The procedure described in this paper provides a sensitive and rapid method for the determination of peptides containing phosphoseryl cluster(s). Under the conditions employed, a good separation by RP-HPLC and a good quality of ESI-MS spectra were obtained and allowed us to determine and characterize iron-phosphopeptide β-CN(1–25) complexes. It would be interesting to detect phosphopeptides derived from other phosphoproteins as α_{s1}-, α_{s2}-caseins, and phosvitin using the same procedure.

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