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Effect of dephosphorylation on bovine casein

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Abstract

Bovine whole casein, α- and β-casein were dephosphorylated by potato acid phosphatase (EC 3.1.3.2); the extents of dephosphorylation were as follows: whole casein 71.6%, α-casein 89.2% and β-casein 73.7%. SDS-PAGE, urea-PAGE, RP-HPLC and ESI-MS demonstrated effects of dephosphorylation on the caseins; α- and β-casein showed both proteolysis and dephosphorylation while whole casein showed only dephosphorylation. Urea-PAGE and ESI-MS confirmed the identities of the individual fractions. ESI-MS established (a) the MW for α- and β-casein as 23612 and 24017 kDa, respectively, (b) random removal of 1, 2, 4, 6, 7 and 8 phosphate groups during dephosphorylation of α-casein, (c) random removal of 1, 2, 3, 4 and 5 phosphate groups during dephosphorylation of β-casein and (d) limited dephosphorylation of both α-casein (1 and 2 phosphates) and β-casein (1 phosphate) in the absence of the phosphatase.

Keywords: Bovine casein; α-Casein; β-Casein; Dephosphorylation

1. Introduction

Milk protein products are recognized for their superior nutritional, organoleptic and functional properties as food protein ingredients (Singh, 2003). Milk caseins precipitate by acidification of milk; the whey proteins remain soluble (Wong, Caminard, & Pavlath, 1996). α_s- and β-caseins are the major protein components of milk; they contain phosphate groups bound to serine and threonine (Fox & McSweeney, 1998). The caseins exhibit microheterogeneity with varying extents of phosphorylation and glycosylation (Farrell et al., 2004). The phosphate groups of caseins are known to affect many characteristics of the proteins (West, 1986), including casein digestion and bioavailability of divalent cations (Li-Chan & Nakai, 1989; Solomons, 1982). Additionally, caseins may influence the immune system causing allergenic reactions (Wal, 2002). Otani, Hori, and Hosono (1987) discussed the role of α_{s1}-casein and its phosphate on allergenicity; the cross-reactivity of the calcium-sensitive caseins (α_s-caseins, β-casein), especially as the phosphoseryl residues are considered to be immunoreactive and resistant to digestion. Casein phosphopeptides are also known to exhibit biological properties (Miquel et al., 2005). Phosphate groups of caseins are directly involved in the micelle–micelle interactions which occur during coagulation and syneresis in the conversion of milk to cheese (West, 1986), and affect the amphiphilic character of caseins. Milk casein assumes a compact micelle with most of the non-polar side chains buried in the interior, and most of the phosphorylated sites located in clusters in the N-terminal region, forming a flexible loop (Holt & Sawyer, 1988). In whole casein, the individual casein fractions associate based on their hydrophobicity and charge properties; this results in the casein micelle having an unique arrangement which contributes to many of its properties (Rollema, 1992).

Modification of caseins without compromising their functional properties is desirable for many food applications. One approach to minimizing the undesirable biological effects of phosphorylated caseins is to remove the phosphate groups; this dephosphorylation can be achieved

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both chemically and enzymatically (Bingham, Farrell, & Dahl, 1976; Ward & Bastian, 1998). In many food applications, enzymatic dephosphorylation of casein proteins is preferred over chemical dephosphorylation (Rolle, 1998). Chemical dephosphorylation under severe conditions (pH 12, 120 °C) resulted in release of phosphate and formation of a dehydroalanine residue by a β-elimination mechanism, with the likelihood of further intermolecular or intramolecular reactions with lysine to form lysinoalanine (Ward & Bastian, 1998). Enzymatic dephosphorylation, or mild chemical dephosphorylation, results in release of phosphate groups leaving the dephosphorylated serine residue intact on the protein. Although there have been several studies on enzymatic dephosphorylation of caseins (Bingham et al., 1976; Li-Chan & Nakai, 1989) there is relatively little information on the identification of the dephosphorylated casein species. The objectives of this work were to study the dephosphorylation of whole casein and its α- and β-casein fractions, to investigate the extents of dephosphorylation, to determine casein molecular changes which occur during dephosphorylation and to use electrospray ionization mass spectrometry (ESI-MS) to identify the dephosphorylated casein species.

2. Materials and methods

2.1. Materials

Commercial dry skim milk powder was purchased and reconstituted with distilled water (370 g/l). Whole casein was precipitated at 20 °C, pH 4.6 with 1 M ammonia acetate buffer. The casein was washed with acetone, air dried, ground and stored in a desiccator until further analysis (Veloso, Teixeira, & Ferreira, 2002). Commercial bovine α- and β-casein standards and potato acid phosphatase (EC 3.1.3.2; activity: 1.2 units/mg) were purchased from Sigma Chemicals Co. (St. Louis, MO). The α-casein was not separated further into its α_{s1}- and α_{s2}-casein fractions.

2.2. Protein and phosphorus contents

Total phosphorus was determined by dry-ashing (600 °C, 5 h; Furnatrol II Furnace, Thermolyne Co., Iowa) followed by colorimetric analysis (823 nm, Ultrospec 100 UV/Vis; Biochrom Ltd., Cambridge, UK) using the method of Pulliainen and Wallin (1994). For measurement of free phosphate, the dry-ashing step was excluded; only the colorimetric step was necessary. Protein content of whole casein and dephosphorylated whole casein was determined using the method of Lowry, Rosebrough, Farr and Randall, as modified by Hartree (1972); bovine serum albumin was used as standard.

2.3. Caseins dephosphorylation

Dephosphorylation of whole casein with potato acid phosphatase was carried out using the method of Bingham et al. (1976) with modification (citrate buffer 0.1 M, pH 5.8, 37 °C). Controls were prepared using whole casein but without the addition of the enzyme. The enzyme reaction was performed in duplicate using different enzyme concentrations; the total reaction mixture was 10 ml and contained 25 mg casein. The reaction was carried out at time intervals of 1 h for 8 h and stopped by the addition of 5 ml 15% TCA. The precipitated protein was recovered by centrifugation (3000*g*, 5 min) and analyzed for phosphorus content as described before. Based on this preliminary experiment an enzyme concentration of 0.2 mg/ml was selected to establish optimum reaction time of 6 h for whole casein, α- and β-casein.

2.4. Electrophoresis

Whole casein, α- and β-caseins and their dephosphorylated products were subjected to slab gel polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS) (Laemmli, 1970), using a Bio-Rad Minipro-

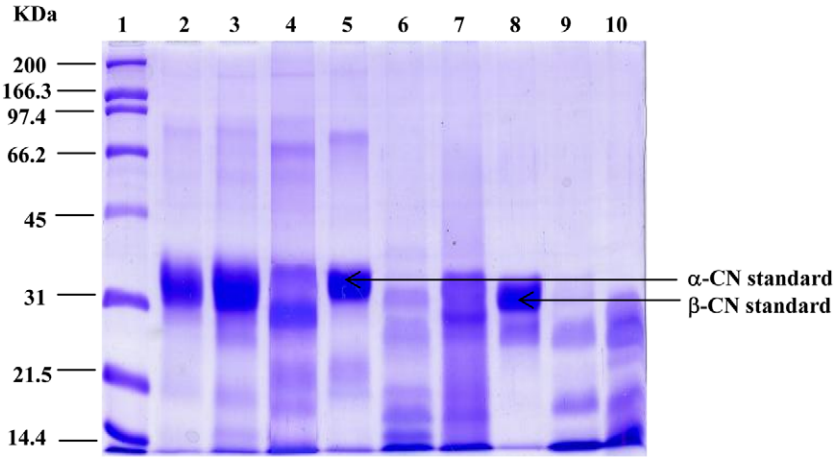

Fig. 1. SDS-PAGE electrophoretic patterns of caseins. (1) Standards, (2) whole casein standard, (3) control whole casein, (4) dephosphorylated whole casein, (5) α-casein standard, (6) control α-casein, (7) dephosphorylated α-casein, (8) β-casein standard, (9) control β-casein and (10) dephosphorylated β-casein (controls: incubated without phosphatase).

tean® II dual slab cell electrophoresis unit (Richmond, CA). The conditions used were: staking and separation gel 4% and 12% acrylamide, respectively and sample injection volume of 20 μl in the gels. Estimation of molecular weight was done by SDS-PAGE broad range molecular weight standards (Bio-Rad Hercules; CA). Slab gel at constant current, 15 mA/gel; run time approximately 2 h. After electrophoresis, gels were fixed for 1 h in a mixture of methanol (20%, v/v) and acetic acid (10%, v/v). Coomassie Brilliant Blue R-350 (0.1%, w/v in fixing solution) was used to stain protein bands. Destaining was done using the fixing solution. Urea-PAGE was performed to achieve complete separation of whole, α- and β-caseins (Coker, 1991). The conditions used were: 6% and 20% acrylamide, respectively. Sample injection volume was 20 μl in the gels. Slab gel at constant voltage, 100 V/gel; run time approximately 1.5 h. Protein bands were stained with Coomassie Brilliant Blue R-350 (0.1%, w/v in fixing solution). Destaining was done using the fixing solution.

2.5. Reverse-phase high performance liquid chromatography

RP-HPLC was used to separate caseins and their dephosphorylated products with a liquid chromatograph (Beckman; CA, USA) equipped with a Programmable Solvent Module (model 126) for high pressure solvent delivery, a manual injector (20 μl loop), a Programmable Detector Module (model 166) and an Ultra C4 reverse phase column 5 μm, 250 × 4.6 mm column (Restek Co., USA). Chromatographic data were analyzed by the Gold System (version V810), translated into PRN format for Microsoft Excel© manipulation. Gradient elution was carried out with a mixture of two solvents. Solvent A consisted of 0.1% trifluoracetic acid (TFA) in water and solvent B was 0.04% acidified water (TFA) in acetonitrile. Elution was performed at a flow rate of 1 ml/min with a 50 min linear gradient starting at 25% of solvent B and increasing up to 45% of solvent B over 40 min, then the linear conditions were re-established over 10 min. Detection was at 220 nm. Fractions eluting from the column were collected by Water Fraction Collector (NE, USA), then pooled and concentrated, freeze-dried and stored at room temperature for further analysis.

2.6. Electrospray ionization mass spectrometry

α-casein and β-casein standards were dissolved in 10% acetic acid and subjected to ESI-MS directly. For the

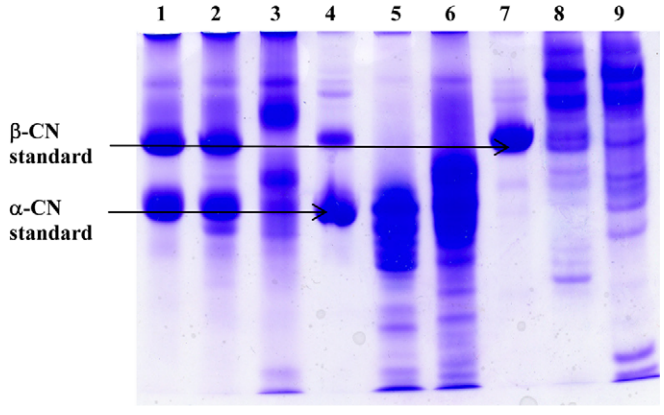

Fig. 2. Urea-PAGE electrophoretic patterns of caseins. (1) Whole casein standard, (2) control whole casein, (3) dephosphorylated whole casein, (4) α-casein standard, (5) control α-casein, (6) dephosphorylated α-casein, (7) β-casein standard, (8) control β-casein and (9) dephosphorylated β-casein (controls: incubated without phosphatase).

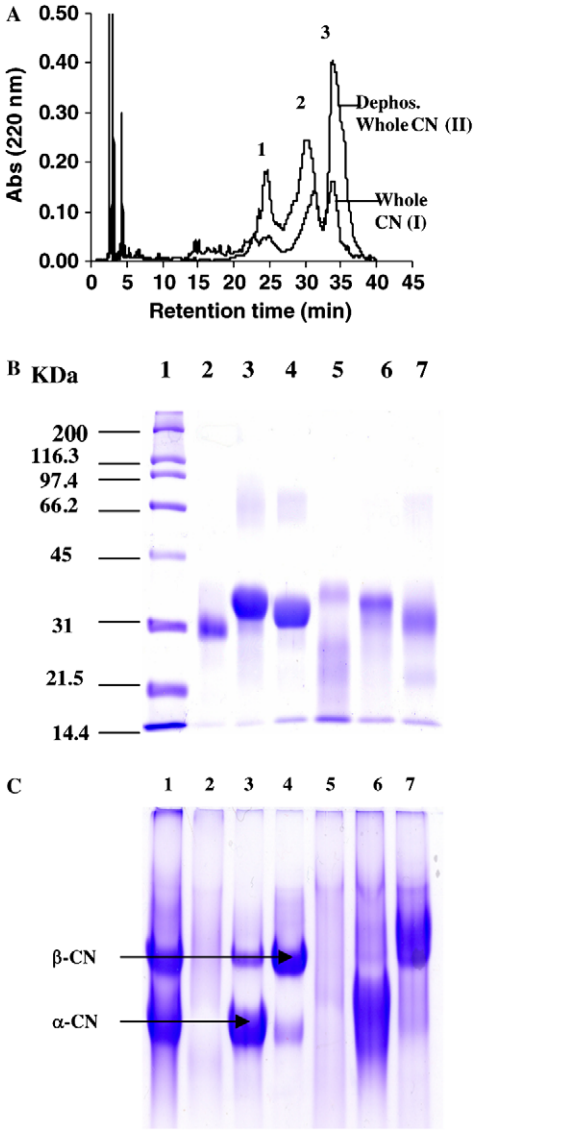

Fig. 3. (A) RP-HPLC chromatograms of (I) standard whole casein and (II) dephosphorylated whole casein. Peak identity: (1) κ-casein, (2) α-casein and (3) β-casein. (B) Casein profiles obtained by SDS-PAGE. Fractions collected from RP-HPLC separation. (1) Standards, (2) κ-casein, (3) α-casein, (4) β-casein, (5) dephosphorylated κ-casein, (6) dephosphorylated α-casein and (7) dephosphorylated β-casein. (C) Casein profiles obtained by urea-PAGE. Fractions collected from RP-HPLC separation. (1) Whole casein standard (2) κ-casein, (3) α-casein, (4) β-casein, (5) dephosphorylated κ-casein, (6) dephosphorylated α-casein and (7) dephosphorylated β-casein.

controls (incubated without phosphatase), dephosphorylated α-casein and dephosphorylated β-casein, dialysis against distilled water (4 °C) was used to remove buffer salts that can suppress the analyte signal during the electrospray ionization. A Waters Micromass QTOF Ultima Global (Micromass; Manchester, UK) hybrid mass spectrometer equipped with a nanoflow electrospray source was used. It was operated in positive ionization mode (+ESI) at 3.80 kV; with source temperature of 80 °C and desolvation temperature of 150 °C. The TOF was operated at an acceleration voltage of 9.1 kV, a cone voltage of 100 V and collision energy of 10 eV (for MS survey). For the MS survey mass range, m/z was 400–1990, scanned continuously over the chromatographic run. The mass spectrometer was tuned and calibrated with [Glu]-Fibrinopeptide B (Sigma Chemicals Co; St. Louis, MO). Instrument control and data analysis were carried out by MassLynx V4.0 software.

3. Results and discussion

3.1. Extent of dephosphorylation

Protein and total phosphate content of whole casein were 94.6% and 0.8%, respectively, and 92.3% protein for dephosphorylated whole casein. The free phosphate before enzymatic dephosphorylation represented 7.7% of the total phosphate; this free phosphate may be due to the presence of indigenous phosphatases in milk. These results agree with the values reported by Fox and McSweeney (1998). The extent of dephosphorylation for whole casein, α- and β-casein were 71.6%, 89.2% and 73.7%, respectively. Previous work by Bingham et al. (1976) showed a maximum dephosphorylation of approximately 70% for α_{s1}-casein at pH 7 and 38 °C. Li-Chan and Nakai (1989) indicated 97%, 70% and 99% of dephosphorylation for whole casein, α_{s1}-casein and β-casein, respectively. van Hekken and Strange (1993) obtained 40% partially dephosphorylated whole casein at pH 6.5, 37 °C. The different extents of dephosphorylation reported in different studies could be related to phosphate end-product inhibition; Bingham et al. (1976) suggested that phosphate is a competitive inhibitor of potato acid phosphatase activity. West and Towers (1976) reported that β-casein with three phosphoseryl residues can resist the action of phosphatase; this resistance was attributed to intermolecular interactions involving the phosphate groups.

3.2. Effects of dephosphorylation on molecular characteristics

Fig. 1 shows the SDS-PAGE patterns for whole casein, α- and β-caseins standards, controls (whole casein, α- and β-caseins incubated without phosphatase), and dephosphorylation products of the caseins; the controls allowed us to determine if experimental incubation conditions affected the casein fractions in the absence of the phosphatase enzyme. No attempt was made to estimate molecular weights for the caseins because of the known anomalous behaviour of caseins in SDS-PAGE (Creamer & Richardson, 1984). The identification of the casein fractions was done by comparison of migration distance with those of the known standards. The high intensity band of whole

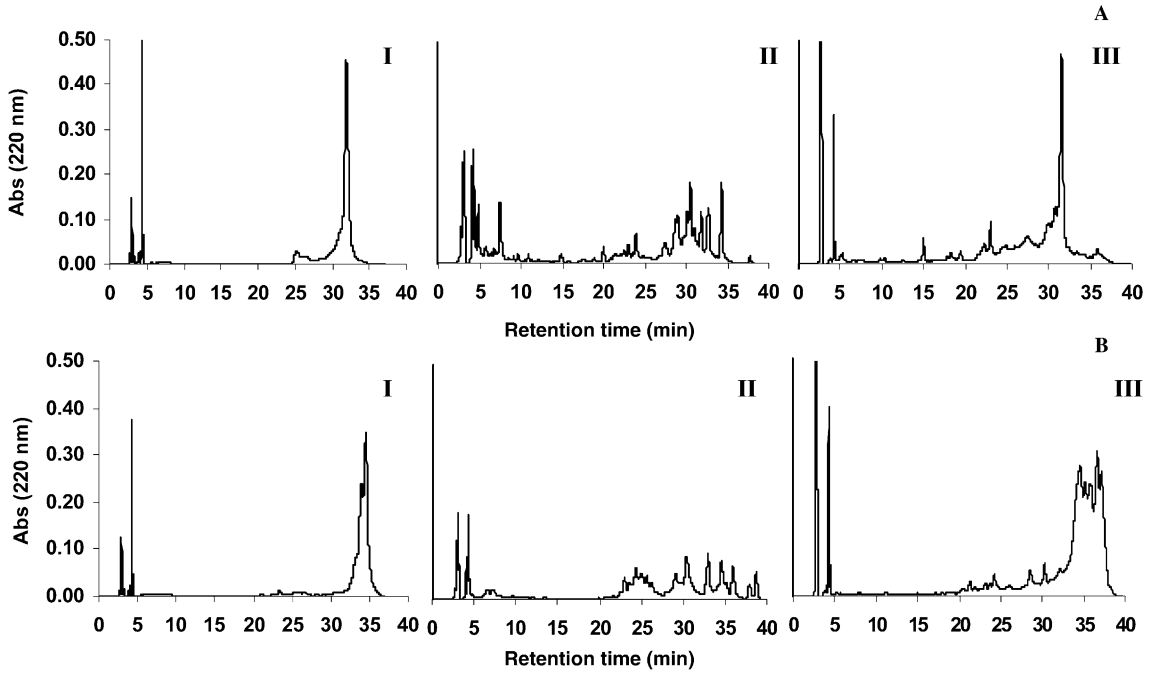

Fig. 4. (A) Chromatographic profiles of (I) α-casein standard, (II) control α-casein and (III) dephosphorylated α-casein. (B) Chromatographic profiles of (I) β-casein standard, (II) β-casein control and (III) dephosphorylated β-casein obtained by RP-HPLC (controls: incubated without phosphatase).

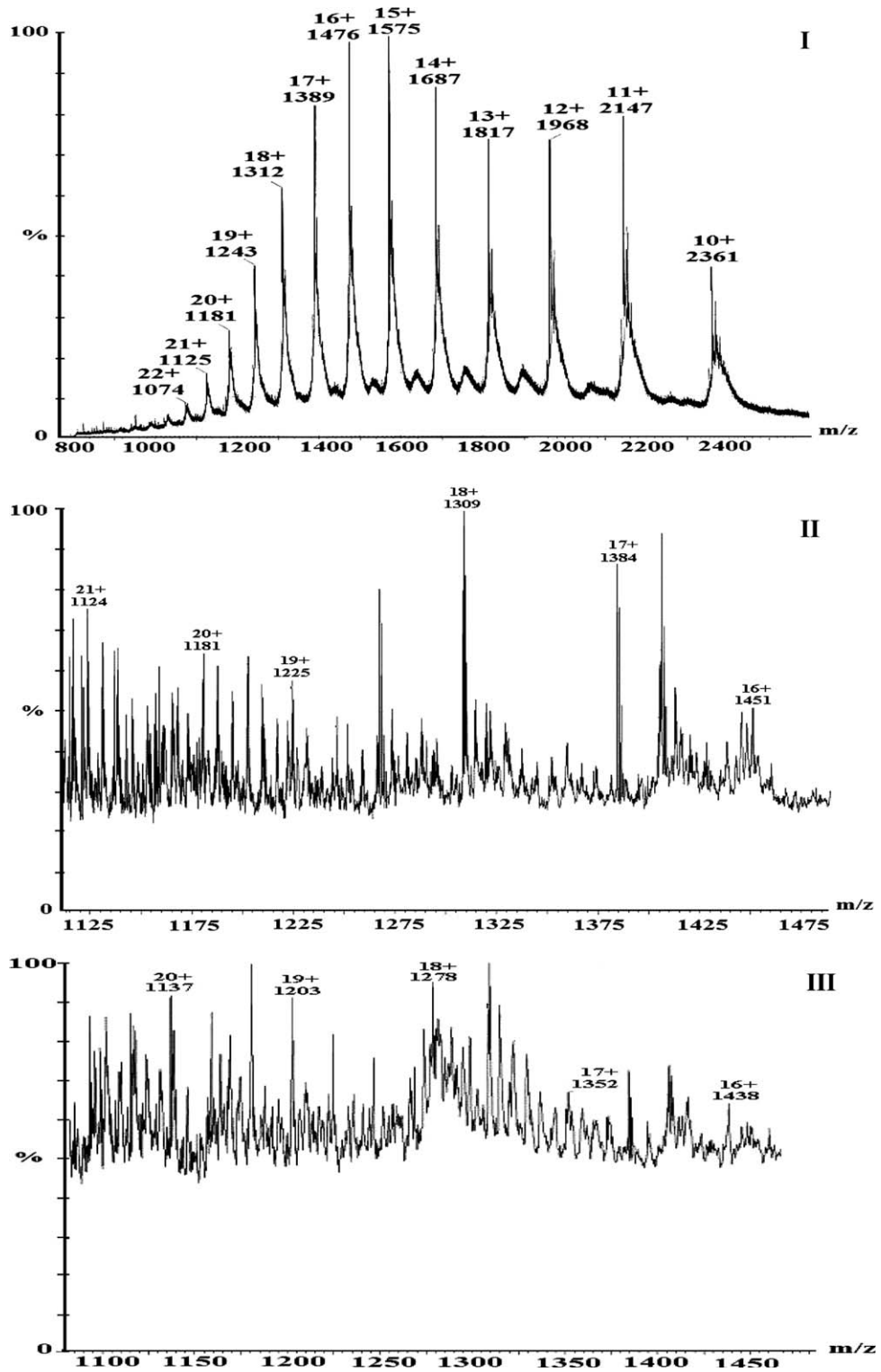

Fig. 5. ESI-MS spectra of (I) α-casein standard, (II) control α-casein (incubated without phosphatase) and (III) dephosphorylated α-casein indicating the net charge of the multiprotonated ions.

casein standard (lane 2) indicates that it was not separated into its individual α- and β-casein fractions as their theoretical individual molecular weights are very similar (23.6 kDa, α_{s1}-casein, 24 kDa β-casein; Farrell et al., 2004). Lane 4 shows dephosphorylated whole casein with two bands which are likely the separated dephosphorylated α- and β-casein fractions (comparison with whole casein, lanes 2 and 3). The differences between the control and the phosphatase incubated α-casein (lanes 6 and 7) and β-casein (lanes 9 and 10) reflect the effect of dephosphorylation of these fractions. The SDS-PAGE results suggest changes in structures of α-casein (lanes 5 and 6) and β-

Table 1
Molecular Species of α-, β- and dephosphorylated α-, β-caseins identified by ESI-MS, after deconvolution of mass spectra

α-Casein				β-Casein			
Exp MW in standard (kDa)	Theoretical MW after dephosphorylated (kDa)	Exp MW in control (kDa)	Exp MW after dephosphorylated (kDa)	Exp MW in standard (kDa)	Theoretical MW after dephosphorylated (kDa)	Exp MW in control (kDa)	Exp MW after dephosphorylated (kDa)
23612	–	23618 (+6)	–	24017	–	24023 (−6)	–
–	23532 (−1 HPO_3^-)	23534 (−78)	23536 (−76)	–	23937 (−1 HPO_3^-)	–	23942 (−75)
–	23452 (−2 HPO_3^-)	23459 (−153)	23458 (−154)	–	23857 (−2 HPO_3^-)	23852 (−165)	23855 (−162)
–	23292 (−4 HPO_3^-)	–	23287 (−325)	–	23777 (−3 HPO_3^-)	–	23774 (−243)
–	23132 (−6 HPO_3^-)	–	23140 (−472)	–	23697 (−4 HPO_3^-)	–	23687 (−330)
–	23052 (−7 HPO_3^-)	–	23050 (−562)	–	23617 (−5 HPO_3^-)	–	23615 (−402)
–	22972 (−8 HPO_3^-)	–	22975 (−637)	–	–	–	–

casein (lanes 8 and 9) in the absence of the phosphatase (control); however, there was no change in the structure of whole casein in the absence of the phosphatase (lanes 2 and 3). Fig. 2 shows the urea-PAGE patterns for whole casein, α- and β-caseins standards, controls (whole casein, α- and β-caseins incubated without phosphatase), and dephosphorylation products of the caseins. For whole casein standard (lane 1) and whole casein control (no phosphatase, lane 2), two intense bands corresponding to α- and β-casein were observed. Urea-PAGE separated the casein fractions present in whole casein that was not obtained with SDS-PAGE. For dephosphorylated whole casein (lane 3), the two main bands were identified as dephosphorylated α-casein and dephosphorylated β-casein; the migration of the dephosphorylated casein fraction is reduced as a result of the removal of the negative charge of the phosphate group (Li-Chan & Nakai, 1989). The presence of several bands with α-casein (lane 6) and β-casein (lane 9) suggests that proteolysis also occurred during dephosphorylation in the presence of phosphatase. The differences between the control and the phosphatase incubated α-casein (lanes 5 and 6) and β-casein (lanes 8 and 9) reflect the actual effect of dephosphorylation of these fractions; these effects were also evident with SDS-PAGE (Fig. 1). Urea-PAGE also suggested that proteolysis occurred in the control experiments (incubation without phosphatase) with α-casein (lane 5) and β-casein (lane 8). Li-Chan and Nakai (1989) also reported that both proteolysis and dephosphorylation of α_{s1}-casein occurred in the presence of an alkaline phosphatase. It is likely that the observed proteolysis in the α-casein and β-casein controls (no phosphatase) was the effect of the incubation conditions on these proteins. Swaisgood (1982) reported that α- and β-casein showed greater susceptibility to proteolysis when they were separated from the casein micelle. Additionally, α- and β-casein exhibit more chain flexibility than typical globular proteins, which increases susceptibility to hydrolysis (Church, Catignani, & Swaisgood, 1981).

Fig. 3A shows the RP-HPLC chromatograms for whole casein standard (I) and dephosphorylated whole casein (II). From the chromatogram of whole casein, the three peaks were assigned as the κ-, α- and β-casein fractions; the identities of these fractions were confirmed by SDS-PAGE (Fig. 3B) and urea-PAGE (Fig. 3C). Bobe, Beitz, Freeman, and Lindberg (1998) reported similar elution pattern for the three casein fractions from whole casein. The RP-HPLC results showed slight changes in the retention times of the α- and β-casein as a result of the dephosphorylation. Removal of the phosphate groups is expected to increase hydrophobicity of the casein fractions, compared with the fully phosphorylated caseins (Strange, van Hekken, & Thompson, 1991; Bernard, Meisel, Creminon, & Wal, 2000). Fig. 4A shows the chromatograms for α-casein standard (I), control α-casein (II) and dephosphorylated α-casein (III). α-Casein was observed as a major peak with retention time of 31.9 min. The chromatogram of the control α-casein (no phosphatase, Fig. 4A, II) also gave the α-

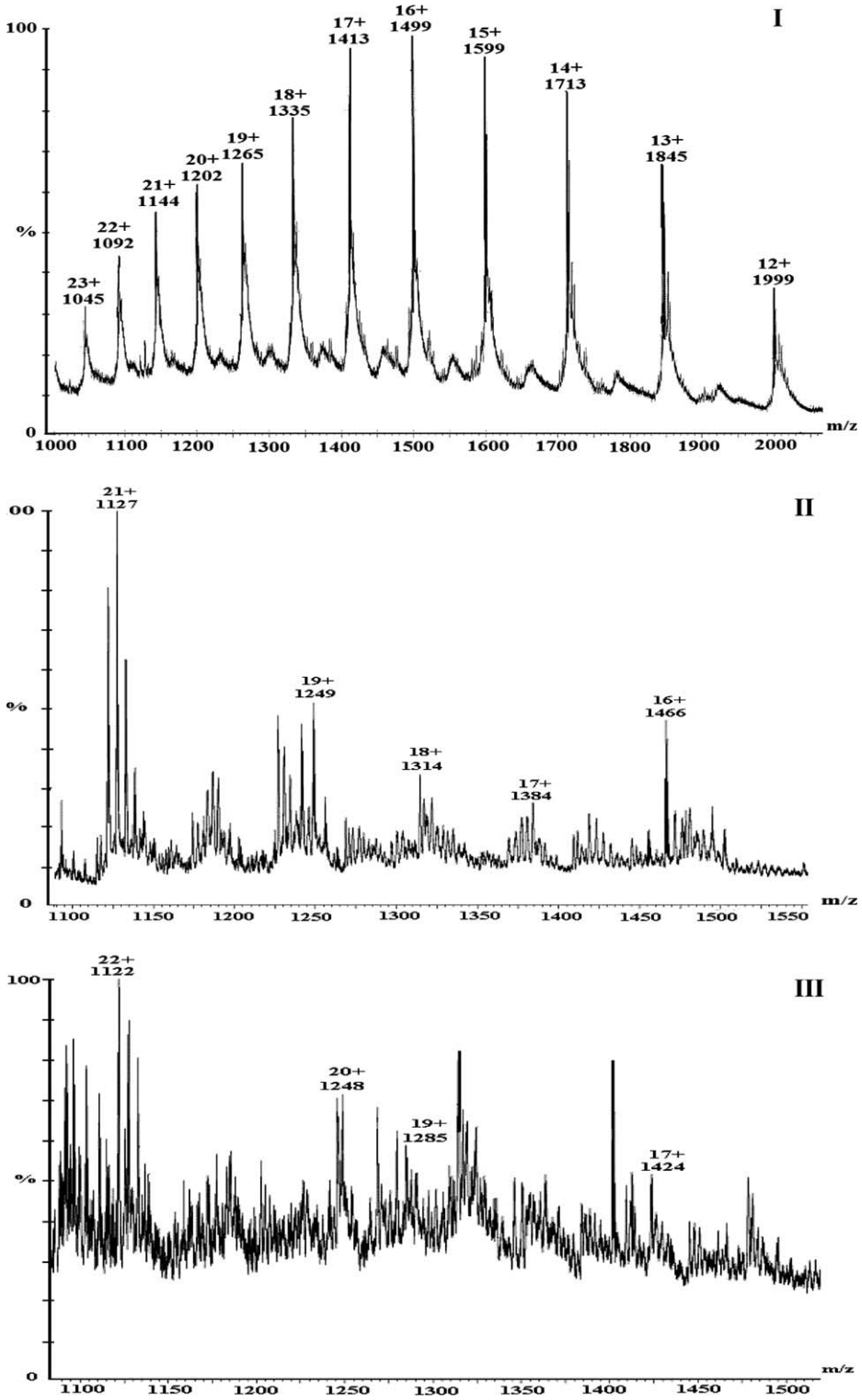

Fig. 6. ESI-MS spectra of (I) β-casein standard, (II) control β-casein (incubated without phosphatase) and (III) dephosphorylated β-casein indicating the net charge of the multiprotonated ions.

casein peak at 31.9 min, as well as several peaks which could be hydrolysis products of the α-casein; these results support our findings from electrophoretic analysis, which suggested that incubation conditions in the absence of the phosphatase resulted in some proteolysis of α- and β-casein. Dephosphorylated α-casein (Fig. 4A, III) gave a major peak at 32.3 min; it is likely that this shift in retention time from 31.6 min is due to the increased hydrophobicity of the dephosphorylated α-casein (Bernard et al., 2000). Fig. 4B shows the chromatogram for β-casein standard (I), control β-casein (II) and dephosphorylated β-casein (III). The β-casein standard was observed as a dou-

blet peak likely due to self-association of this casein (Bobe et al., 1998) with retention time 34.6 min. Control β-casein (no phosphatase, Fig. 4B, II) shows the β-casein peak of 34.6 as well as several peaks which could be hydrolysis products of the β-casein; similar observations were made from the previous in electrophoretic analysis. Dephosphorylated β-casein showed several overlapping peaks in the retention time range of 34.8 and 36.7 min. It is likely that these peaks represent dephosphorylated β-casein and dephosphorylated hydrolysis products of β-casein; similar results were observed with dephosphorylated α-casein.

Fig. 5 shows the mass spectra of standard α-casein (I), control (no phosphatase) α-casein (II) and dephosphorylated α-casein (III). Using ESI-MS data to identify dephosphorylation in proteins, it can be expected that a mass decrease of 80 kDa will be observed for removal of each phosphate group (Hirschberg et al., 2004); with ESI tandem MS of phosphopeptides, loss of a phosphate group results in a decrease of 98 kDa from a phosphoserine residue (Lund & Ardö, 2004). However, in this particular work a difference of 80 kDa is considered since dephosphorylation with acid phosphatase leaves intact the serine residue and only HPO_3^- is removed. MS techniques have been used to identify the presence of phosphate groups in phosphoproteins and in phosphopeptides resulting from enzymatic or chemical degradation of the phosphoproteins (Lee et al., 2001; Zhong, Zhang, Wen, & Li, 2004). MW species corresponding to removal of phosphate groups form α-casein are identified in Table 1; in addition MW species not assigned to removal of phosphate by added phosphatase but present in the control α-caseins are identified. Standard α-casein gave a MW of 23 612 kDa; this compares well with both the theoretical MW of α_{s1}-casein B variant (23 618 kDa) calculated from the cDNA sequence as reported by Stewart, Willis, and Mackinlay (1984) and the observed MW (23 614 kDa) reported by Léonil et al. (1995). Based on the comparison of expected MW from theoretical dephosphorylation of α-casein and observed MW from the dephosphorylated α-casein (Table 1), six dephosphorylated species (MW 23 536, 23 458, 23 287, 23 140, 23 050 and 22 975 kDa) were identified, corresponding to loss of 1, 2, 4, 6, 7 and 8 phosphate groups during dephosphorylation. Four of the phosphoseryl residues occur at the Ser_{64}–Ser_{68} region of the α_{s1}-casein molecule, with three of the phosphates on adjacent serine residues but separated from a fourth by a single Ile_{65} residue; the other four serilphosphates are distributed in the remainder of the molecule (Ng-Kwai-Hang, 2003). The control α-casein (no phosphatase) showed the presence of the original α-casein (MW 23 618), as well as MW species (MW 23 534 and 23 459 kDa) corresponding to loss of 1 and 2 phosphate groups respectively; this suggests partial dephosphorylation of α-casein in the absence of added phosphatase; Léonil et al. (1995) also observed with ESI-MS, the occurrence of partially dephosphorylated casein with the loss of 1 phosphate group in the absence of added enzyme.

Fig. 6 shows the mass spectra of standard β-casein (I), incubated (no phosphatase) β-casein (II) and dephosphorylated β-casein (III); Table 1 shows the MW species identified from these spectra. MW species corresponding to removal of phosphate groups from β-casein are summarized in Table 1; in addition MW species not assigned to removal of phosphate but present in the control β-caseins are identified. Standard β-casein gave a MW of 24 017 kDa, which compares well with the values of 24 024 and 24 028 kDa reported for variant A^1-5P (Jimenez-Flores, Kang, & Richardson, 1987; Swaisgood, 1992). Based on the comparison of expected MW from theoretical dephosphorylation of β-casein and observed MW from the dephosphorylated β-casein (Table 1), five dephosphorylated species (MW 23 942, 23 855, 23 774, 23 687 and 23 615 kDa) were identified, corresponding to loss of 1, 2, 3, 4 and 5 phosphate groups during dephosphorylation. West and Towers (1976) reported that dephosphorylation of β-casein was non-random with an alkaline phosphatase from *E. coli*, but was different from the action observed with a bovine spleen phosphatase. The control β-casein showed the presence of the original β-casein (MW 23 023 kDa), as well as a MW species of 23 852 kDa corresponding to loss of 2 phosphate groups; this suggests partial dephosphorylation of β-casein in the absence of added phosphatase.

Whole casein and its separated α- and β-casein fractions showed substantial (>70%) dephosphorylation by an acid phosphatase. During the dephosphorylation the whole casein did not show the non-specific proteolysis which was observed with α- and β-casein. Random dephosphorylation was observed with both α- and β-casein. Limited dephosphorylation was observed with both α- and β-casein in the absence of the phosphatase.

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