JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

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Detection of Two Minor Phosphorylation Sites for Bovine κ-Casein Macropeptide by Reversed-Phase Liquid Chromatography−Tandem Mass Spectrometry

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ABSTRACT: This work addresses the characterization of phosphopeptides in bovine κ-casein macropeptide by reversed-phase liquid chromatography−electrospray ionization−tandem mass spectrometry (RPLC−ESI−MS^2). Two different mass spectrometers, equipped with an ion trap (IT) or a quadrupole time-of-flight (Q-TOF) analyzer, were used to perform an accurate phosphorylation site assignment. A total of 8 phosphopeptides from 26 identified peptides were characterized. MS^2 spectra of phosphopeptides were dominated by the neutral loss of a phosphoric acid molecule (H_3PO_4) and sufficient informative fragment ions resulting from peptide backbone cleavages enabling the elucidation of the phosphopeptide sequence. A higher number of sequence informative b and y ions were detected using a Q-TOF instead of an IT analyzer. In addition to the well-established phosphorylation sites at Ser^{149} and Ser^{127}, this study also revealed the presence of two minor phosphorylation sites at Thr^{145} and Ser^{166}. These findings indicate that RPLC−ESI−MS^2 on a Q-TOF analyzer is a useful technique for identifying low-abundance phosphorylation sites in caseins.

KEYWORDS: Caseinomacropeptide, phosphorylation, LC−MS, ion trap, quadrupole time-of-flight, tandem mass spectrometry

INTRODUCTION

Phosphorylation is one of the most important post-translational modifications occurring in proteins and can play a crucial role in their structure and biological properties.[1] Phosphoproteins are particularly abundant in milk, and caseins are the main phosphoproteins found in milk of most mammalian species.[2] Basically, bovine caseins are a mixture of four different phosphoproteins, α_{s1}-, α_{s2}-, β-, and κ-caseins, in the approximate proportions 4:1:4:1, respectively.[3] Phosphorylation is a key event for the primary function of the caseins, i.e., the formation of large micelles upon binding of Ca^{2+}.[4] Likewise, it has been described that casein phosphopeptides may possess anticariogenic properties, and they are also important in the bioavailability of Ca^{2+} from milk and dairy products by increasing its passive absorption and other trace elements in the distal small intestine.[5]

The most common type of casein phosphorylation involves the formation of phosphate ester bonds with the hydroxyl side chains of serine and, although much less frequently, threonine. Thus, bovine caseins are phosphorylated at different levels, and the common genetic variants of α_{s1}-, α_{s2}-, β-, and κ-caseins normally contain 8−9, 11−13, 5, and 1−2 phosphate groups, respectively.[6] In the case of κ-casein, several posterior studies revealed the presence of up to three phosphate residues per molecule, with the estimated abundance of the triphosphorylated form of caseinomacropeptide (CMP) being around 1.2−2%.[7,8] This low abundance can probably explain that only two phosphorylation sites have clearly been determined, i.e., the residue Ser^{149}, which is fully phosphorylated, and Ser^{127}, as the second most important phosphorylation site. However, significant advances of mass spectrometry-based proteomics techniques, especially in terms of sensitivity and dynamic range, are enabling the detection and fragmentation of phosphorylated peptides at lower and lower levels, and consequently, a wide number of novel *in vivo* phosphorylation sites is being characterized.[9,10] In this context, evidence for a third phosphorylation site being occupied at Thr^{145} has recently been published, following a study based on the characterization of bovine κ-casein by two-dimensional gel electrophoresis after a cysteine-tagging enrichment strategy.[11]

The main aim of this work was the identification of phosphopeptides and the characterization of phosphorylation sites in bovine κ-casein by reversed-phase liquid chromatography−electrospray ionization−tandem mass spectrometry (RPLC−ESI−MS^2) using two different mass analyzers [ion trap (IT) and quadrupole time-of-flight (Q-TOF)] to make an accurate phosphorylation site assignment. For that purpose, the 64 C-terminal amino acids of κ-casein, also called CMP, was selected because phosphorylations, as well as all of the other post-translational modifications, are found exclusively in this C-terminal portion of κ-casein.

Received: August 1, 2011
Revised: September 12, 2011
Accepted: September 12, 2011
Published: September 12, 2011

dx.doi.org/10.1021/jf203089n | *J. Agric. Food Chem.* 2011, 59, 10848–10853

MATERIALS AND METHODS

Hydrolysis of Bovine CMP. CMP (mixture of A and B variants) was kindly provided by Davisco Foods International (Le Sueur, MN). CMP was subjected to a combined trypsin/chymotrypsin proteolysis (overnight at 37 °C and pH 7) at 1:0.05:0.025 CMP/trypsin/chymotrypsin ratios (w/w/w).[12] Enzymatic activities of porcine pancreas trypsin (EC 3.4.21.4, type IX-S) and bovine pancreas α-chymotrypsin (EC 3.4.21.1, type I-S) were 13 000−20 000 and ≥40 u/mg of protein, respectively.

RPLC−ESI−MS^2 Analyses. Two different LC−MS^2 instruments were used in this study to analyze the hydrolyzed CMP. The first one was a Finnigan Surveyor pump with a quaternary gradient system coupled to a Finnigan LCQ Deca IT mass spectrometer using an ESI interface. Sample injections (5 μL) were carried out by a Finnigan Surveyor autosampler. All instruments were from Thermo Fisher Scientific (San Jose, CA).

The electrospray voltage was set at 4.5 kV. The heated capillary temperature was at 200 °C. Nitrogen (99.5% purity) was used as a sheath (0.6 L min^{-1}) and an auxiliary (6 L min^{-1}) gas, and helium (99.999% purity) was used as the collision gas. Mass spectra were recorded in the positive-ion mode between m/z 450 and 2000. MS^2 data were obtained by collision-induced dissociation (CID) of the $[M + H]^+$ ion and acquired in the automatic data-dependent mode with a relative collision energy of 35%, using a total cycle time of approximately 5 s. The ion selection threshold was set at 5×10^5 counts. Data acquisition and processing were managed by Xcalibur software (1.2 version, Thermo Fisher Scientific).

The second LC−MS^2 instrument was an Agilent 1200 Series LC system (equipped with a binary pump, an autosampler, and a column oven) coupled to a 6520 Q-TOF mass spectrometer using an ESI interface working in the positive-ion mode. Sample injections (20 μL) were carried out by the autosampler. All instruments were from Agilent Technologies (Santa Clara, CA). The electrospray source parameters were adjusted as follows: spray voltage, 4.5 kV; drying gas temperature, 300 °C; drying gas flow rate, 6 L min^{-1}; nebulizer pressure, 30 psi; and fragmentor voltage, 150 V. Nitrogen (99.5% purity) was used as a drying and a nebulizer gas, while nitrogen of a higher purity (99.999%) was used as the collision gas. MS^2 spectra were obtained by CID, selecting the target masses (see the Results) and applying collision energies ranging between 30 and 80 eV. Data acquisition and processing were performed using Agilent Mass Hunter Workstation Acquisition version B.02.00 software.

In both cases, the hydrolyzed CMP was dissolved in 5% acetonitrile (v/v) at a concentration of 2.0 mg mL^{-1}, and their separation performed at 25 °C on a Hypersil HyPurity C_{18} (100 mm × 2.1 mm, 3 μm) column (Thermo Fisher Scientific) at a flow rate of 100 μL min^{-1}. A gradient of two eluents was used: eluent A consisted of 0.1% (v/v) formic acid (analytical grade, Merck, Darmstadt, Germany) in water and eluent B consisted of acetonitrile (LC−MS Chromasolv grade, Sigma-Aldrich, St. Louis, MO) containing 0.1% (v/v) formic acid. The elution program was as described by Moreno et al.[13]

Data Treatment. Full-scan mass spectra obtained in the IT LC−MS system were used for an initial assignment of observed ions to the corresponding amino acid sequences. It was based on the known sequence of κ-casein[14] using the protein database Swiss-Prot and TrEMBL and the tools Peptide Mass and FindPept available at www.expasy.org. Parameters for the search were the following: (i) monoisotopic peptide masses were indicated as $[M + H]^+$ with cysteines treated with nothing; (ii) as enzymes, trypsin/chymotrypsin were chosen; (iii) two missed cleavages were allowed; (iv) peptides with a mass larger than 500 u were displayed; (v) the mass tolerance was kept at 0.5 u; (vi) potential phosphorylation on serine and threonine residues was considered. To confirm the sequence of these assigned peptides, their experimental MS^2 spectra from the IT LC−MS^2 system were compared to the theoretical fragmentation of the putative peptides, obtained from the MS-Product software (Protein Prospector, http://prospector.ucsf.edu/). This program was also used for the identification of the phosphopeptides from the MS^2 spectra obtained by the Q-TOF LC−MS^2 system.

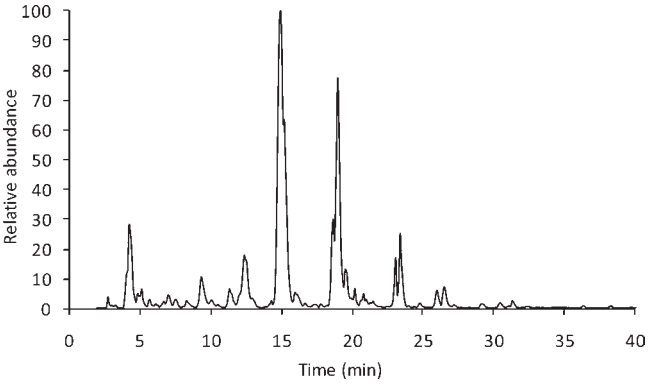

Figure 1. RPLC−ESI−MS base peak profile monitorized on an IT at positive ionization mode of hydrolyzed (trypsin/chymotrypsin) bovine CMP.

RESULTS

Phosphopeptide Characterization by RPLC−ESI−MS^2 on an IT Mass Spectrometer. The initial use of an IT mass spectrometer was based on its robustness, ease of use, high sensitivity in scan, and ability to perform multi-stage fragmentation. The resulting RPLC−ESI−MS base peak chromatogram for bovine CMP after hydrolysis revealed the presence of a complex mixture of peptides eluting between 3 and 33 min, with no traces of the intact CMP (Figure 1). This profile indicated that bovine CMP was efficiently hydrolyzed at neutral pH by trypsin and chymotrypsin, which is in good agreement with previous findings reported by Shammet et al.[15] The presence of a large number of hydrophobic amino acids (6 Val, 7 Ile, and 6 Ala) together with 3 Lys residues in the CMP sequence explains the extensive hydrolysis of CMP with trypsin and chymotrypsin.[15]

The RPLC−ESI−MS^2 characterization of the non-glycosylated fraction of CMP confirmed the presence of 26 peptides with molecular masses ranging between 504 and 1848 u (Table 1). The identified peptides covered the whole CMP sequence, with the exception of the NH_2-terminal methionine. CID MS^2 spectra of phosphothreonine- and, especially, phosphoserine-containing peptides normally show an intense neutral loss of H_3PO_4 (−98 u) from the precursor ion because of the lability of the phosphate group.[16] In addition to this neutral loss, it is necessary to detect sufficient informative fragment ions resulting from peptide backbone cleavages to identify the phosphopeptide sequence. Thus, 6 of these 26 peptides (^{124}Thr-Thr^{135}, ^{124}Thr-Glu^{137}, ^{149}Ser-Asn^{160}, ^{147}Glu-Asn^{160} genetic variants A and B, and ^{145}Thr-Asn^{160}) resulted in monophosphorylation at Ser residues located at positions 149 or 127 (Table 1; spectra not shown). This is in good agreement with the well-established determination of the phosphorylation sites of bovine κ-casein, where Ser^{149} is fully phosphorylated and Ser^{127} is the second most important phosphorylated site.[6]

Nonetheless, there were two additional MS^2 spectra that unambiguously indicated the presence of two monophosphorylated

Table 1. Non-glycosylated Peptides Identified in Hydrolyzed Bovine CMP by RPLC−ESI−MS^2 Using Either an IT or a Q-TOF Mass Spectrometer

t_R (min)	peptide sequence	position[a]	experimental mass $[M + H]^{+}$[b]	genetic variant
3.3	NQDK	113−116	504.1	A and B
4.0	AIPPK	107−111	525.1	A and B
4.0	IASGEPTSTPTTE	125−137	1290.5	A
4.1	TVQVT	161−165	547.0	A and B
4.2	AVESTVA	138−144	676.2	A and B
4.6	TIASGEPTSTPTTE	124−137	1391.4	A
4.9	STPTIE	132−137	647.1	B
5.0	TIAS^{c}GEPTSTPT	124−135	1241.3	A and B
5.2	TIAS^{c}GEPTSTPTTE	124−137	1471.3	A
7.0	TVQVTSTAV	161−169	905.1	A and B
7.1	TVQVTS^{c}TAV	161−169	985.3	A and B
7.8	IEAVESTVA	136−144	918.2	B
8.4	IASGEPTSTPTIE	125−137	1302.6	B
9.4	STVATL	141−146	591.1	A and B
11.9	TIASGEPTSTPTIE	124−137	1403.4	B
12.5	VIESPPEIN	152−160	997.3	A and B
13.2	AVESTVAT^{c}L	138−146	970.4	A and B
15.0	TEIPTIN	117−123	787.2	A and B
16.0	AVESTVATL	138−146	890.2	A and B
16.6	IEAVESTVATL	136−146	1132.3	B
16.7	VIESPPEINTVQ	152−163	1325.5	A and B
18.2	S^{c}PEVIESPPEIN	149−160	1390.3	A and B
18.4	EAS^{c}PEVIESPPEIN	147−160	1590.4	B
19.0	EDS^{c}PEVIESPPEIN	147−160	1634.4	A
20.2	VIESPPEINTVQVT	152−165	1525.5	A and B
21.5	TLEDS^{c}PEVIESPPEIN	145−160	1848.4	A

[a] Residue numbering is based on the Swiss-Prot entry for the mature form of bovine κ-casein (accession number P02668). [b] Monoisotopic mass values. [c] Phosphorylated residue.

peptide matching sequences, ^{138}Ala-Leu^{146} and ^{161}Thr-Val^{169}, which did not contain any of the two described phosphorylation sites (Figure 2). Furthermore, peptide ^{138}Ala-Leu^{146} could have three potential phosphorylation sites at Ser^{141}, Thr^{142}, and Thr^{145}, while peptide ^{161}Thr-Val^{169} contains Thr^{161}, Thr^{165}, Ser^{166}, and Thr^{167} as potential phosphorylation sites. Thus, the MS^2 spectrum of the precursor ion of $[M + H]^+$ at m/z 970.2 (^{138}Ala-Leu^{146}) was characterized by the presence of fragments derived from the neutral loss of H_3PO_4 at m/z 872.2 with one and two subsequent dehydrations, as well as the detection of abundant fragment ions b_6 and b_7 and minor ion b_5 and their corresponding singly and doubly dehydrations. The mass values corresponding to the fragment ions b_5−b_7 were unmodified, indicating that these peptide fragments are not phosphorylated (Figure 2A). This fact indicated that Ser^{141} and Thr^{142} could be ruled out as phosphorylation sites, leaving Thr^{145} as the only residue susceptible to be phosphorylated. This was further confirmed by the detection of the abundant ion at m/z 741.2 derived from the loss of a phosphoric acid molecule (H_3PO_4) of b_8 ion that contains the residue Thr^{145}. Likewise, detection of b_8 and y_4 minor ions containing the phosphate group (corresponding to +80 u compared to the unmodified peptide) further corroborated the residue Thr^{145} as the phosphorylation site.

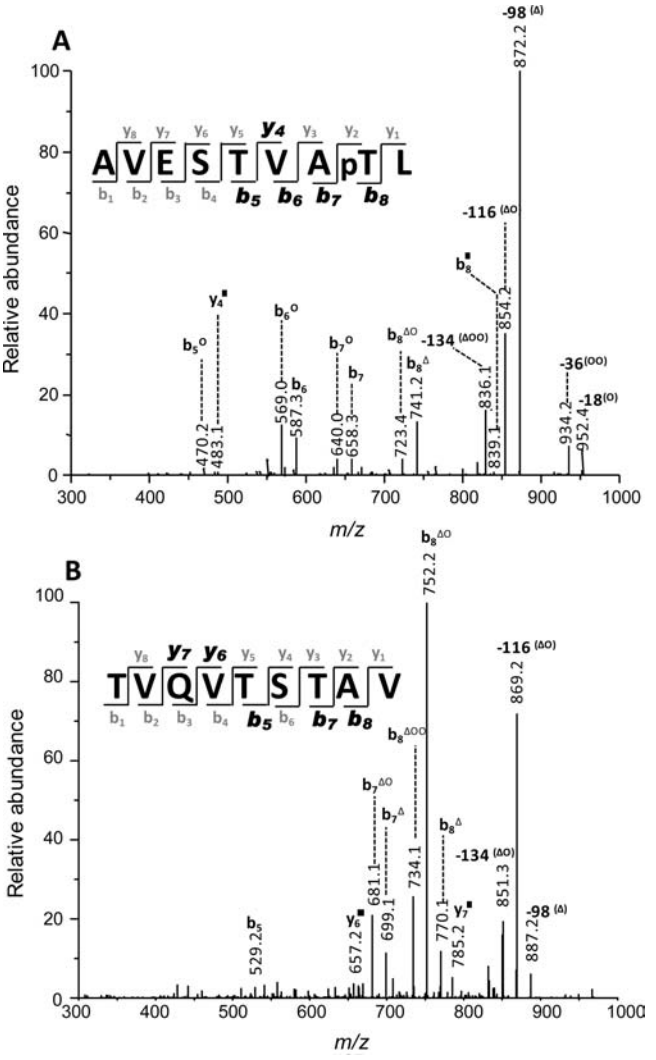

Figure 2. MS^2 spectra of singly charged ions corresponding to κ-casein monophosphorylated peptides (A) ^{138}AVESTVApTL^{146} and (B) ^{161}TVQVTSTAV^{169} acquired using an IT mass spectrometer. Phosphorylation of ^{161}TVQVTSTAV^{169} could be at S^{166} or T^{167}. (■) +80 u (+HPO_3), (△) −98 u [−H_3PO_4 or −(H_2O + HPO_3)], and (○) = −18 u (−H_2O). The observed y_n and b_n fragment ions are highlighted in bold in the peptide sequence.

The MS^2 spectrum of the precursor ion of $[M + H]^+$ at m/z 985.2 corresponding to peptide ^{161}Thr-Val^{169} was dominated by the presence of fragment ions at m/z 869.2, 851.3, and 887.2 corresponding to the neutral loss of a phosphoric acid molecule (H_3PO_4) and singly and doubly dehydrations and by the detection of b_8 and b_7 ions after losing H_3PO_4. These main fragments ions were not sufficient to exclude any potential phosphorylation site. Nevertheless, detection of less abundant fragments corresponding to unmodified b_5 and phosphorylated y_7 and y_6 ions left residues Ser^{166} and Thr^{167} as possible phosphorylation sites (Figure 2B). Unfortunately, no additional ions that could resolve this ambiguity were detected.

Finally and considering the capacity of IT analyzers to perform multiple stages of fragmentation, MS^3 spectra obtained from a collisionally generated ion derived from the loss of H_3PO_4 were performed, with the aim of detecting additional peptide backbone fragmentation. However, the effect of another fragmentation

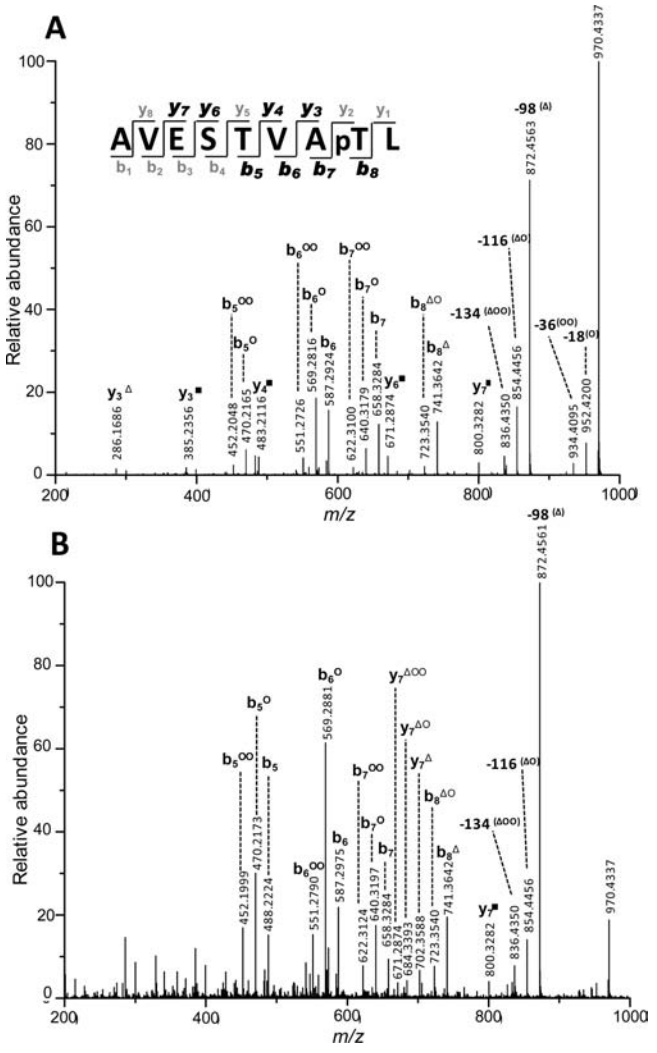

Figure 3. MS^2 spectra of singly charged ions corresponding to κ-casein monophosphorylated peptide ^{138}AVESTVApTL^{146} acquired using a Q-TOF mass spectrometer. (A) CID = 30 eV and (B) CID = 40 eV. (■) +80 u (+HPO_3), (△) −98 u [−H_3PO_4 or −(H_2O + HPO_3)], and (○) −18 u (−H_2O). The observed y_n and b_n fragment ions are highlighted in bold in the peptide sequence.

stage on phosphopeptide characterization was not significant in identifying additional peptide fragments (data not shown).

Phosphopeptide Characterization by RPLC−ESI−MS^2 on a Q-TOF Mass Spectrometer. The use of a second type of mass spectrometer was carried out to unambiguously assign the correct phosphosite localization in peptides containing multiple potential sites of phosphorylation and, thus, to confirm the determination of new and/or minor phosphorylation sites in the bovine κ-casein macropeptide. In this context, it is noteworthy to mention that a Q-TOF mass spectrometer may represent advantages, such as a high sensitivity in MS^2 for parent product scans, as well as its ability to give accurate mass measurements for fragment identification.

On the one hand, Palumbo and Reid[17] indicated that the application of multi-stage mass spectrometry (MS^2 and MS^3) in an IT is valid for routine phosphopeptide identification but not for unambiguous phosphorylation site localization in peptides containing multiple potential phosphorylation sites. These authors reported that, in linear and conventional 3D ITs under typical CID MS^2 conditions, the phosphate group might be gas-phase-rearranged and transferred prior to fragmentation to another serine, threonine, or tyrosine residue present in the precursor peptide and not originally phosphorylated, because of the relatively long (millisecond) time scales associated with ion activation in these instrument platforms. In contrast, using tandem-in-space instruments, such as triple quadrupole (microsecond activation time scale), no product ions resulting from the gas-phase phosphate group rearrangement reactions were observed. Conversely, recent works carried out using CID on IT analyzers have indicated that this phenomenon is negligible.[18−20] Thus, evidence for the gas-phase phosphate group rearrangements were observed specifically for doubly charged species, although the extent of the transfer reaction was insufficient to mislead the phosphorylation site assignment.[18]

On the other hand, it has also been described that IT mass spectrometers generate a higher level of neutral loss than tandem-in-space instruments,[21] which is attributed to the difference in collision energy used and the time frame allowed for fragmentation.[16] This fact implies that the fragment corresponding to the neutral loss of H_3PO_4 (−98 u) should be less abundant in triple quadrupole or Q-TOF mass spectrometers, allowing for the detection of more fragments ions derived from peptide backbone cleavages and, consequently, enabling a more accurate phosphopeptide sequencing. For this reason, the eight phosphorylated peptides previously identified on the IT were also analyzed in the Q-TOF mass spectrometer (a tandem-in-space instrument) at three different collision energy values. MS^2 spectra confirmed the phosphorylation at Ser^{149} or Ser^{127} of the six peptides previously identified on the IT mass spectrometer (data not shown). Q-TOF MS^2 spectra corresponding to peptides ^{138}Ala-Leu^{146} and ^{161}Thr-Val^{169} are shown in Figures 3 and 4, respectively. MS^2 spectra of phosphopeptide ^{138}Ala-Leu^{146} clearly confirmed that Thr^{145} was phosphorylated. Thus, fragment ions b_5−b_7 and their corresponding singly and doubly dehydrations were detected in their unmodified form, while the b_8 ion was detected after losing H_3PO_4. Furthermore, fragment ions y_7, y_6, y_4, and y_3 were also detected in their phosphorylated form, being the two latter ones indicative of the phosphorylation at Thr^{145} (Figure 3A).

Q-TOF MS^2 spectra corresponding to peptide ^{161}Thr-Val^{169} showed the detection of fragment ions b_3−b_5 in their unmodified form, excluding residues Thr^{161} and Thr^{165} as phosphorylation sites. In addition, ions b_6−b_8 were detected after losing H_3PO_4, and they were also detected with a much less abundance containing the phosphate group. The detection of the fragment ion b_6 in its phosphorylated form and after losing H_3PO_4 could allow for the unambiguous localization of the phosphorylation at Ser^{166}. In addition, fragment ions y_5, y_6, and y_7 with and without the phosphate group could also be detected (Figure 4).

■ DISCUSSION

The determination of the minor phosphorylation site at Thr^{145} conforms to the sequence motif, Ser/Thr-X-Glu/Ser-(phosphorylated), recognized by the casein kinase from the lactating bovine mammary gland (also called Golgi apparatus casein kinase or G-CK). Thus, our data support the findings by Holland et al.,[11] who pointed out Thr^{145} as the third phosphorylation site. Nevertheless, the phosphorylation of Thr residues instead of Ser residues in caseins is much more uncommon, and this might reflect alternative kinase activities.[11]

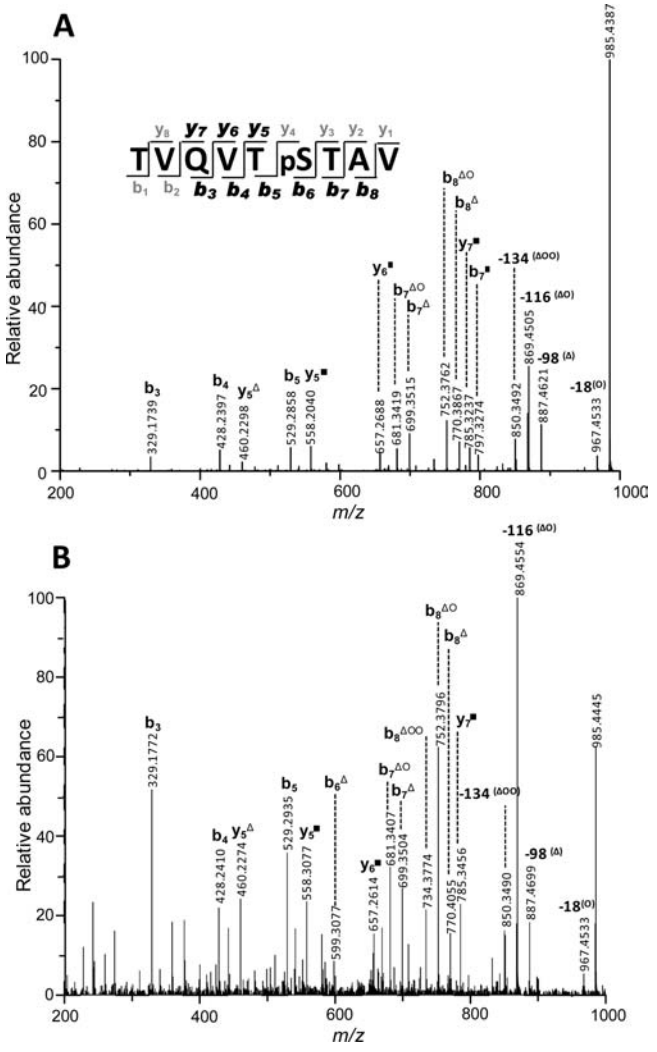

Figure 4. MS^2 spectra of singly charged ions corresponding to κ-casein monophosphorylated peptide ^{161}TVQVTpSTAV^{169} acquired using a Q-TOF mass spectrometer. (A) CID = 30 eV and (B) CID = 40 eV. (■) +80 u (+HPO_3), (△) −98 u [−H_3PO_4 or −(H_2O + HPO_3)], and (○) −18 u (−H_2O). The observed y_n and b_n fragment ions are highlighted in bold in the peptide sequence.

With regard to the determination of the previously unrecognized phosphorylation site, Ser^{166}, this is localized within a sequence that apparently does not fulfill any phosphorylation site motifs recognized by G-CK or even other casein kinases, such as CKI or CKII.[22] Therefore, the determination of Ser^{166} as a new phosphorylation site might be explained by either a possible implication of a novel or uncharacterized protein kinase, in which the sequence motif is fulfilled by the sequence surrounding Ser^{166}, or that G-CK is also able to recognize consensus sequences different from the well-established Ser/Thr-X-Glu/Ser(phosphorylated). In this sense, Brunati et al.[23] reported a novel consensus sequence in proline-rich protein 1 (PRP1), Ser-X-Gln-X-X-(Asp/Glu)^3, which is phosphorylated by G-CK with an efficiency similar to the canonical efficiency. Another example could be the human osteopontin, which is a milk integrin-binding highly phosphorylated glycoprotein that also contains a phosphorylated serine residue located within a sequence atypical of those normally recognized by G-CK or CKII.[24] Furthermore, to the best of our knowledge, it is striking that, despite its importance, the genes encoding for G-CK are still unknown and their activity has to be ascribed to the category of "orphan" enzymes.[25,26] Likewise, the determination of a fourth phosphorylation site in bovine κ-casein might lead to the occurrence of a four phosphorylated form in a very low abundance, because the three phosphorylated form was estimated to be present as low as 1.2−2%.[7,8] Another possibility could be that the three phosphorylated form of bovine κ-casein could contain either Thr^{145} or Ser^{166} as the third phosphorylation site.

On the other hand, Ser^{168} in the caprine and ovine κ-casein sequences, which is equivalent to Ser^{166} in the bovine sequence, has previously been described as a major phosphorylation site,[27−29] and this behavior was attributed to the fact that the C-terminal sequences of bovine and caprine/ovine κ-caseins differ by the substitution of Ala (bovine) by Glu (caprine/ovine), thus leading to the occurrence of the consensus sequence for G-CK (^{168}Ser-Thr-Glu^{170}) in the caprine and ovine species.[6] However, our data point out that this residue is also a minor phosphorylation site in the bovine counterpart.

Finally, these results highlight the complex heterogeneity of milk caseins, despite the large number of studies performed on milk proteins. Likewise, although the biological or structural role of these minor phosphorylation sites, if any, remain to be determined, data presented in this work indicate that RPLC−ESI−MS^2 on a Q-TOF mass spectrometer is a useful technique for the identification of low-abundance phosphorylation sites in caseins. This is particularly important if it is considered that even a single phosphorylation event can have a significant impact on protein activity.[30]

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Funding Sources

This work has been funded by projects Consolider Ingenio 2010 FUN-C-FOOD CSD2007-00063 (MICINN), ALIBIRD S2009/AGR-1469, and ANALISYC-II S2009/AGR-1464 (CAM).

ACKNOWLEDGMENT

Oswaldo Hernández-Hernández thanks the CSIC for a JAE-Predoc Ph.D. Grant. We thank Laurie Davis from Davisco Foods International, Inc. for kindly providing us with CMP.

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