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Identification of Multiphosphorylated Peptides in Milk

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Supporting Information

ABSTRACT: Multiphosphorylated peptides endogenously present in milk exert anticariogenic activity due to their calcium binding capacity. This study performed comprehensive analysis of multiphosphorylated peptides in raw milk using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Since phosphopeptides are often negatively discriminated during ionization, putative phosphopeptides were identified by three different methods: (i) selective detection in 4-chloro-α-cyanocinnamic acid MALDI matrix compared to α-cyano-4-hydroxycinnamic acid; (ii) higher relative signal intensity in negative compared to positive ionization mode; and (iii) detection of signal pairs with mass differences of −80 Da or multiples thereof before and after enzymatic dephosphorylation. Thus, 18 putative phosphopeptides from raw milk were annotated. Peptide structures were then determined by product ion spectra from targeted liquid chromatography electrospray ionization tandem-MS analysis. Thus, β-casein$_{33-48}$, β-casein$_{29-48}$, β-casein$_{1-21}$, β-casein$_{2-25}$, β-casein$_{1-25}$, β-casein$_{1-27}$, β-casein$_{1-28}$, β-casein$_{1-29}$, β-casein$_{1-32}$, α_{S2}-casein$_{1-21}$, and α_{S2}-casein$_{1-24}$ were revealed as major peptides with one or four phosphorylation sites in raw milk.

KEYWORDS: *milk, native multiphosphorylated peptides, casein phosphopeptides, mass spectrometry, MALDI-TOF-MS*

INTRODUCTION

Peptides, which are endogenously present in milk or released from milk proteins either by biotechnological processes or by digestion, may exert various physiological functions. Besides others, the oral intake of bioactive milk peptides may affect the cardiovascular, digestive, immune, and nervous systems.[1,2] Casein phosphopeptides (CPP) are a subgroup of bioactive milk peptides, which are obtained, for example, by tryptic hydrolysis of caseins and further enrichment of phosphorylated peptides.[3] Major components of different CPP preparations are α_{S2}-casein$_{1-32}$, β-casein$_{1-28}$, α_{S1}-casein$_{59-79}$, or β-casein$_{1-25}$.[4,5] Furthermore, CPPs are released by gastrointestinal digestion after the consumption of milk proteins.[6] CPPs readily bind and solubilize bivalent metal ions such as Ca^{2+} and, thus, may enhance the mineral absorption from some matrices in the gastrointestinal tract.[7] In the oral cavity, CPPs have a beneficial influence on tooth enamel remineralization, delay of demineralization, and buffering of plaque pH.[5] Additionally, in vitro studies suggested immunostimulatory and antioxidative activity of phosphopeptides.[3,8,9]

In addition to CPPs released in the gastrointestinal tract or prepared by enzymatic hydrolysis, phosphopeptides can be endogenously present in milk. Native milk phosphopeptides may be of particular interest for the impact of milk on dental health. Furthermore, whey, which is available in large amounts as a byproduct of cheese production, may contain native milk phosphopeptides and could be considered as a source to generate bioactive phosphopeptides. However, endogenous phosphopeptides are difficult to monitor by conventional peptide profiling techniques. Mass spectrometric analysis of phosphopeptides is challenging, because the phosphate groups lead to a low ionization efficiency of the peptide, which especially hampers the detection of multiphosphorylated peptides.[10] Thus, the ionization of phosphopeptides can be suppressed by nonphosphorylated peptides in complex peptide mixtures.[11]

Different approaches have been adopted to overcome these problems. The chemical modification of phosphopeptides by β-elimination of phosphoric acid and subsequent derivatization with nucleophiles results in increased ionization and allows the detection of phosphopeptides among nonphosphorylated peptides.[12] Alternatively, enzymatic dephosphorylation by phosphatases can be applied.[10,13,14] For the analysis of low abundant phosphopeptides, specific enrichment techniques are available, for example using immobilized metal ion affinity chromatography (IMAC)-Fe resins, TiO_2-based resins, or hydroxyapatite.[15−19] Because of the poor ionization properties of phosphopeptides, special matrices have been developed facilitating their detection by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Thus, improved detection of phosphopeptides was achieved by the combination of 2,5-dihydroxybenzoic acid with phosphoric acid[20] or by using 4-chloro-α-cyanocinnamic acid (ClCCA) as MALDI matrix.[21] In MALDI-TOF-MS, phosphopeptides show higher relative signal intensity in the negative mode than nonphosphorylated sequences. Consequently, phosphorylated and nonphosphorylated peptides can be distinguished by the ratio of relative signal intensities in positive and negative mode.[22] Applying liquid chromatography electrospray ionization (LC-ESI)-MS, the loss of PO_3^- in collision-induced dissociation (CID) is characteristic for peptides containing phosphoserine or phosphothreonine residues. Therefore, the loss of 79 Da can be used by a

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precursor ion scan in the negative mode.[23] Alternatively to the precursor ion scan in negative mode, a neutral loss scan was used, where the loss of H_3PO_4 (m/z 98) indicates the presence of phosphoserine and -threonine residues.[24] Finally, the peptide structure and phosphorylation sites can be unambiguously determined by LC−ESI-MS/MS analysis.[25]

The aim of the present study was the comprehensive analysis of endogenous multiphosphorylated peptides in milk by combining different mass spectrometric approaches.

MATERIALS AND METHODS

Milk Samples. Raw milk was obtained from a local farm directly after morning milking and cooled immediately on ice. In order to reduce the influence of individual differences, a pooled sample from 30 animals was used [Fleckvieh cattle, with an average age of 4.5 years; about one-third of the animals were in the first lactation period and 10% in the fifth lactation period; the average somatic cell count of the herd was 168 ± 71 × 10^3 cells/mL (mean ± SD, n = 10 analyses/year) indicating a good health status]. After centrifugation at 1100g for 60 min at 4 °C, the fat layer was removed, and aliquots of the defatted milk were frozen at −80 °C.

Sample Preparation. Defatted milk was subjected to peptide purification either directly or after removal of the caseins (=whey), or after removal of all proteins (=ultrafiltrate). For the preparation of whey, 5 mL of the milk sample was acidified with 1% (v/v) acetic acid solution to reach a pH of 4.6. Precipitated caseins were removed by centrifugation (1100g, 15 min, 4 °C) and filtration through a 0.22 μm membrane filter (Carl Roth, Karlsruhe, Germany). For the preparation of ultrafiltrate, whey was subjected to ultrafiltration (Pall Nanosep centrifugal devices with Omega membrane and a molecular weight cutoff of 10 kDa, Pall, Dreieich, Germany) at 10000g and 10 °C for 45 min. The ultrafiltrate was used for analysis by UHPLC−ESI-MS/MS. MALDI-TOF-MS analysis was performed of milk and, additionally, of whey for comparison of signal intensities in positive and negative ionization mode and for dephosphorylation experiments, since the use of whey resulted in higher signal intensity compared to milk. In all figures, MALDI-TOF mass spectra from milk matrix are shown.

For MALDI-TOF analysis, peptides were purified from the defatted milk or whey with C18-ZipTip (Millipore, Billerica, USA) pipet tips. All solutions were applied by aspirating and dispensing the solutions with a variable piston pipettor. The tips were wetted with 3 × 10 μL of acetonitrile and equilibrated with 3 × 10 μL of 0.1% trifluoroacetic acid (TFA) in water. After application of 10 × 10 μL of sample, the tip was washed with 5 × 10 μL of 0.1% TFA and the peptides were eluted by aspirating and dispensing 10 × 5 μL of 60% acetonitrile, containing 0.1% TFA. The use of ZipTip extraction under these conditions made it possible to recover relatively hydrophilic peptides with four phosphorylation sites. However, we cannot fully exclude that some highly hydrophilic peptides were lost during this step.

Peptide analysis by ultrahigh-performance liquid chromatography (UHPLC) ESI-MS/MS required protein removal, so that the ultrafiltrate was used for analysis. The ultrafiltrate was further purified by stage tip extraction according to Rappsilber et al.,[26] with some modifications (see also Supporting Information). For the assembly of a stage tip, four small layers with a diameter of 1 mm each were punched out of a C-18 Empore Disk (3M, Neuss, Germany) with a biopsy punch (KAI Medical, Solingen, Germany) and inserted into a 1−20 μL epTIPS pipet tip (Eppendorf, Hamburg, Germany). The tip was then placed into the perforated cap of a 2 mL test tube (Eppendorf, Hamburg, Germany). For peptide extraction, 50 μL of acetonitrile/0.1% formic acid was pipetted into a stage tip and spun down with centrifugation at 1840g and 25 °C for 30 s. This procedure was repeated once, followed by two spin steps with 50 μL of 0.1% formic acid each. In the same way, 50 μL of ultrafiltrate of the milk sample was loaded to the stage tip and spun down for 3 min. Subsequently, the tips were washed twice each time applying 50 μL of 0.1% formic acid to the tip and centrifuging for 3 min. Prior to elution, the stage tip was removed from the 2 mL test tube and placed in a 0.1 mL glass microinsert for HPLC vials (VWR, Darmstadt, Germany), which in turn was placed in a 1.5 mL test tube. Subsequently, 10 μL of 60% acetonitrile/0.1% formic acid was pipetted into the tip and spun down for 3 min.

Dephosphorylation of Milk Peptides. The pH of the sample (ultrafiltrate for LC−MS, whey or milk for MALDI-TOF) was adjusted to 7.9 ± 0.05 with ammonia (1% v/v). An aliquot of 200 μL of the sample was then mixed with 5 μL of calf intestine alkaline phosphatase (CIAP grade I; Roche, Mannheim, Germany) or 5 μL of water (control). After incubation for 60 min in a thermomixer (Eppendorf, Hamburg, Germany) at 37 °C and 550 rpm, the sample was cooled on ice and then subjected to further purification with ZipTips and subsequent MALDI-TOF measurement or processing to ultrafiltrate, followed by stage tip purification and UHPLC−ESI-MS as described above.

MALDI-TOF Analysis. The eluate of ZipTip purification was mixed 1:1 with matrix (C1CCA or α-cyano-4-hydroxycinnamic acid (HCCA), 5 mg/mL in 60% acetonitrile, containing 0.1% TFA (v/v)), spotted onto a ground steel target and air-dried before analysis in a Bruker Autoflex MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) in positive reflector mode (acceleration voltage 20 kV, 140 ns delay) with a mass range from 700 to 5000 m/z. The same settings were used for phosphopeptide analysis in negative reflector mode. For each spectrum, at least 300 single subspectra were averaged. External calibration was performed with Bruker peptide calibration standard II (Bruker Daltonics, Bremen, Germany).

Analysis by UHPLC−ESI-MS/MS. UHPLC was performed on a Dionex UltiMate 3000 RS system (Dionex, Germering, Germany) coupled to an API 4000 QTrap mass spectrometer (AB Sciex, Foster City, CA, USA) fitted with an ESI source (Applied Biosystems, Foster City, CA, USA). An aliquot of 5 μL of the stage tip peptide extract diluted 1:1 with eluent A (0.1% formic acid in water) was injected into a Waters 1.7 μm Acquity BEH 300 C18 (2.1 mm × 100 mm) column with a flow rate of 0.3 mL/min and a column temperature of 30 °C. The peptides were separated applying a gradient of 0.1% formic acid (eluent A) and LCMS-grade acetonitrile (eluent B) (−8 min 5% B, 0 min 5% B, 60 min 60% B, 60.1 min 95% B, 65 min 95% B). By using a two-position valve, only eluate between 2 and 60 min was led into the mass spectrometer. The ion source was operated with a voltage of 5500 V and a declustering potential of 50 V. The solvent was dried with nitrogen at 500 °C.

To determine the retention time and charge state of the phosphopeptides previously detected by MALDI-TOF-MS, mass scans (250 amu/s, target mass ± 5 m/z) were performed using the third quadrupole of the hybrid mass spectrometer as linear ion trap for enhanced resolution and sensitivity.[27,28] Tandem mass spectra were then acquired in enhanced product ion (EPI) mode with collision energy of 30 V and collision energy spread of 10 V. Spectra with insufficient fragmentation were acquired again with a collision energy of 40 V and a collision energy spread of 10 V. Analyst software version 1.5.1 was used for data acquisition and processing.

Database Search. Spectra from ESI-MS/MS were searched against SwissProt Database by Mascot (Matrix Science, London, U.K.). Search parameters were as follows: taxonomy, other mammals; enzyme, semiTrypsin; variable modifications, phosphorylation (serine, threonine); mass tolerance 0.2 Da for precursor ions and 0.5 Da for product ions obtained by ESI-MS/MS.

Since search with the parameter semiTrypsin may not reveal all peptides, subsequent search against a FASTA-database consisting of α_{S1}- (P02662), α_{S2}- (P02663), β- (P02666), and κ-casein (P02668) as well as α-lactalbumin (P00711), β-lactoglobulin (P02754), and lactophorin (P80195) was performed by Proteome Discoverer software without enzyme restriction. Phosphorylation of serine and threonine were allowed as variable modifications. Mass tolerance was set to 0.2 amu for precursor ion spectra and 0.5 amu for product ion spectra of ESI-MS/MS. Due to some lower ion scores in Mascot, the spectra were verified by manual evaluation.

All experiments were performed in triplicate using pooled milk samples from different days. Only peptides that were detected in all three samples were considered.

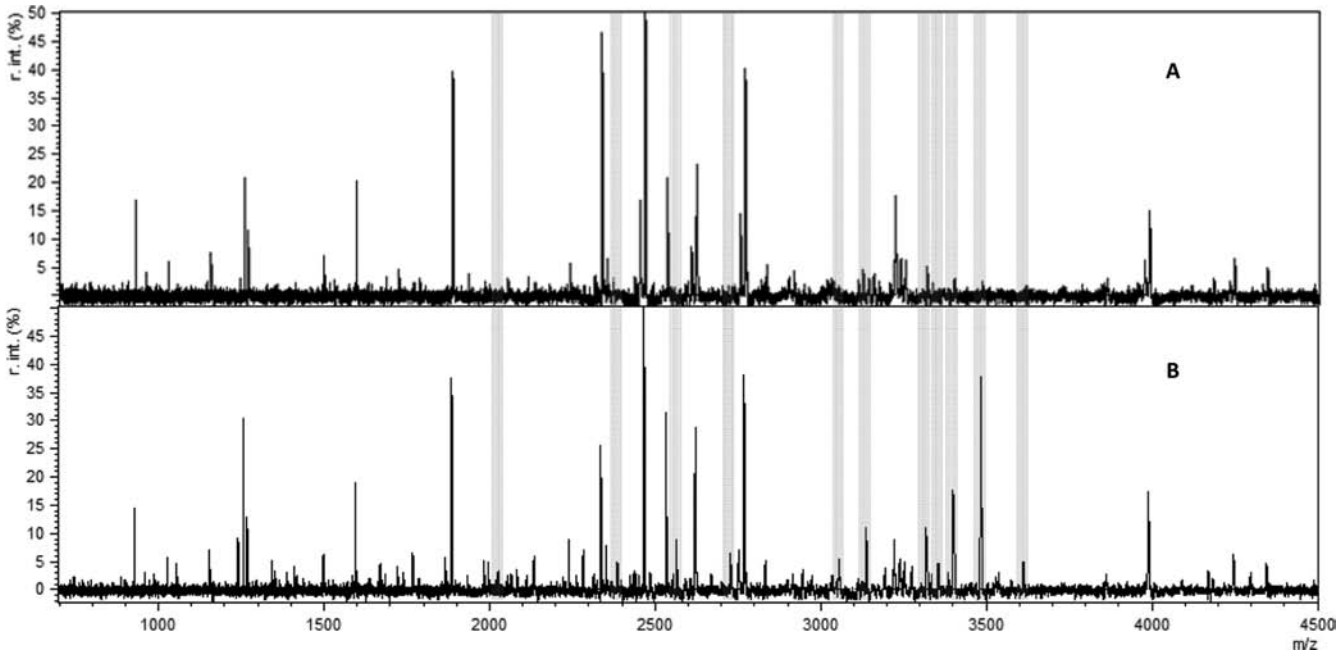

Figure 1. MALDI-TOF spectra of the peptide fraction acquired with (A) HCCA and (B) ClCCA matrix after C-18 ZipTip extraction of raw milk. Additional signals in the spectrum acquired with ClCCA matrix are marked with gray bars.

RESULTS AND DISCUSSION

Commonly applied peptide profiling techniques often fail to detect multiphosphorylated peptides because they are negatively discriminated during mass spectrometric analysis. The phosphate groups result in low ionization efficiency of phosphopeptides, so that especially the detection of multiphosphorylated peptides presents a challenge.[10] Phosphopeptides can therefore be suppressed by nonphosphorylated peptides in complex peptide mixtures.[11] Thus, the present study aimed at the comprehensive analysis of endogenous multiphosphorylated peptides in raw milk. For this purpose, (multi)phosphorylated peptides were identified during MALDI-TOF-MS analysis using a combination of different MS modes; the structure of putative phosphopeptides was then determined by targeted UHPLC−MS/MS analysis.

Following reports that ClCCA, a newly developed MALDI-TOF-MS matrix, yields better ionization of phosphopeptides[21,29] than the common HCCA matrix for peptide analysis,[30] MALDI-TOF-MS spectra of the raw milk peptide fraction were recorded using both matrices. The MALDI-TOF-MS spectra of milk peptides acquired with ClCCA matrix revealed 11 additional signals, which were not observed using HCCA (Figure 1). The new signals were quite abundant, indicating that their appearance was the consequence not only of the lower detection limit of the method but also of a selective improvement of ionization efficiency for previously poorly ionized (multi)phosphorylated peptides. Thus, the 11 signals that could be exclusively observed in the milk peptide spectrum obtained with ClCCA matrix indicated the presence of phosphopeptides (Table 1).

Additionally, two further methods were used for the assignment of putative phosphopeptides. For a differentiation between phosphorylated and nonphosphorylated peptides, samples were analyzed in negative MALDI-TOF mode. Figure 2 shows the comparison of MALDI-TOF-MS spectra of milk peptides obtained in positive and negative mode (ClCCA matrix). A high quotient of the relative ion signal in negative mode to the relative ion signal in positive mode can indicate the presence of a phosphopeptide.[22] This approach allows a very fast overview, because spectra in negative and positive mode can be generated from the same spot. Although fewer peptides were ionized in the negative mode than in positive mode and the absolute signal intensities were generally lower (Figures $2A_1$ and $2B_1$), 14 signals showed higher relative signal intensity in

Table 1. MALDI-TOF-MS Signals of Endogenous Milk Peptides Recorded after C-18 ZipTip Extraction of Raw Milk or Whey[a]

signal	ClCCA $[M+H]^+$	negative mode $[M-H]^-$	$[M+H]^+$ dephosphorylated	mass diff [amu]	theor phosphorylation degree
1	2061.9	2059.9	1981.9	80	1
2	2391.2	2389.1			
3	2560.2	2558.1	2480.2	80	1
4		2579.4			
5		2663.0			
6	2747.0	2745.0	2427.0	320	4
7		2964.1	2646.2	320	4
8	3049.4	3047.4			
9		3120.2	2802.4	320	4
10	3132.2	3130.2	2812.2	320	4
11			3142.5		
12			3179.5		
13	3313.7				
14	3349.4	3347.4	3029.4	320	4
15	3394.5	3392.5			
16	3477.5	3475.5	3157.5	320	4
17	3605.6	3603.6	3285.6	320	4
18			3655.8		

[a]The presence of phosphopeptides is indicated by selective detection using ClCCA matrix, higher ionization efficiency in the negative mode, and/or mass shifts of $n \times -80$ Da after enzymatic dephosphorylation. Theoretical phosphorylation degree was calculated by the detected mass difference after phosphorylation.

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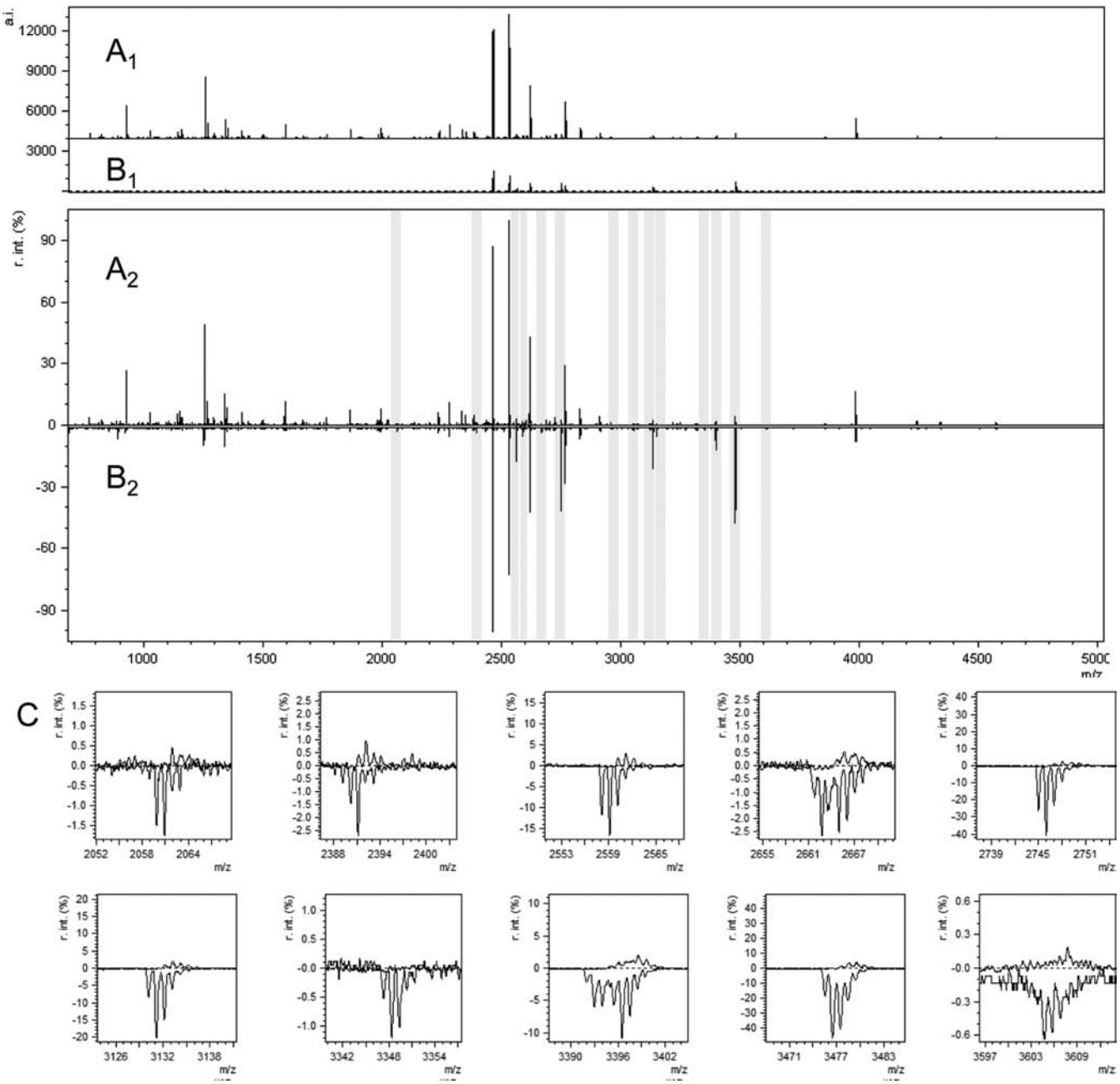

Figure 2. MALDI-TOF spectra of the peptide fraction acquired with ClCCA matrix after C-18 ZipTip extraction of raw milk in the positive (A_1, A_2) and negative mode (B_1, B_2). The number and intensities of the signals are generally higher in the positive mode than in the negative mode (A_1 and B_1). Normalization of the spectra (A_2 and B_2, spectrum B_2 flipped for better comparability) revealed signals that show higher relative intensities in negative mode (marked by gray bars). Exemplarily, ten signals, which show higher relative signal intensity in the the negative compared to the positive mode, are zoomed in in panel C.

negative mode than the corresponding signals in the positive mode spectrum after normalization of the spectra (Figures $2A_2$ and $2B_2$, Table 1). Except the peptide signal at m/z 3313.7, all signals specifically detected in ClCCA matrix also showed higher relative intensity in the spectra acquired in the negative mode, supporting the assumption that they were actually phosphopeptides. Besides, four new signals were observed that showed better relative ionization efficiency in negative mode.

Further confirmation was then obtained by dephosphorylation experiments, which allow indirect identification of phosphopeptides.[13] Dephosphorylation of milk peptides results in the loss of $H_1PO_3^-$ and leads to mass shifts of −80 Da or multiples thereof, according to the phosphorylation degree (recorded in positive mode). Thus, phosphopeptides can be identified and the phosphorylation degree can be determined in one experiment.

The spectra of the samples after dephosphorylation, which was performed with calf intestine alkaline phosphatase, showed significant differences compared to the original spectra (Figure 3) and revealed 12 new signals (Table 1).The appearance of 9 out of those 12 new signals was accompanied by the loss of signals that had been putatively assigned to phosphopeptides in the native samples. The typical m/z differences of 80 Da or multiples thereof could indeed be detected between the missing

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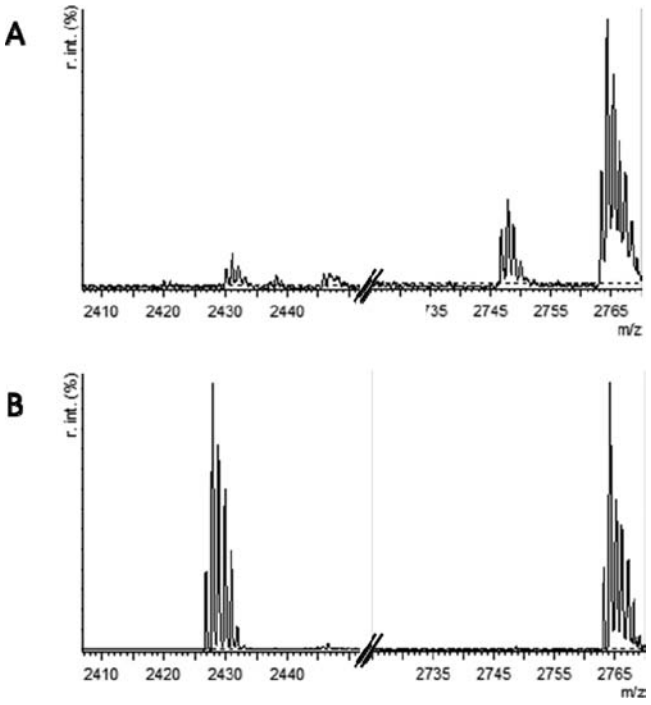

Figure 3. MALDI-TOF spectra of the peptide fraction acquired with ClCCA matrix after C-18 ZipTip extraction of raw milk. The dephosphorylation of peptides is exemplarily shown for m/z 2747.0. Spectrum A is an untreated control; spectrum B was acquired after dephosphorylation with calf intestine alkaline phosphatase (CIAP). The peak at m/z 2747.0 disappears after dephosphorylation, while a new peak arises after dephosphorylation at m/z 2427.0. The mass difference of the signal pair is 320 Da, which indicates the loss of four phosphate groups (80 Da each).

nine signals and the new signals which appeared after dephosphorylation (Figure 3, Table 1). Two signal pairs showed a mass shift of 80 Da indicating a single phosphorylation. The other seven signal pairs differed by 320 Da representing four phosphate groups.

With the exception of the peak at m/z 2391.2, which could also be observed after dephosphorylation and, therefore, apparently did not refer to a phosphorylated peptide, all signals that appeared in the first experiment with ClCCA matrix were lost after dephosphorylation.

Five of the signals that disappeared after treatment with phosphatase could not be matched to a dephosphorylated peptide by the characteristic mass difference. On the other hand, three signals which appeared after dephosphorylation could not be matched to a phosphorylated precursor peptide.

In total, 18 peptides were annotated as putative phosphopeptides by one or more of the applied MS methods (selective detection by ClCCA matrix, higher relative signal intensity in the negative mode and mass analysis before and after dephosphorylation). For structure assignment, enhanced product ion spectra of putative phosphopeptides were recorded by UHPLC–ESI-MS/MS (Figure 4). In most cases the product ion spectra of the phosphopeptides could be used for database search. However, the quality of the product ion spectra of three phosphopeptides was not sufficient for structure analysis. Therefore, the corresponding dephosphorylated form was subjected to structure analysis.

In contrast to untargeted peptide analysis, which is commonly performed using ESI-LTQ-Orbitrap MS technology, targeted sequence analysis could be carried out using tandem-MS with lower mass resolution, namely, ESI-QTRAP-MS/MS. Thus, 11 phosphopeptides could be identified, which exclusively were fragments of β- and α_{S2}-casein (Table 2). The detected phosphorylation sites were in accordance with those reported in literature for the intact precursor proteins.[31] The two breakdown products of α_{S2}-casein were derived from the N-terminal sequence. Seven peptides were released from the N-terminal sequence of β-casein and two peptides from the positions 33–48 and 29–48 of β-casein.

The peptide with signal m/z 2391.2, which appeared in ClCCA matrix and showed similar ionization behavior as phosphopeptides but no mass shift after dephosphorylation, was identified by UHPLC–ESI-MS/MS as the nonphosphorylated β-casein$_{106-125}$ fragment with the sequence HKEMPFPKYPVEPFTESQSL. Although this peptide does not contain a phosphorylation site and possesses only a slightly acidic pI of 5.5, its ionization properties are most likely caused by three glutamic acid residues in the sequence.

The sequence of the other six signals, which had been annotated as putative phosphopeptides, is still unknown. The quality of the product ion spectra was probably insufficient for database search or the peptides contained post-translational modifications, such as glycosylation, which were not considered in the Mascot-database search.

The recent study applied three different MS methods to annotate phosphopeptides. Selective detection using ClCCA over HCCA matrix correctly predicted 6 out of the 11 identified phosphopeptides, whereas relative signal intensity ratio in positive and negative mode correctly predicted 10 out of 11 phosphopeptides. Dephosphorylation provided nine signal pairs which were correctly identified as phosphopeptides later. Additionally, one peptide was identified as phosphopeptide due to its appearance after dephosphorylation, whereas its phosphorylated precursor was not detected by any of the methods (β-casein$_{1-32}$). Since the MS signal appeared only after dephosphorylation and since its precursor protein contains four phosphorylation sites in the sequence, however, it was deduced that the 4-fold phosphorylated form was originally present in the milk sample.

Thus, comparison of the ratio between positive and negative ionization mode in ClCCA matrix was the most successful single method to annotate putative phosphopeptides. This method is very easy and fast, because it allows direct MALDI-TOF-MS analysis of the samples without further sample preparation. However, an additional dephosphorylation step can provide optimal coverage. Overall, the combination of MS annotation of multiphosphorylated peptides with targeted sequence analysis was similarly effective as the use of selective enrichment techniques combined with MALDI-TOF-MS analysis. Very recently, casein phosphopeptides were analyzed in milk after selective enrichment with hydroxyapatite.[18] Seven out of the eleven phosphopeptides, which were identified in the present study, have also been detected in the previous study after hydroxyapatite enrichment.[18] In contrast, two phosphopeptides were only detected after hydroxyapatite enrichment, but did not appear in the present study.[18] Thus, it can be concluded that the present combined mass spectrometric method and the analysis of multiphosphorylated peptides after hydroxyapatite enrichment represent complementary approaches for the comprehensive analysis of multiphosphorylated peptides in native milk.

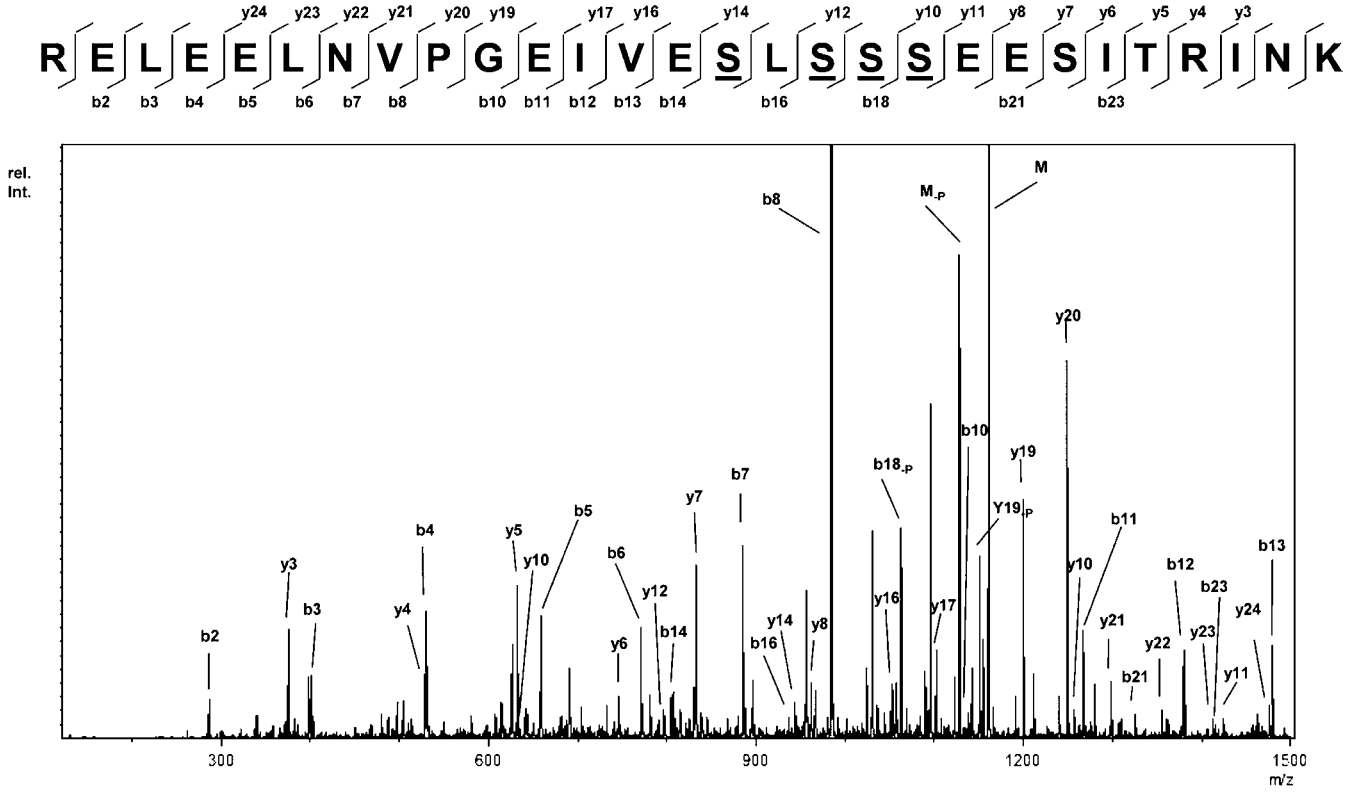

Figure 4. Product ion spectrum of the peptide RELEELNVPGEIVESLSSSEESITRINK with a precursor mass of m/z 1159.8 and a charge state of 3. Detected fragment ions are annotated in the spectrum and also shown in the sequence. The b- and y-ion series are singly and doubly charged. Phosphoserine residues are underlined (S). "−P" added to an ion indicates the loss of a phosphate group during fragmentation.

Table 2. Endogenous Milk Peptides, Identified by UHPLC−ESI-MS/MS after Stage Tip Extraction of Milk Ultrafiltrate[a]

No.	$[M+H]^+$	protein precursor	position	phosphorylation degree	amino acid sequence	Mascot score	$[M+H]^+$ dephosphorylated
1	2061.90*	β-Casein	33-48	1	K.FQSEEQQQTEDELQDK.I	51	1981.93
2	2391.15*	β-Casein	106-125	-	K.HKEMPFPKYPVEPFTESQSL.T	40	
3	2560.15*	β-Casein	29-48	1	K.KIEKFQSEEQQQTEDELQDK.I	46	2480.18
4	2664.99*	β-Casein	1-21	4	RELEELNVPGEIVESLSSSEE.S	21	2345.12
5	2747.00*	α_{S2}-Casein	1-21	4	KNTMEHVSSSEESIISQETYK.Q	32	2427.13
6	2966.10*	β-Casein	2-25	4	R.ELEELNVPGEIVESLSSSEESITR.I	10	2646.23
7	3122.26	β-Casein	1-25	4	RELEELNVPGEIVESLSSSEESITR.I	85	2802.39*
8	3132.19*	α_{S2}-Casein	1-24	4	KNTMEHVSSSEESIISQETYKQEK.N	19	2812.32
9	3349.39	β-Casein	1-27	4	RELEELNVPGEIVESLSSSEESITRIN.K	69	3029.52*
10	3477.48*	β-Casein	1-28	4	RELEELNVPGEIVESLSSSEESITRINK.K	47	3157.61
11	3605.58*	β-Casein	1-29	4	RELEELNVPGEIVESLSSSEESITRINKK.I	40	3285.71
12	3975.80	β-Casein	1-32	4	RELEELNVPGEIVESLSSSEESITRINKKIEK.F	41	3655.93*

[a]Where applicable, the amino acids from the parent protein upstream or downstream of the detected peptide sequence are shown in grey. Phosphorylated serine residues are underlined (S), and cleavage sites are indicated by dots. Peaks indicated with the symbol "*" were used for identification.

Sequence analysis revealed α_{S2}- and β-casein as major precursors for endogenous multiphosphorylated milk peptides (Figure 5). Phosphopeptides derived from α_{S1}-casein or lactophorin, which contain 9 and 5 phosphoserine residues, were not detected.[31,32] β-Casein contains five phosphorylation sites at serine residues 15, 17, 18, 19, and 35. All of them are located relatively close to the N-terminus and could be recovered in the detected phosphopeptides. α_{S2}-Casein, in contrast, contains 12 phosphorylation sites at serine residues 8, 9, 10, 16, 31, 56, 57, 58, 61, 129, 131, and 143. The four sites that are located closest to the N-terminus were recovered in endogenous peptides.

Plasmin is a major endogenous milk protease and responsible for the formation of the γ- and λ-caseins and proteose peptones,

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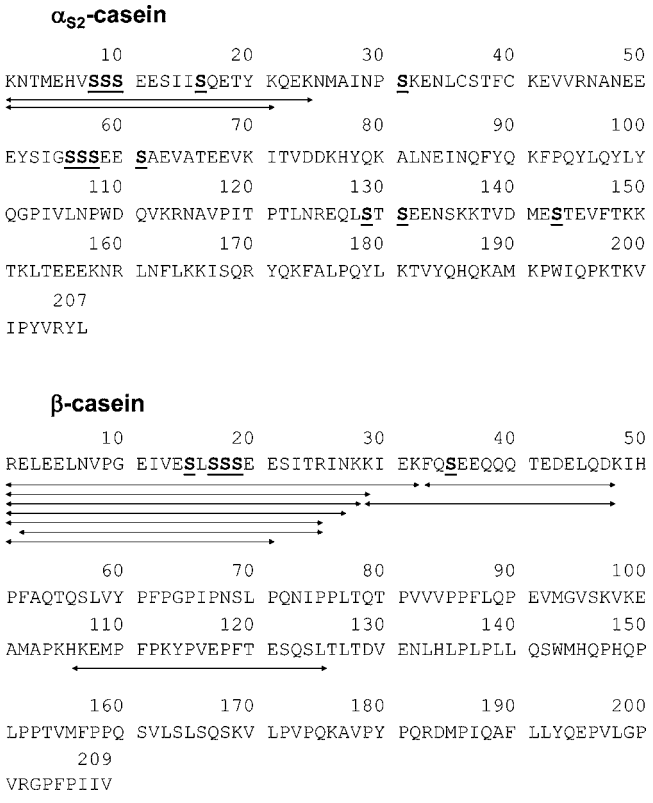

Figure 5. Sequence of α_{S2}-casein variant A and β-casein variant A2 in single letter code. Phosphoserine residues are marked by bold, underlined letters. Endogenous (phospho-)peptides identified by MS/MS are marked by horizontal black double arrows.

proteolytic fragments of α_{S1}-, α_{S2}-, and β-casein, including PP8F/β-casein$_{1-28}$, while κ-casein seems to be resistant to plasmin proteolysis.[33] In milk, plasmin is associated with casein micelles. It cleaves Lys−X and Arg−X bonds, but prefers Lys−X.[34] In our experiments, we found mostly plasmin cleavage sites. The cleavage between positions leucine$_{125}$ and threonine$_{126}$ in β-casein indicates the action of cathepsin D.[35] The enzyme that is responsible for the cleavage between glutamic acid$_{21}$ and serine$_{22}$, as well as asparagine$_{27}$ and lysine$_{28}$, is not clear. It is possible that these peptides are further breakdown products of β-casein$_{1-28}$, which is extremely abundant, representing 8−12% of the proteose-peptone fraction.[33]

The structures of the endogenous, multiphosphorylated milk peptides were used to evaluate the possible bioactivity of this fraction of raw milk. Phosphopeptides have the ability to bind minerals. For this function, the sequence motif "-pSer-pSer-pSer-Glu-Glu-" (pSer = phosphoserine) is required, which is found in the sequence of α_{S1}-, α_{S2}-, and β-casein.[36] Nine of the detected phosphopeptides contained this sequence motif, indicating that they may efficiently bind to minerals. Mineral binding of casein phosphopeptides may exert anticariogenic activity on the one hand and, on the other hand, increase calcium bioavailability.[7] A commercial casein phosphopeptide preparation, which consisted mainly of β-casein$_{1-28}$ and β-casein$_{1-32}$, additionally showed an immunostimulatory effect.[3]

The present study combined different mass spectrometric approaches to analyze the profile of multiphosphorylated peptides in raw milk without additional enrichment steps. Consequently, the newly established method proved to be complementary to profiling of multiphosphorylated milk peptides after enrichment with hydroxyapatite leading to the identification of novel peptides. The sequence and phosphorylation sites of the annotated peptides were then identified by targeted UHPLC−MS/MS analysis. MALDI-TOF-MS analysis in the negative and positive mode using ClCCA matrix proved to be an easy and fast screening method to identify multiphosphorylated peptides in a complex matrix such as milk. Thus, 11 phosphopeptides could be identified originating from the N-terminal regions of β-casein and α_{S2}-casein, possibly with beneficial physiological properties. The observed cleavage pattern indicates that peptides are mainly released by the action of plasmin and other endogenous milk proteases. The method can now be applied to analyze the profiles of multiphosphorylated peptides in other matrices, such as fermented milk products.

■ ASSOCIATED CONTENT

Supporting Information

Scheme S1, a schematic explanation of the stage tip extraction. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS USED

CPP, casein phosphopeptides; MALDI-TOF-MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; C1CCA, 4-chloro-α-cyanocinnamic acid; LC, liquid chromatography; ESI, electrospray ionization; TFA, trifluoroacetic acid; UHPLC, ultrahigh-performance liquid chromatography; HCCA, hydroxycinnamic acid

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