Journal of Chromatography B, 878 (2010) 2669–2678

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Hydroxyapatite as a concentrating probe for phosphoproteomic analyses

Gabriella Pinto[a,*], Simonetta Caira[b], Marina Cuollo[a], Sergio Lilla[a], Olga Fierro[b], Francesco Addeo[a]

[a] Department of Food Science, Faculty of Agriculture, University "Federico II" via Università 100, Parco Gussone, 80055 Portici, Italy
[b] Food Science and Technology Institute of the National Research Council (C.N.R.), Via Roma, 83100 Avellino, Italy

ARTICLE INFO

Article history:
Received 21 April 2010
Accepted 27 July 2010
Available online 4 August 2010

Keywords:
Direct MALDI detection
Hydroxyapatite enrichment
Phosphopeptides (CPP)
Phosphoproteins
Proteomics

ABSTRACT

A novel method for the selective enrichment of casein phosphoproteins/phosphopeptides (CPP) from complex mixtures is reported herein. This method employs ceramic hydroxyapatite (HA) as a solid-phase adsorbent to efficiently capture phosphoproteins and CPP from complex media. Casein was chosen as the model phosphoprotein to test the protocol. CPP immobilized on HA microgranules formed a complex that was included in the matrix-assisted laser desorption/ionization mass spectrometry (MALDI) matrix before desorbing directly from the well plate. Casein fractions with different levels of phosphorylation were desorbed based upon the specific concentration of trifluoroacetic acid (TFA) included in the MALDI matrix. The HA-bound casein enzymolysis was performed in situ with trypsin to remove non-phosphorylated peptides and isolate the immobilized CPP. The latter were recovered by centrifugation, dried, and co-crystallized with a 1% phosphoric acid (PA) solution in the matrix that was appropriate for detecting CPP in MALDI-MS spectra. This approach for the selection of casein/CPP resulted in the identification of 32 CPP by MALDI-time of flight (TOF). The analytical process involved two steps requiring ∼2 h, excluding the time required for the enzymatic reaction. The alkaline phosphatase (AP)-assisted de-phosphorylation of tryptic CPP allowed the phosphorylation level of peptides to be calculated concurrently with MALDI-TOF MS and liquid chromatography-electrospray ionization–mass spectrometry (LC–ESI–MS/MS). The effectiveness of the extraction procedure assayed on eggshell phosphoproteins resulted in the identification of 5 phosphoproteins and 14 derived phosphopeptides with a phosphoprotein global recovery of ∼70% at least.

© 2010 Elsevier B.V. All rights reserved.

1. Introduction

Several reviews have described phosphoproteomics, which attempts to study protein phosphorylation on a large scale by elucidating the phosphorylation status of all proteins present in cells or organisms. One of the most significant phosphoproteomic studies described the "phosphokinome" of human cancer cell lines, identifying almost 1000 phosphorylation sites on 219 protein kinases [1]. Given their often substoichiometric concentration, phosphopeptides frequently escape detection because of a poor mass spectrometric (MS) precursor response and the low m/z values of MS2 fragment ions [2]. Moreover, proteins/peptides containing multiple phosphorylation sites are detected with low efficiency by MALDI-MS because of their attenuated or suppressed ionization [3,4]. Prior enrichment of phosphoproteins/phosphopeptides is one of the important methods to enhance the MS detection of phosphoanalyte signals. The objective of any separation and enrichment procedure from protein or peptide complex media is the quantitative recovery of phosphorylated peptides free of the non-phosphorylated components. To date, several strategies have been developed to fulfill this objective, most of which are based upon enrichment and/or down-scaling strategies. These strategies both reduce the amount of starting non-phosphorylated material and increase the intensity of the phosphoanalyte peaks in MS. Immobilized metal affinity chromatography (IMAC) [5–7], which chelates phosphoanalytes on metal ions (e.g., Zn^{+2}, Ga^{+3}, and Fe^{+3}) through metal-phosphate ion–pair interactions, has been suggested to be one of the best procedures for enriching phosphorylated proteins and peptides. Another method utilizes either titanium dioxide [8–11] (TiO_2) or zirconium dioxide (ZrO_2) [12–14] to capture phosphoanalytes through bidentate interactions [15], providing complementary information to IMAC [16]. Each affinity capture method has a particular specificity for the phosphoproteins/phosphopeptides that it can isolate and, consequently, isolates a different set of phosphopeptides. A comparative study of three affinity methods concluded that different, partially overlapping segments of the phosphoproteome were detected by each method and that, at present, no single method is sufficient for a comprehensive phosphoproteomic analysis [16]. In addition, the extent of phosphopeptide identification with any approach is highly dependent upon the sample and the laboratory performing the analysis [17]. The affinity techniques developed for the

* Corresponding author. Tel.: +39 081 2539346; fax: +39 081 7762580.
E-mail address: gabriella.pinto@unina.it (G. Pinto).

1570-0232/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2010.07.024

selective enrichment of phosphoproteins/phosphopeptides from complex samples gave reproducible but incomplete results [16] due to the poor binding of phosphopeptides present in low concentrations and the insufficient recovery of peptides with multiple phosphorylation sites [18]. IMAC also binds non-phosphorylated peptides because the metal ions also chelate protein carboxylate groups. To overcome this drawback, binding and washing are performed at low pH, at which carboxylic acid groups are not ionized, while elution is subsequently accomplished by raising the pH [19]. Esterification of acidic residues can also reduce the binding of non-phosphorylated peptides [20]. At the present time, which of these affinity isolation procedures would yield the best results is unclear. We developed a hydroxyapatite (HA)-based procedure for the chromatographic isolation of casein fractions according to the increasing phosphate content [21]. Since then, there have been a few reports on the use of HA for the enrichment of phosphoproteins/CPP [22,23]. Recent work has compared the efficiency of HA- and TiO_2-based methods, demonstrating that both showed an almost equal performance for CPP enrichment [22]. Basically, the HA-based protocol immobilizes CPP on HA microgranules after elution with a phosphate-containing buffer, which are then identified by off-line MALDI-MS [22]. To speed up this procedure for its routine application on a variety of samples, we investigated the possibility of using MALDI-TOF for direct analysis of the HA-bound phosphoproteins/CPP in a solution free of non-phosphorylated analytes. The efficiency of an analytical method is usually determined using model proteins. Whole casein was chosen as the reference protein for this study because it is formed by four protein families each having a known sequence and well documented phosphorylation sites [24]. Because a true standard for how these CPP are reported has already been established, there is no need to validate the assignment of peptides. Not all predicted masses, however, actually correspond to CPP. To reduce the misidentification rate, the assignment and sequence of peptides can be accomplished by MALDI-TOF post source decay and LC–MS/MS, which are both able to identify phosphorylated amino acids in the resultant mass spectra [25]. Because progress in mass spectrometry in the field of proteomics is outside the scope of this report, we mainly focus on the analysis of our hydroxyapatite-based method, which specifically captures both phosphoproteins and phosphopeptides as well as eliminates the sample loss that can occur with elution prior to MALDI analysis [22]. This hydroxyapatite (HA)-based method provides a very efficient means to specifically analyze microgranule-bound phosphoproteins and phosphopeptides.

2. Materials and methods

2.1. Materials and reagents

HA (Macro-Prep Ceramic Hydroxyapatite TYPE I) was purchased from Bio-Rad (Milan, Italy). Tris(hydroxymethyl) aminomethane hydrochloride (Tris–HCl), potassium chloride (KCl), urea, TFA, ACN for HPLC, 85% orthophosphoric acid (PA), and ammonium bicarbonate buffer (AMBIC) were purchased from Carlo Erba (Milan, Italy). TPCK-treated trypsin from bovine pancreas was purchased from Sigma (St. Louis, MO, USA). Sinapinic acid, sodium acetate trihydrate and 2,5-dihydroxybenzoic acid (DHB) were obtained from Fluka (St. Louis, MO, USA). Acetic acid was purchased from Baker Chemicals B.V. (Deventer, Netherlands). Alkaline phosphatase (Grade I, 4000 U) from calf intestine was supplied by Roche (Roche Diagnostics, GmbH, Mannheim, Germany). Dithiothreitol (DTT) was purchased from Applichem (Darmstadt, Germany). Water was prepared using a Milli-Q system (Millipore, Bedford, MA, USA).

2.2. Sample preparation

Raw whole milk was collected from local dairy farms. The milk was skimmed by centrifugation at 4000 rpm at 4 °C for 30 min. Isoelectric casein was prepared by the addition of 10% (v/v) acetic acid to the resulting skim milk, followed by incubation for 30 min at 35 °C and the subsequent addition of 1 M NaOAc to produce a final pH of 4.6. After an additional 30-min incubation, the suspension was recentrifuged as above, the supernatant was discarded, the casein was washed twice with buffer diluted 1:4 and twice with Milli-Q water, and the sample was subsequently freeze-dried. The shell proteins from fresh commercial egg chicken were recovered according to the procedure previously described [26] from a local store. The calcified layer was finely grinded and demineralized in 10% acetic acid at 4 °C for 24 h with constant stirring. Insoluble material was removed by centrifugation and the supernatant was dialyzed against 5% acetic acid and lyophilized.

2.3. HA-based phosphoprotein/CPP enrichment

Lyophilized casein (10 mg) was dissolved in 100 μL of buffer containing 50 mM Tris–HCl, 0.2 M KCl, 4.5 M urea, and 10 mM DTT, pH 7.8. The casein solution was loaded onto 10 mg of HA that was previously packed with loading buffer. The HA-bound casein was incubated for 15 min at room temperature and centrifuged for 5 min at 4000 rpm. The resin was successively washed with three different buffers: 1 mL of loading buffer, 1 mL of 50 mM Tris–HCl, pH 7.8 (washing buffer), and 1 mL of buffer containing 20 mM Tris–HCl, pH 7.8 and 20% ACN (v/v). The resin was washed with 1 mL of Milli-Q water and freeze-dried with a SpeedVac concentrator system (Thermo Electron, Milford, MA, USA). For eggshell proteins, 5 mg/100 μl buffer were loaded onto 5 mg HA.

2.4. HA-based CPP enrichment

The HA-bound phosphoproteins/CPP enzymolysis was performed in situ with trypsin, which was added to the suspension at an enzyme/substrate ratio of 1:50 (w/w) in 50 mM Tris–HCl buffer, pH 7.8 containing 0.2 M KCl, 4.5 M urea, and 10 mM DTT. The reaction was carried out at 37 °C overnight and stopped by centrifuging the HA-CPP microgranules for 5 min at 4000 rpm. The microgranules were then washed as described above for the phosphoproteins. After washing with Milli-Q water, the microgranules were dried with a SpeedVac apparatus.

2.5. MALDI-TOF MS

MALDI-TOF mass spectra were recorded using a Voyager DE-PRO mass spectrometer (Applied Biosystems, Framingham, MA, USA). Mass spectra were acquired in positive linear mode due to the known instability of CPP in the reflector mode [27]. This situation is amplified by the multiply phosphorylated peptides generating dephosphorylated peptide in the metastable decomposition [28]. In addition, the co-presence in high number of phosphorylated peptides would complicate the reflector spectrum. Instead, interpretation of the linear spectrum is straightforward, since it contains only the $[M+H]^+$ ion for each species. Moreover, higher molecular weight peptides and phosphoproteins also show lower sensitivity in the reflector mode than in the linear mode due to longer flight path and action of reflector [29]. Finally, the LC–ESI–MS/MS experiments were carried out to confirm the assignment of CPP identity [30]. The spectra were acquired in the range of 15–30 kDa (for proteins) and 1–5 kDa (for peptides) with the following settings: an accelerating voltage of 25 kV (for proteins) or 20 kV (for peptides) and a grid voltage of 93% (for proteins) or 95% (for peptides) of the accelerating voltage, a guide wire of 0.15% (for proteins) or

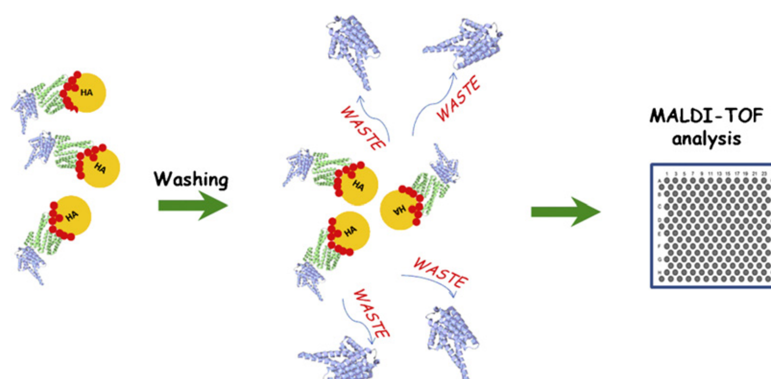

Fig. 1. Schematic representation of the HA-based technique for phosphoprotein enrichment. Phosphoproteins bind to HA through their phosphate groups, while the non-phosphorylated components are removed using washing buffers. The dried HA-phosphoprotein microgranules are then analyzed directly by MALDI-TOF after co-crystallization with sinapinic acid in an appropriate quantity of trifluoroacetic acid. ● = phosphate group.

0.05% (for peptides), and a delayed ion extraction time of 485 ns (for proteins) or 175 ns (for peptides). The laser power was set just above the ion generation threshold to obtain peaks with the highest possible signal-to-noise ratio. All spectra were acquired with 200 shots in three replicates. Two different matrix solutions were freshly made each day from stocks of the required solvents and sonicated for 15 min in an ultrasonic water bath (Bransonic 220, Mazian Med Equip, Toronto, Canada) prior to use. The sample/wash (SW) method was based on the sample/matrix/wash (SMW) method described by Zhang et al. [31], with the exclusion of the wash step.

The HA-phosphoprotein microgranules ($n = \sim 1000$) were deposited onto the MALDI plate, covered with the matrix solution (10 mg/mL sinapinic acid (SA) in 50% ACN, 1 μL) to promote analyte/matrix co-crystallization and air-dried at room temperature in the presence of increasing TFA concentrations (0.01%, 0.05%, 0.1% and 0.5%). For HA-CPP, 1% PA was included in the matrix solution (10 mg/mL 2,5-dihydroxybenzoic acid (2,5-DHB) in 50% ACN) [32].

2.6. LC–ESI–MS/MS analysis

HA-CPP complexes (1 mg) were solubilized with 120 μL of 0.5% PA and analyzed by a CapLC nano-flow high-pressure pump system (Waters/Micromass, Manchester, UK) interfaced with a QTOF Micromass spectrometer (Waters/Micromass) operating in the positive ion mode. Chromatographic separations were performed on a reverse phase Atlantis dC18 capillary column (75 μm i.d.). The mobile phase was water (A) and ACN (B) with 0.1% FA. A linear gradient from 5% to 70% of B was applied to the pre-column and column over a 45-min period at a flow-rate of approximately 300 nL/min pre-column splitting using a pump operating at 5 μL min^{-1}. The source conditions were as follows: capillary voltage, 2.6 kV; cone voltage, 100 V; and RF1 lens, 40 V. Argon was used as the collision gas in the ESI-MS/MS experiments for peptide sequencing. Raw data were processed using MassLynx 4.0 ProteinLynx software.

2.7. Microscopic observation

The HA-CPP microgranules deposited onto a 96-well MALDI target were observed by stereomicroscopy before and after co-crystallization with the DHB matrix. The microscope (PBI International, Milan, Italy) was equipped with a binocular head illuminated with a 6-V, 20-W halogen lamp and a 100–230-V, 50/60-Hz power supply.

2.8. CPP de-phosphorylation

The HA-bound tryptic CPP complex (1 mg) was treated with 15 μL of a 5% aqueous PA solution to render soluble both the HA and phosphorylated components. The solution was then loaded on a Zip Tip C18 to obtain the salt-free phosphopeptide mixture and freeze-dried in a SpeedVac apparatus. The residue was then dissolved in a solution composed of 50 μL 0.4% AMBIC, pH 9 and 1 μL alkaline phosphatase (ALP) and incubated at 37 °C overnight. The de-phosphorylated CPP diluted 10 times with 0.1% H_2O/TFA (1 μL) was deposited in a MALDI well, covered with DHB matrix (10 mg/mL of the H_2O/ACN/PA = 49/50/1 solution) and allowed to crystallize. Spectra were acquired as indicated above.

2.9. Phosphoprotein workflow

An integrated strategy for the experimental analysis of the phosphoproteome combining MALDI-TOF analysis with the phosphoprotein enrichment steps is schematically illustrated in Fig. 1. The suspension of HA microgranules in the loading buffer was mixed with the protein solution to bind the target phosphorylated components to the adsorbent. The analytical method (Fig. 1) requires six consecutive steps: (1) enriching and separating phosphoproteins using HA microgranules (HA: protein, 1:1 [w/w]); (2) washing with the dilution/washing buffer to remove any non-phosphorylated proteins from the casein-HA microgranule; (3) drying the protein-HA microgranules; (4) loading the HA microgranules ($n = \sim 1000$) onto the multi-well MALDI target plate; (5) covering the microgranules with a sinapinic acid matrix (10 mg/mL in 50% ACN) to promote co-crystallization of the analyte/TFA (0.01–0.5%) mixture; and (6) in situ desorption of the phosphoproteins by direct MALDI-MS analysis (Fig. 2).

2.10. Phosphopeptide workflow

Whole casein (or eggshell proteins) was converted to tryptic digests for the selective enrichment of phosphorylated peptides using HA chromatography (Fig. 3). Preliminary experiments revealed that the highest CPP yield was obtained using a 1:1 HA-to-protein ratio (w/w), a 1:50 trypsin-to-protein ratio (w/w) and an 18-h digestion period. After addition of trypsin to the protein-HA microgranules, the supernatant, which contained non-phosphorylated tryptic peptides and trypsin, was discarded. The residue was sequentially washed with a loading/washing buffer/water solution, recovered by centrifugation and freeze-dried. The resultant dried phosphopeptide-HA microgranules, mixed with a DHB and 1% PA solution, were left to co-crystallize on a MALDI target plate and directly analyzed by MALDI-TOF. This version of the protocol could further speed up our previously developed procedure requiring phosphopeptide elution from HA before MS analysis [22]. Briefly, microgranules deposited on the target were covered by matrix solution containing 1% H_3PO_4, which

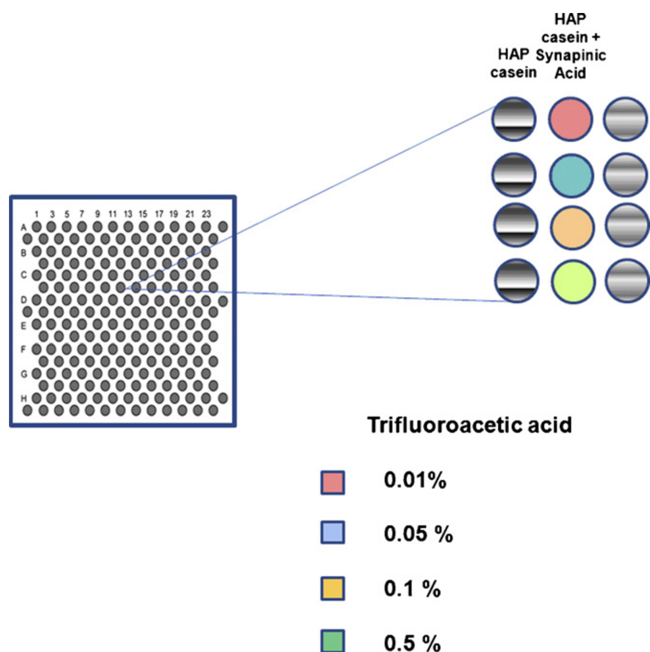

Fig. 2. Schematic representation of the co-crystallization of HA-bound casein with the sinapinic matrix on the MALDI target plate in the presence of increasing concentrations of TFA (0.01%, 0.05%, 0.1% and 0.5%).

dissolved the HA-captured CPP or eggshell phosphopeptides and making their analysis by MALDI possible.

3. Results

The identification of phosphoproteins by MALDI-TOF is not as straightforward as the identification of proteins due to their relatively low abundance, the variance in phosphorylation sites and the possible presence of phosphatases. Given that de-phosphorylation could occur even as the sample is processed, the results also depend on the specific activity of alkaline phosphatase. Therefore, phosphatase-free isoelectric casein was selected to study the specificity of interactions with HA. HA possesses pairs of positively charged C-sites and negatively charged P-sites [33] that are capable

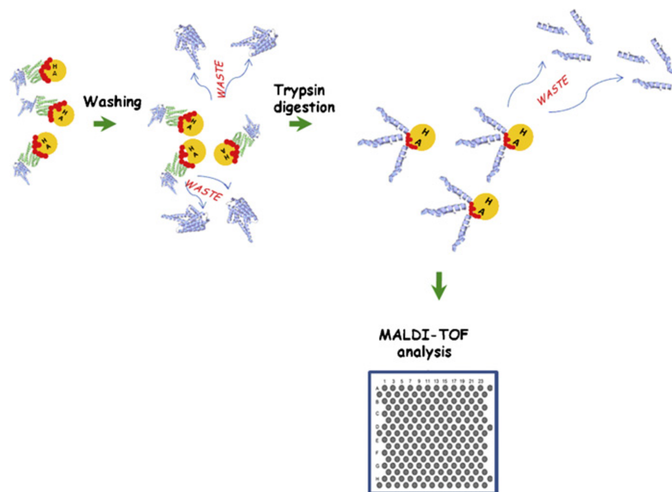

Fig. 3. Schematic representation of the HA-based CPP enrichment technique. HA-bound phosphoprotein enzymolysis is performed in situ with trypsin, and non-phosphorylated peptides are released in the flow-through. The dried HA-CPP microgranules included in the co-crystallization matrix are analyzed by MALDI-TOF directly on the MALDI plate, as indicated in Fig. 2.

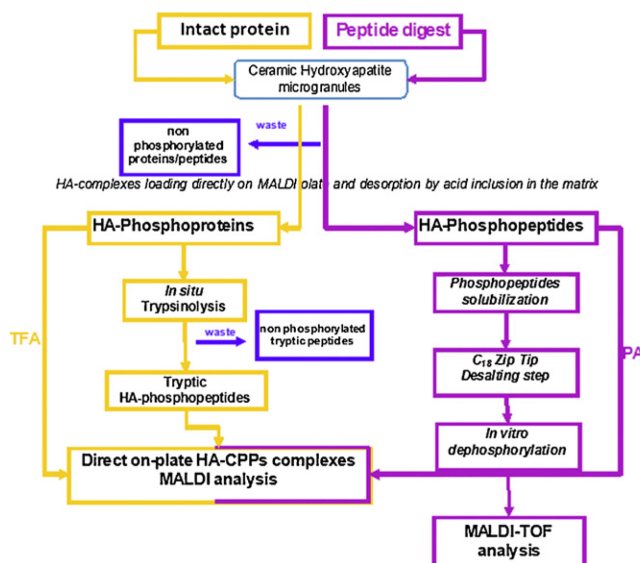

Fig. 4. Experimental scheme for the direct MALDI-MS analysis of phosphoproteins and CPP bound to HA microgranules. The samples were analyzed as follows. An aliquot of the solution containing casein and/or its derivative peptides was mixed with the HA microgranules to capture the phosphorylated components. Another aliquot containing HA-bound casein or tryptic CPP was spotted on the target, covered with the MALDI matrix solution containing exogenous acids (TFA for phosphoproteins, shown in yellow; PA for CPP, shown in violet) and the protein/peptide ions desorbed from the MALDI-TOF matrix. The HA-based protein/peptide enrichment procedure was tested using MALDI-TOF to discriminate the phosphoanalytes in two distinct mass ranges. The presence of phosphoanalytes in the mixture was verified through phosphate-specific reactions (e.g., sensitivity to alkaline phosphatase). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

of interacting, under a wide range pH levels, with the side chain groups of proteins/peptides. These characteristics allow the HA surface to bind proteins with different p*I* [33]. In this study, HA was first tested for its ability to bind the four major casein families through their variable number of phosphate groups present in concentrations ranging from 1 to 13 moles/molecule [21]. The suspension of HA-bound casein complexes was then assayed for its susceptibility to trypsin in comparison to native casein in solution. The detailed procedure for the phosphoprotein/phosphopeptide enrichment and optimization is outlined in Fig. 4.

3.1. Analysis of phosphoproteins

Using the strategy depicted in Figs. 1 and 3, hydroxyapatite was tested for its ability to chelate casein and be used in the subsequent characterization of captured proteins using MALDI-TOF. Using 0.01% TFA, those casein components with a low number of phosphate groups were desorbed first from the on-bead HA microgranules. The resulting spectral signals were assigned to the corresponding κ-CN 1P variant A and β-CN (Fig. 5, panel a; Table 1), while those with multiple phosphorylation sites were desorbed by the addition of 10-fold concentrated TFA (Fig. 5, panel b). The results indicate that HA captured all casein fractions. Among the weakly HA-bound fractions, the caseins with a low number of phosphorylation sites were desorbed first. Almost exclusively κ-CN could be desorbed by the inclusion of 0.005% TFA in the MALDI matrix (Supplemental Figure S1). In addition, κ-CN had been previously found to elute first from a HA column using a buffer with a low phosphate concentration (5 mM) [21]. Based on the rationale that the casein can be fractionated according to the phosphate concentration of the eluent, a HA-based chromatographic method was developed [22] to separate low- and high-phosphorylated CPP. If this fractionation does not really apply to such CPP, however, the

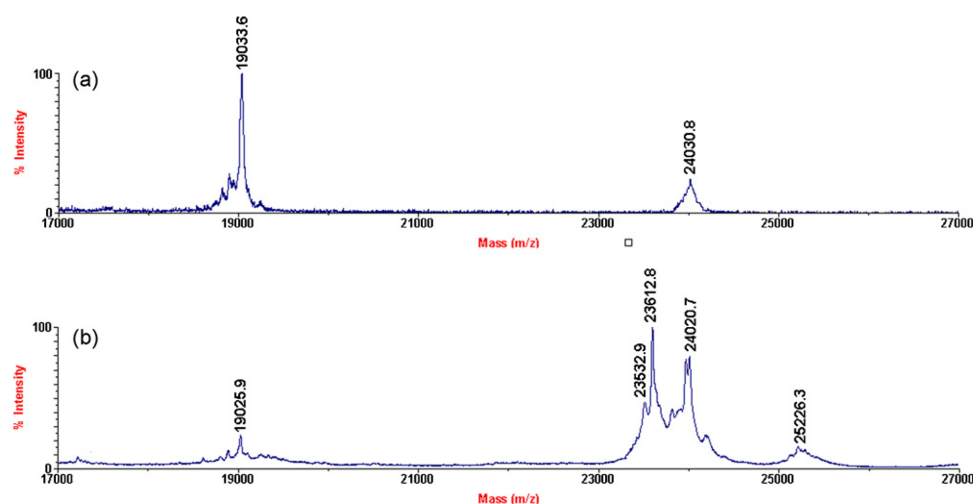

Fig. 5. MALDI-TOF analysis of phosphoproteins desorbed from HA microgranules by 0.01% TFA (a) or 0.1% TFA (b).

HA-bound peptides might all be eluted simultaneously [22]. Indeed, HA-bound proteins can all be recovered by an adsorbent collapse through TFA addition to the MALDI matrix. Because this procedure does not require any prior chromatographic separation of the phosphoproteins, it can be assumed to enable a single-step identification of the HA-bound phosphoproteins by comparing the measured mass to the theoretical mass (Table 1). More precisely, four coupled casein fractions, each with differing numbers of phosphate groups were identified (Table 1). β-CN A^1 and A^2 differed by the additional amino acid substitution His^{67} (β-CN A^1) → Pro^{67} (β-CN A^2). Notwithstanding the presence of β-CN in amounts three-fold higher than those of the heavily phosphorylated α_{s2}-CN, the ionization of the latter is not completely suppressed with respect to that of β-CN. The results demonstrate that MALDI-TOF MS is capable of identifying on-bead, immobilized casein fractions with single and multiple phosphorylation sites. Although casein is a well known model phosphoprotein, the method could present some limitations in more complex biological matrices. We tested the analytical power of our procedure by submitting the eggshell proteins to HA enrichment as shown in Fig. 10. Herein, the MALDI spectrum of the HA-bound eggshell phosphoproteins was more complex than that of casein showing several overlapping signals (Fig. 10). Undoubtedly, the procedure described herein has advantages over previous methods due to its simplicity, straightforwardness and reduced work-up. Using this method, analytical tests for the detection of multiple phosphoprotein markers can be developed.

3.2. Tryptic digestion and HA-based CPP enrichment

The casein digestion yielded the expected ratio of mono- to penta-phosphorylated CPP, irrespective of protein weight (1–10 mg), bound to the same quantity of HA (1 mg, Supplemental Figure S2). In complex mixtures, the signals of highly phosphorylated peptides are usually suppressed by those of non-phosphorylated peptides [4]. The analysis of the tryptic casein digest without any prior enrichment (Fig. 6, panel a) revealed a number of prominent signals that were assigned to non-phosphorylated tryptic peptides. Four signals represented the non-phosphorylated peptides α_{s1}-CN (f91–100; m/z 1267), α_{s1}-CN (f8–22; m/z 1759), α_{s1}-CN (f23–34; m/z 1383.7) and α_{s1}-CN (f133–151; m/z 2316.1). The high level of these peptides in the washes (Fig. 6, panel b) accounts for their scarce affinity for HA. This result confirms once again the low interaction of non-phosphorylated peptides and the attenuation of C-sites by the inclusion of 20% ACN in the HA loading/washing buffer [22]. In vitro, a solution of salts in milk concentrations containing β-casein (f1–25) 4P tryptic CPP caused the spontaneous formation of calcium phosphate nanoclusters [34]. In milk, nanometer-sized clusters of calcium phosphate are sequestered within the casein micelles that have a larger colloidal dimension [35]. Therefore, CPP would be expected to compete in a similar fashion as phosphoproteins with regard to the adsorption on HA C-sites. Indeed, the MALDI-TOF mass spectra confirmed that the phosphorylated peptides were selectively sequestered by the adsorbent (Fig. 7). Trials were conducted to determine the best methods for sample and matrix preparation. The microscopic images of HA-CPP on the MALDI well (Fig. 8, panel a) did not show signs of structural modification in detectable amounts with respect to HA (data not shown). Moreover, microgranule aggregation in the matrix solution was effectively prevented by employing dried HA-CPP. The matrix solvent rapidly evaporated, yielding crystals with a large surface area. As a consequence, the HA-CPP microgranules distributed within the DHB crystal matrix showed homogeneously sized needles (Fig. 8, panel b). HA-bound CPP complexes were checked by direct MALDI-TOF analysis of either microgranules internalized into the acidified matrix or in solution. The resultant spectra showed CPP peaks at the expected m/z with only a few changes in their heights (Supplemental Figure S3). Furthermore, ionization was optimized by the presence of Arg and Lys in C-terminal positions, which facilitated the identification of tryptic peptides by MALDI-TOF. The inclusion of 1% PA in the MALDI matrix solution improved the ionization efficiency for phosphorylated peptides in the mixture [32]. MALDI signals were improved by the use of 1% PA in this study compared to 0.1% TFA. Spectra were manually annotated, and the identification of tryptic CPP was confirmed by using the FindPept tools available at the

Table 1
List of the casein fractions identified in the MALDI-TOF spectra shown in Fig. 5.

Phosphoprotein	Theoretical mass (Da)	Measured mass (Da)
κ-CN 1 P[a,b]	19,037.6	19,025.7
α_{s1}-CN 8P[a]	23,614.9	23,612.8
α_{s1}-CN 7P[a]	23,534.9	23,532.9
β-CN A^1 5 P[a,b]	24,022.4	24,020.7
β-CN A^2 5P[a]	23,983.4	23,980.3
β-CN A^1 4P[a]	23,942.4	23,940.0
β-CN A^2 4P[a]	23,903.4	23,900.9
α_{s2}-CN 12P[a]	25,308.5	25,306.5
α_{s2}-CN 11P[a]	25,228.5	25,226.3

[a,b]Signals occurring in the MALDI spectra from Fig. 5, panels a and b, respectively.
A^1 and A^2, bovine β-casein A^1 and A^2 variants, respectively.

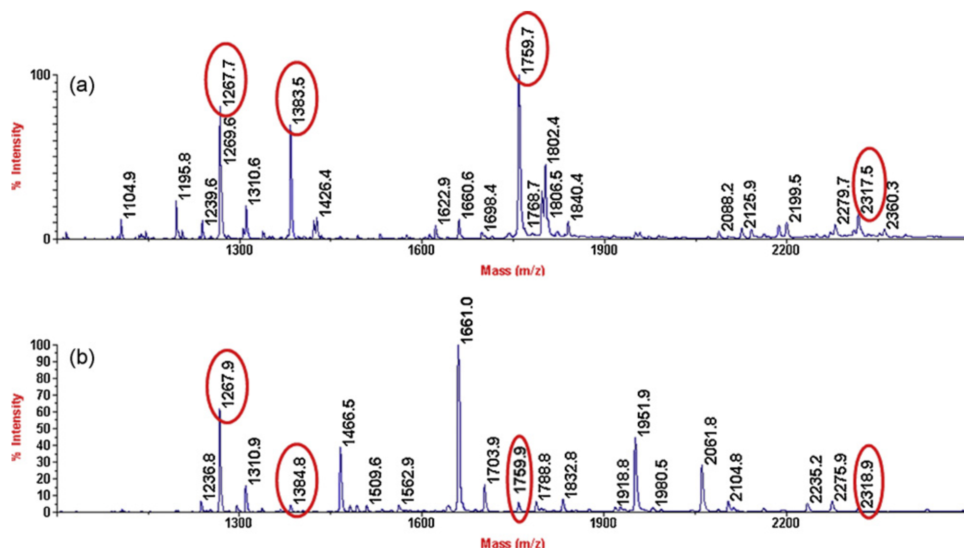

Fig. 6. Mass spectra of tryptic digests obtained after in situ hydrolysis of the HA-bound casein (a) and water soluble peptides from the last wash with 20 mM Tris–HCl, pH 7.8 containing 20% ACN (v/v) (b).

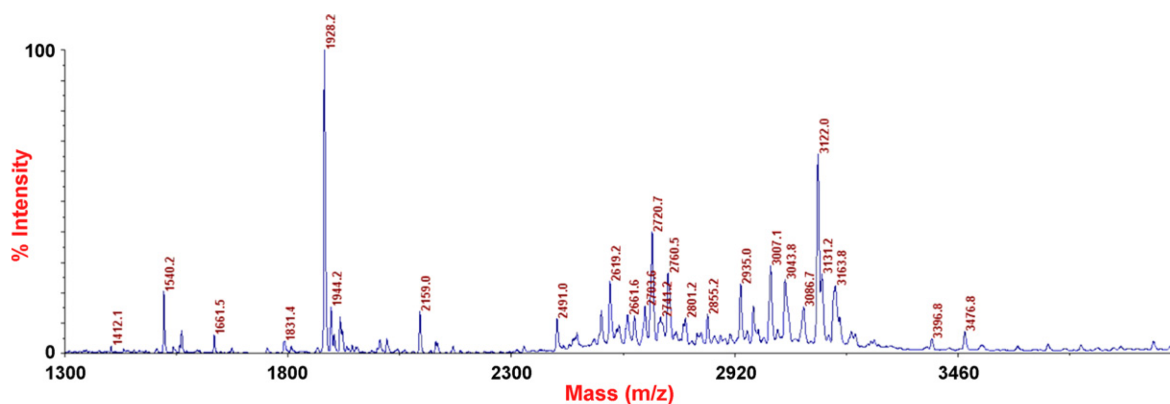

Fig. 7. Mass spectra of HA-bound CPP co-crystallized with the DHB matrix containing 1% PA. The components are identified in Table 2.

www.expasy.ch site (http://www.expasy.org/tools/findpept.html). As expected, the enriched CPP fraction contained a large number of peptides containing multiple phosphorylation sites (up to five phosphate groups/molecule, Fig. 9 and Table 2) and no non-phosphorylated peptides. The data shown in Table 2 emphasize the importance of the enrichment step for the identification of tryptic CPP. The MALDI-TOF spectra (Fig. 9) definitely confirmed the identity of casein-derived tryptic CPP complexes on the basis of the expected masses. Alternatively, alkaline phosphatase action reduced the number of candidate peptides, leading to the characteristic 80-Da mass shift that corresponds to $-HPO_3$ group loss or multiple integers. Thus, a simplified MALDI spectrum containing exclusively non-phosphorylated peptides was obtained (Fig. 9, panel b). The number of P groups was calculated by the *n* (80-Da) mass shift. The masses of native peptides were missing after the alkaline phosphatase treatment (Fig. 9, panel a), and the tryptic CPP annotated with respect to those expected (in parentheses) (Table 2) that were raised from β-, α_{s1}-, and α_{s2}-CN were 6 (2), 7 (3), and

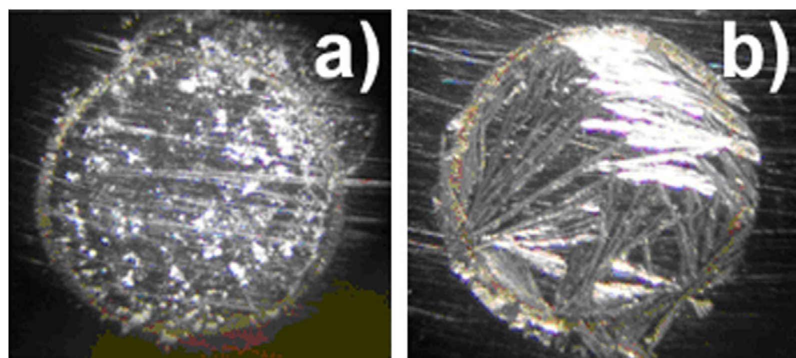

Fig. 8. Stereoscopic microscope images of HA-CPP complexes on the MALDI plate before (a) and after co-crystallization (b) of the HA-CPP sample with the DHB matrix in PA solution.

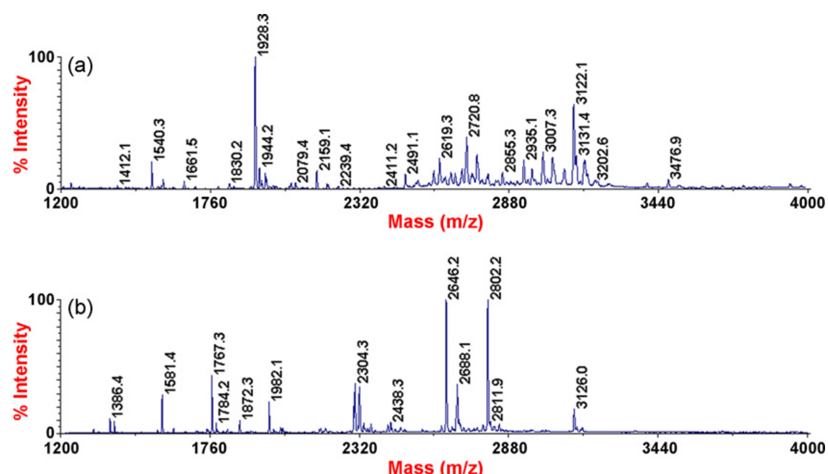

Fig. 9. Mass spectra of HA-bound CPP (a) co-crystallized with the MALDI DHB matrix containing 1% PA or (b) fully de-phosphorylated using alkaline phosphatase.

10 (5), respectively. Neither GMP nor other tryptic κ-CN peptides were detected in the casein digest. The peptide modification (e.g., partial de-phosphorylation, oxidization and N-terminal residue pyroglutamination) that occurred in the tryptic digests could explain the increased number of CPP complexes that are derived from the same phosphoprotein. For egg phosphoproteins in situ trypsinolysis allowed to obtain HA-bound phosphorylated tryptic peptides as shown in MALDI spectra (Fig. 11, Table 3). Fourteen HA-bound tryptic phosphopeptides derived from 5 phosphoproteins were identified and some were partially dephosphorylated as apparent from the characteristic 80-Da mass difference. The number of peptides is partially consistent with the long list of tryptic eggshell phosphopeptides recently reported [26]. Identification of ovocleidin-116- and osteopontin-derived phosphopeptides confirmed the co-presence of eggshell phosphoproteins with multiple phosphorylation consensus sequences. The function of these modifications is unknown although the phosphorylation sites are preserved in the multiple phosphorylated proteins [26].

Table 2
List of the HA-bound tryptic native and de-phosphorylated CPP complexes identified in the MALDI spectra shown in Fig. 9.

Molecular mass (Da) HA-bound CPP		De-phosphorylated CPP		Number of phosphate groups	CPP identity
Expected	Measured	Expected	Measured		
1410.1	1411.1	1251.3	1252.3	2	α_{s2} (f126–136)
1466.1	1467.1	1385.6	1386.4	1	α_{s2} (f138–149)
1539.4	1540.0	1378.6	1379.4	2	α_{s2} (f126–137)[a]
1660.7	1661.1.	1580.7	1581.4	1	α_{s1} (f106–119)
1846.1	1847.0.	1766.7	1767.4	1	α_{s1} (f43–58)
1927.8	1928.0.	1766.7	1767.4	2	α_{s1} (f43–58)
1943.8	1944.2	1782.7	1783.4	2	α_{s1} (f43–58) + M (O)
1952.0	1952.0	1872.1	1872.3	1	α_{s1} (f104–119)
2060.1	2062.1	1981.8	1982.2	1	β (f33–48)
2158.8	2159.1	1998.8	1998.8	2	α_{s2} (f42–59)[a]
2238.8	2239.4	1998.8	1998.8	3	α_{s2} (f42–59)[a]
2410.8	2411.3	2170.9	2170.9	3	α_{s2} (f2–20)
2432.4	2432.1	2352.1	2352.0	1	β (f30–48)[a]
2490.8	2491.2	2170.9	2170.9	4	α_{s2} (f2–20)
2539.0	2539.1	2299.0	2299.2	3	α_{s2} (f2–21)
2598.0	2598.0	2438.1	2438.3	2	α_{s1} (f37–58)[a]
2618.1	2619.0	2299.0	2299.2	4	α_{s2} (f2–21)
2678.0	2678.0	2438.1	2438.3	3	α_{s1} (f37–58)[a]
2703.1	2703.1	2304.0	2304.3	5	α_{s1} (f59–79) pyroQ
2720.1	2720.1	2321.0	2321.3	5	α_{s1} (f59–79)
2746.1	2747.1	2427.1	2427.7	4	α_{s2} (f1–21)[a]
2759.6	2760.5	2439.2	2439.5	4	α_{s1} (f62–83)[a]
2855.0	2855.0	2695.2	2695.8	2	α_{s1} (f35–58)[a]
2886.0	2886.0	2646.3	2646.2	3	β (f2–25)
2935.0	2935.0	2695.2	2695.8	3	α_{s1} (f35–58)[a]
2966.0	2966.0	2646.3	2646.2	4	β (f2–25)
3007.1	3007.0	2688.2	2688.2	4	α_{s2} (f46–70)
3088.0	3087.0	2688.2	2688.2	5	α_{s2} (f46–70)
3042.0	3043.1	2802.4	2802.2	3	β (f1–25)[a]
3122.0	3122.0	2802.4	2802.2	4	β (f1–25)[a]
3132.0	3131.4	2812.3	2812.5	4	α_{s2} (f1–24)[a]
3448.3	3445.9	3128.4	3126.4	4	α_{s2} (f1–27)[a]
3477.1	3476.9	3157.6	3157.0	4	β (f1–28)[a]
3605.1	3605.0	3285.7	3285.4	4	β (f1–29)[a]

[a] Peptide with tryptic cleavage sites that were missed.
M, methionine oxidation.

Table 3
List of the HA-bound tryptic phosphopeptide complexes from eggshell phosphoproteins identified in the MALDI spectra shown in Fig. 9.

Theoretical mass	Measured mass M[H+] (Da)	Identification	Phosphate number
1806.9	1807.4	Ovocleidin-17 (f47–62)	1P
2275.2	2276.8	Ovocleidin-17 (f53–74)	2P
2089.1	2090.1	Ovalbumin (f340–359)	1P
1826.7	1827.0	Ovocleidin-116 (f704–719)	1P
1905.6	1906.6	Ovocleidin-116 (f704–719)	2P
1986.6	1986.7	Ovocleidin-116 (f704–719)	3P
2148.3	2148.6	Ovocleidin-116 (f389–407)	1P
3137.1	3138.7	Ovocleidin-116 (f690–719)	2P
3217.1	3218.7	Ovocleidin-116 (f690–719)	3P
3297.1	3298.6	Ovocleidin-116 (f690–719)	4P
1887.9	1888.0	Osteopontin (4–19)	1P
1966.8	1967.5	Osteopontin (4–19)	2P
1957.8	1958.7	Osteopontin (8–22)	3P
2147.0	2148.6	Ovocalyxin-32 (221–241)	1P

These authors have identified 39 phosphoproteins including low abundance components containing at least 155 different phosphorylation sites with a ratio of pS/pT/pY of approximately 90:10:1. We were able to identify only 5 high abundance phosphoproteins and 14 derived phosphopeptides each containing only the phosphorylation site pS. In-depth study using fresh material and use of alternative trypsin enzymes able to access hindered sites to trypsin could afford the enrichment of more low abundance peptides.

3.3. Localization of phosphate residues in CPP

The determination of phosphorylated site(s) could be important for the establishment of the functional consequences of the phosphorylation. In many cases, peptide flexibility depends on the phosphorylated sequence (e.g., participation of the phosphorylated loop in a protein-protein interaction or reposition of loops after de-phosphorylation). Fragmentation of the MS1 precursor ions by LC–ESI–MS/MS for the selective detection of CPP ordinarily exhibits a strong neutral loss, although not for all phosphopeptides, when collisionally activated in positive mode [36]. For all chromatographic peaks, the fragmentation provided the sequence information that was used to ultimately identify the phosphorylated peptide. Supplemental Figures S4–S9). The α_{s2}-CN (f138–149) 1P peptide, which contains the residues Thr^{138} and Ser^{143} as candidates for phosphorylation, only had Ser^{143} phosphorylated (Supplemental Figure S4). Moreover, the α_{s2}-CN (f126–137) 2P peptide, which contains Ser^{129}, Thr^{130} and Ser^{131} residues, had $PSer^{129}$ and $PSer^{131}$, but not Thr^{130}, phosphorylated (Supplemental Figure S5). This result was confirmed by the presence of $PSer^{129}$, $PSer^{131}$ and non-phosphorylated Thr^{130} in the peptide α_{s2}-CN(f126-136) 2P, which was longer by one residue. Further examples of peptide sequences with a lower number of phosphorylated Ser than was expected were α_{s1}-CN (f43–58) 1P and 2P, in which the candidate residues were Ser^{46} and Thr^{49}, with the phosphorylation of both resulting in the 2P form or phosphorylation of only Thr^{49} resulting in the 1P form (Supplemental Figures S7 and S8). The co-presence of α_{s1}-CN(f35–58) 2P and 3P, α_{s1}-CN (f37–58) 2P and 3P, and β-CN (f1–25) 4P and 3P peptides (Supplemental Figure S9) provided confirmatory evidence of partially phosphorylated parent casein fractions. Given these data, the application of tandem MS/MS represents a major improvement in peptide identification with respect to probability-based Databank searches by resolving the problem posed by the presence of multiple phosphorylation sites for proper peptide characterization.

Whether the MALDI or LC–MS/MS technique was applied to large-scale phosphoproteomics, a high number of phosphorylated peptides immobilized on HA microgranules could be detected.

3.4. Quantification and recovery of CPP

In order to quantify the casein-derived tryptic CPP using HA beads, two synthetic peptides, a natural and the modified counterpart that differed by single amino acid substitution, were used as internal standards (IS) (Table 4). The bovine α_{s1}-CN (f43–58) 2P, the most abundant CPP in the phosphopeptide mixture, was chosen to evaluate the recovery of CPP. The $Ile^{44} \rightarrow Gly^{44}$ amino acid substitution in the peptide analogues were introduced to give rise to a distinct mass MALDI signal, without appreciable perturbation of the ionization efficiency. The 0.9/1.0 quasi-equimolar peptide mixture yielded a very similar MALDI ionization efficiency (Supplemental Figure S10). In this manner, the natural peptide analogues were able to function appropriately as IS. A constant concentration (10 μg/μl) of synthetic modified peptide was used to spike solutions containing different concentration of synthetic natural peptide (Supplemental Figure S11). Using synthetic natural/modified area ratio, obtained by integration via software, a calibration curve was built up as a function of their concentration ratio (Supplemental Figure S12). The combined data yielded a linear calibration curve, $y = 1.1147x + 0.1001$ ($R^2 = 0.967$) (mean of 10 replicates) for α_{s1}-CN (f43–58) 2P. Standard deviations for the measurements are indicated by error bars (Supplemental Figure S12). For quantification purposes, IS (1 μg) was added to HA-CPP complex (1 mg) and solubilized with a 0.5% aqueous PA solution. The amount of α_{s1}-CN (f43–58) 2P derived from whole casein (0.05 mg) was found to be 920 ng (Supplemental Figure S13). Therefore, the use of defatted milk tryptic digests in amount as low as 0.1–100 μl/mg dried HA allowed to detect 0.027–27 ng α_{s1}-CN (f43–58) 2P. This means ~70% CPP recovery from either milk or casein (Figs. 10 and 11).

4. Discussion

Using the procedure described above, HA-bound phosphorylated peptides did not contain any non-phosphorylated casein-derived γ_2- and γ_3-CN peptides. The new approach worked

Table 4
List of synthetic native and modified peptides used as internal standard. The amino acid residues differentiating synthetic analogue from natural tryptic CPP are underlined.

α_{s1}-CN peptide	Synthetic native peptide [M+H]+	Native sequence
(f43–58) 2P	1926.7	DIGS(P)ES(P)TEDQAMEDIK
α_{s1}-CN peptide	Synthetic modified peptide [M+H]+	Modified sequence
(f43–58) 2P	1870.6	DGGS(P)ES(P)TEDQAMEDIK

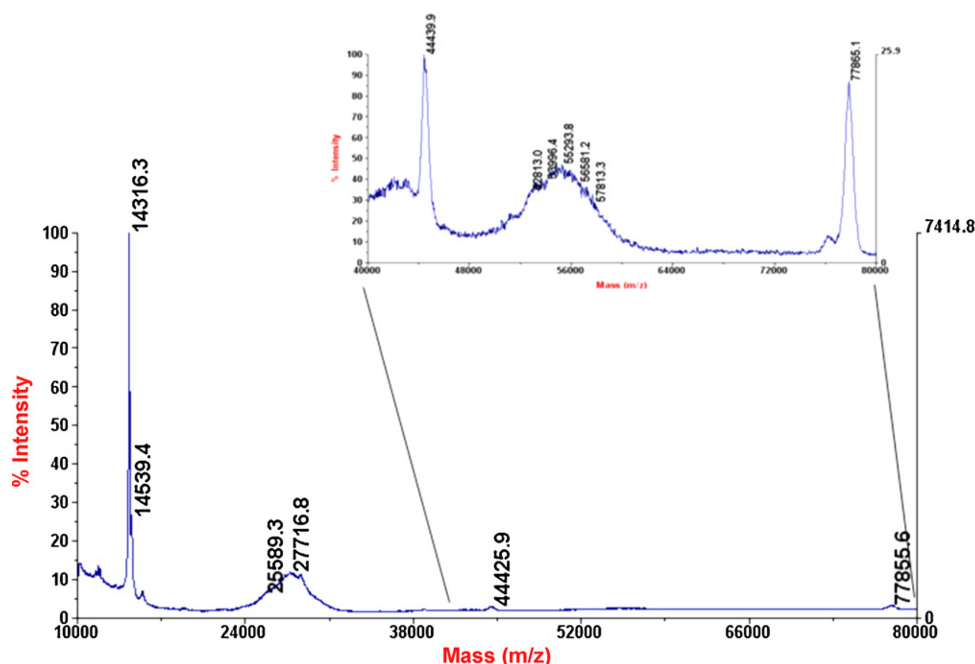

Fig. 10. MALDI-TOF analysis of eggshell phosphoproteins desorbed from HA microgranules.

well with egg proteins as MALDI spectra did not contain non-phosphorylated peptides in appreciable amounts. Therefore, the proposed procedure and our application can accommodate a wide variety of biological matrices.

Various strategies for the enrichment of phosphopeptides based on different chemical strategies have improved the overall recovery of low-abundance proteins. Amongst the strategies for selective enrichment of phosphoproteins/phosphopeptides, the IMAC (Fe^{3+})-based techniques have been claimed to allow recovery up to 90% of phosphoproteins [37]. These methods could be coupled to other fractionation steps to improve the overall recovery of low-abundance proteins. Our method is more selective, more cost-effective and faster than any IMAC-based method as it is based on one-step procedure inclusive of enrichment on HA and MALDI analysis. Using a phosphorylated peptide analogues as internal standard, α_{s1}-casein was recovered up to 70% working either on milk or derived casein.

In addition, when HA was deliberately overloaded with phosphoanalytes to saturate the adsorbent, the number of identified CPP complexes did not vary, demonstrating a lack of preferentiality amongst CPP absorption (Supplemental Figure S2). The role of exogenous acid addition is important because its omission resulted in a complete lack of phosphoprotein signals in the MALDI matrix spectra. To achieve the goal of detecting weakly phosphorylated species, very low TFA concentrations have to be used. In contrast, more tightly bound proteins could be successively eluted by increasing concentrations of TFA. As in the classical method for casein fractionation, the phosphorylated members of this protein family were fractionated into sub-mixture pools by controlling the desorption process on the MALDI plate. Basically, the in situ fractional desorption of casein is controlled by the level of phosphorylation, which also governs the affinity of CPP for HA. Trypsinolysis released CPP according to the reactivity of the lysine residues of the individual caseins that were either HA-bound or in solution in the expected number. Only κ-CN CPP was missing in the HA-bound CPP because hydrolysis of κ-CN by trypsin can be insignificant [38]. Our results demonstrated that CPP could be enriched for MS analysis within only a few minutes, showcasing the outstanding enrichment capacity of HA. Two different mass range acquiring analysis could be performed to demonstrate that the co-existing bound phosphoproteins and CPP can be identified in two separated zones of the MALDI spectra. Conversely, enzyme-mediated CPP have been isolated from cheese and characterized by MS [39,40], but the isolation procedures used in those studies were long, tedious, cumbersome and not suitable for the analysis of a large number samples. Our procedure has the advantage of capturing phosphoproteins on HA and identifying the in situ immobilized CPP by direct MALDI analysis with easy sample manipulation. This procedure circumvents the leakage of CPP by in situ desorption on the MALDI plate. Controlling the desorption process to the equilibrium point at which there are no phosphoproteins/CPP still binding HA, the procedure can be used for quantitative purposes either for CPP or non-phosphorylated peptides concentrated to a known volume, provided that internal standards are available. In summary, for the first time, HA-immobilized phosphoproteins/CPP are directly analyzed on-bead by MALDI-TOF MS without requiring a prior elution of the phosphorylated components. This procedure demonstrates several possibilities: (1) detecting phosphorylated proteins/peptides even in complex mixtures, (2) determining phosphorylated and de-phosphorylated sites, and (3) attaining information about both weakly and heavily phosphorylated peptides. All of our results indicate that this

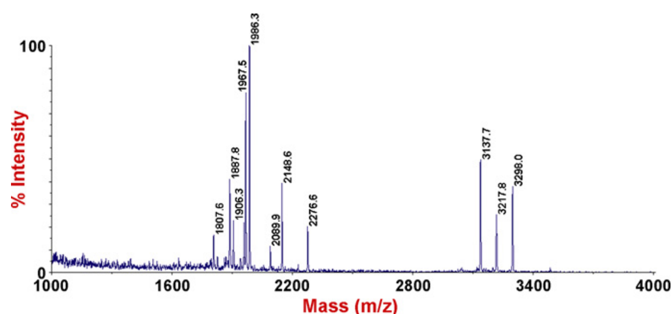

Fig. 11. Mass spectra of tryptic digests obtained after in situ hydrolysis of the HA-bound eggshell proteins. The non-phosphorylated peptides were eluted by washing buffer and the HA-bound CPP identified by FindPept tools and literature data [35].

method will allow proteins/peptides with different level of phosphorylation to be accurately measured. The time required for the HA-based procedure, from the application of the soluble protein mixture to the readout of the MALDI spectra, is less than 2 h (excluding the trypsinolysis step). The development of such a specific strategy opens up the possibility for quantitative applications in phosphoproteomics.

Acknowledgments

Publication in partial fulfillment of the requirements for the degree of PhD in Industrial Biotechnology Sciences -XXII Cycle, University of Naples 'Federico II'. The research carried out was not supported by any specific grant or funding from public or private institutions. The Authors gratefully acknowledge the "Centro di Competenza per le Produzioni Agroalimentari della Regione Campania" for permitting the use of the MS facilities. Text was revised by the American Journal Experts Association (http://www.journalexperts.com/).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2010.07.024.

References

[1] H. Daub, J.V. Olsen, M. Bairlein, F. Gnad, F.S. Oppermann, R. Korner, Z. Greff, G. Keri, O. Stemmann, M. Mann, Mol. Cell 31 (2008) 438.
[2] B. Macek, M. Mann, J.V. Olsen, Annu. Rev. Pharmacol. Toxicol. 49 (2009) 199.
[3] A. Pandey, J.S. Andersen, M. Mann, Sci. STKE 37 (2000) 1.
[4] K. Janek, H. Wenschuh, M. Bienert, E. Krause, Rapid Commun. Mass Spectrom. 15 (2001) 1593.
[5] M.C. Posewitz, P. Tempst, Anal. Chem. 71 (1999) 2883.
[6] M. Kokubu, Y. Ishihama, T. Sato, T. Nagasu, Y. Oda, Anal. Chem. 77 (2005) 5144.
[7] K. Moser, F.M. White, J. Proteome Res. 5 (2006) 98.
[8] M.W. Pinkse, P.M. Uitto, M.J. Hilhorst, B. Ooms, A.J. Heck, Anal. Chem. 76 (2004) 3935.
[9] C.T. Chen, Y.C. Chen, Anal. Chem. 77 (2005) 5912.
[10] S. Rinalducci, M.R. Larsen, S. Mohammed, L. Zolla, J. Proteome Res. 5 (2006) 973.
[11] T.E. Thingholm, T.J. Jørgensen, O.N. Jensen, M.R. Larsen, Nat. Protoc. 1 (2006) 1929.
[12] H.K. Kweon, K. Hakansson, Anal. Chem. 78 (2006) 1743.
[13] H. Zhou, R. Tian, M. Ye, S. Xu, S. Feng, C. Pan, X. Jiang, X. Li, H. Zou, Electrophoresis 28 (2007) 2201.
[14] M. Cuccurullo, G. Schlosser, G. Cacace, L. Malorni, G. Pocsfalvi, J. Mass Spectrom. 42 (2007) 1069.
[15] M.R. Larsen, T.E. Thingholm, O.N. Jensen, P. Roepstorff, T.J.D. Jorgensen, Mol. Cell Proteomics 4 (2005) 873.
[16] B. Bodenmiller, L.N. Mueller, M. Mueller, B. Domon, R. Aebersold, Nat. Methods 4 (2007) 231.
[17] K. Blackburn, M.B. Goshe, Brief. Funct. Genom. Proteom. 8 (2009) 90.
[18] D.T. McLachlin, B.T. Chait, Curr. Opin. Chem. Biol. 5 (2001) 591.
[19] P.A. Grimsrud, D.L. Swaney, C.D. Wenger, N.A. Beauchene, J.J. Coon, ACS Chem. Biol. 5 (2010) 105.
[20] S.B. Ficarro, M.L. McCleland, P.T. Stukenberg, D.J. Burke, M.M. Ross, J. Shabanovwitz, D.F. Hunt, F.M. White, Nat. Biotechnol. 3 (2002) 301.
[21] F. Addeo, J.M. Chobert, B. Ribadeau-Dumas, J. Dairy Res. 44 (1977) 63.
[22] G. Mamone, G. Picariello, P. Ferranti, F. Addeo, Proteomics 10 (2010) 380.
[23] N. Liu, R. Li, J. Walker, T. Wehr, K. Academia, A. Paulus, Alzheimer's Dementia 5 (2009) 488.
[24] J.C. Mercier, Biochimie 63 (1981) 1.
[25] A. Tholey, J. Reed, W. Lehmann, J. Mass Spectrom. 34 (1999) 117.
[26] K. Mann, J.V. Olsen, B. Macek, F. Gnad, M. Mann, Proteomics 7 (2007) 106.
[27] A.V. Vener, A. Harms, M.R. Sussman, R.D. Vierstra, J. Biol. Chem. 276 (2001) 6959.
[28] R.S. Annan, S.A. Carr, Anal. Chem. 68 (1996) 3413.
[29] E. De Hoffmann, V. Stroobant, The Atrium. 3rd ed. Mass Spectrometry: Principles and Applications, John Wiley Sons Ltd., Southern Gate, Chichester, 2007, p. 131.
[30] A.L. Burlingame, S.A. Carr, M.A. Baldwin (Eds.), Mass Spectrometry in Biology & Medicine, E-Publishing, Totowa, NJ, 2000, p. 317.
[31] X. Zhang, L. Shi, S. Shu, Y. Wang, K. Zhao, N. Xu, S. Liu, P. Roepstorff, Proteomics 7 (2007) 2340.
[32] S. Kjellstrom, O.N. Jensen, Anal. Chem. 76 (2004) 5109.
[33] S.R. Schmidt, F. Schweikart, M.E. Andersson, J. Chromatogr. B 849 (2007) 154.
[34] C. Holt, N.M. Wahlgren, T. Drakenberg, Biochem. J. 314 (1996) 1035.
[35] C.G. de Kruif, C. Holt, in: P.F. Fox, P.L.H. McSweeney (Eds.), Advanced Dairy Chemistry, Proteins, vol. 1, 3rd ed., Kluwer Academic/Plenum Publishers, New York, 2003, p. p233.
[36] J.P. DeGnore, J. Qin, J. Am. Chem. Soc. Mass Spectrom. 9 (1998) 1175.
[37] A. Dubrovska, S. Souchelnytskyi, Proteomics 5 (2005) 4678.
[38] O. Diaz, A.M. Gouldsworthy, J. Leaver, J. Agric. Food Chem. 44 (1996) 2517.
[39] P. Ferranti, F. Barone, L. Chianese, F. Addeo, A. Scaloni, L. Pellegrino, P. Resmini, J. Dairy Res. 64 (1997) 601.
[40] M. Lund, Y. Ardö, J. Agric. Food Chem. 52 (2004) 6616.