Original Paper

Characterization of multi-phosphopeptides by $\mu$HPLC–ESI-MS/MS with alkaline phosphatase treatment

The detection of phosphopeptides, especially multi-phosphopeptides, by tandem electrospray ionization mass spectrometry (ESI-MS/MS) is a great challenge due to their low abundance and the poor ionization efficiency of samples. In our recent study, a strategy was proposed for the analysis of trace multi-phosphopeptides which combined selective enrichment of phosphorylated peptides by TiO$_2$ and dephosphorylation by alkaline phosphatase (AP). After separation by $\mu$HPLC, the profiles of enriched peptides before and after AP treatment were compared, and the additional peaks appearing in the latter case hinted at the existence of multi-phosphopeptides. Subsequently, an incomplete dephosphorylation reaction was performed to partially remove the phosphate groups so that the phosphorylation sites of the multi-phosphopeptides might be estimated. Through analysis of the digests of $\beta$-casein and extracted proteins of bovine milk, more information on the multi-phosphopeptides was obtained by $\mu$HPLC–ESI-MS/MS than that obtained without AP treatment, which demonstrated that such a strategy might supply some potential information about trace multi-phosphopeptides lost in shotgun analysis.

Keywords: Alkaline phosphatase / Dephosphorylation / Multi-phosphopeptide / $\mu$HPLC–ESI-MS/MS

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1 Introduction

As one of the most important post-translational modifications (PTMs) in nature, reversible phosphorylation regulates a large number of biological processes, such as cell cycle, regulation, and differentiation. Therefore the characterization of the phosphorylation state of proteins is of great significance for an understanding of biological processes [1–3]. Nowadays, the development of a high throughput method for the detection of phosphorylated proteins and the identification of corresponding phosphorylation sites is a principal assignment in deciphering large numbers of proteins in phosphoproteomics. As a common technique in proteomics research, MS/MS has been more widely used for the identification of phosphorylated proteins than other analytical technologies. Nevertheless, such a technique still remains challenging due to the low abundance and poor ionization efficiency of phosphorylated peptides [4–6].

To overcome the signal suppression caused by non-phosphopeptides present in relatively high concentration, selective isolation of phosphopeptides is indispensable prior to MS/MS identification. Immobilized metal affinity chromatography (IMAC) is commonly used for this purpose [7–9]. The negatively charged phosphopeptides could be enriched by the specific interaction with immobilized metal ions, such as Fe$^{3+}$ [10, 11], Ga$^{3+}$ [12], and Zr$^{4+}$ [13]. However, the co-concentration of acidic non-phosphopeptides undermined the specificity of such a method. Besides, strong cation exchange chromatography (SCX) [14, 15] and immunoaffinity chromatography [16] have also been used for the isolation of phosphopeptides and phosphoproteins. Recently, much attention has been paid to the use of metal oxides such as titania [17–20], zirconia [21, 22], and alumina [23, 24] as a new kind of phosphopeptide-affinity materials displaying higher selectivity than that obtained by conventional IMAC technology.
In addition, chemical modification, such as β-elimination and Michael addition [25, 26], performed before MS/MS analysis is also an effective strategy to improve the detection sensitivity of phosphopeptides. However, disadvantages such as complicated operation and poor reproducibility restricted its further application. Another approach for the indirect detection of phosphopeptides by MS/MS is sample treatment by enzymatic dephosphorylation, which is commonly used in MALDI-based methods [27, 28]. By removing the phosphates of phosphopeptides and phosphoproteins, the mass downshift by 80 Da or 98 Da revealed the existence of potential phosphorylated peptides. MS/MS could also yield more information about the sequence and the phosphorylation sites of the phosphopeptides [29]. Compared to the MALDI mode, MS/MS employed in electrospray ionization (ESI) mode could be preferably coupled with HPLC, since this would overcome the ionization suppression effect by reducing the complexity of the peptide mixture.

More recently, the combination of complete dephosphorylation (ESI) mode could be preferably coupled with HPLC, and MS/MS employed in electrospray ionization (ESI) mode could be preferably coupled with HPLC, and MS/MS employed in electrospray ionization (ESI) mode could be preferably coupled with HPLC, since this would overcome the ionization suppression effect by reducing the complexity of the peptide mixture. More recently, the combination of complete dephosphorylation with detection by ESI-MS/MS for phosphopeptides analysis was reported. Liao et al. [30] took the advantage of the ~80nDa (1 HPO₃ = 80 Da, where n is the number of phosphate groups) mass shift due to the AP dephosphorylation to compile a computing algorithm to mine phosphopeptides signals in HPLC–ESI-MS/MS. Oda et al. [31] presented another strategy based on analyzing the retention time ratio of phosphopeptide to non-phosphopeptide to increase the reliability of phosphopeptides identification. However, in the above-mentioned ESI-MS/MS based approaches, the detection of multi-phosphopeptides before the dephosphorylation treatment was indispensable. For those undetected multi-phosphopeptides, it was difficult to deduce the phosphorylation sites after complete dephosphorylation.

In our previous experiments, we found that the multi-phosphopeptides sometimes could not be detected in the positive ion mode of ESI-MS/MS due to the low abundance and the poor ionization capability. To address this problem, a simple strategy combining selective enrichment by TiO₂ with AP-based dephosphorylation was developed for the indirect detection of the low abundance multi-phosphopeptides. Similar to the previous work [30, 31], the existence of multi-phosphopeptides could be assessed by complete dephosphorylation. Furthermore, with incomplete dephosphorylation of the enriched multi-phosphopeptides, the detection of partially dephosphorylated peptides became possible. Therefore, some information relating to the phosphorylation sites of multi-phosphopeptides could be obtained. Such a strategy was successfully applied to analysis of the digests of β-casein and extracted proteins of bovine milk, and more information about the low-abundance multi-phosphopeptides was obtained by μHPLC–ESI-MS/MS.

2 Experimental

2.1 Reagents

Calf intestinal alkaline phosphatase (1 U/μL) was obtained from Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China), and adjusted to different concentrations with 50 mM ammonium bicarbonate before use. β-Casein (≥90%), trypsin (bovine pancreas), iodoacetamide (IAA), and trifluoroacetic acid (TFA) were obtained from Sigma (St. Louis, MO). Acetonitrile (HPLC grade) was purchased from Merck (Darmstadt, Germany). Water used in the experiments was purified by a Milli-Q system (Millipore Inc. Milford, MA). All other inorganic chemicals were of analytical grade.

2.2 Instrumentation

A SpeedVac concentrator system (Thermo Electron, Milford, MA) was used for lyophilization of samples. A LC-20AD pump, an SPDM-20A ultraviolet detector (Shimadzu, Kyoto, Japan) and a TiO₂ column (1 mm id × 8 mm, Michrom Biosources, Auburn, CA) were assembled as the enrichment system for phosphopeptides. A Paradigm MG4 HPLC system (Michrom Biosources, Auburn, CA) with an in-house-packed reversed phase column (5 μm, 250 μm × 10 mm) and a Finnigan LCQΔ+ ion trap mass spectrometer (Thermo Fisher Scientific, San Jose, CA) equipped with a home-made ESI ion source [32] were hyphenated for the separation and identification of phosphorylated peptides.

2.3 Sample preparation

β-Casein was dissolved in 50 mM NH₄HCO₃, to give a final concentration of 1 mg/mL, and then digested with trypsin solution (1 mg/mL in 1 mM HCl) at 37°C overnight. The substrate:enzyme ratio was 50:1 by mass. After digestion, 10 μL formic acid was added into the solution to stop the reaction.

Milk was purchased from a local supermarket. The proteins were extracted according to the method of Lin et al. [19]. Briefly, the milk was first defatted by centrifugation at 15,000 × g for 30 min at 4°C, and then dried in a speed vacuum centrifuge. Then the lyophilized sample (5.0 mg) was mixed with 100 μL 50 mM ammonium bicarbonate (pH 8.2) containing 8 M urea, and denatured at 37°C for 1 h. Next, DTT solution (1 M, 8 μL) was added to the mixture. After purging with nitrogen for 10 min, the mixture was incubated at 55°C for 4.5 h. Thereafter, the sample was mixed with IAA solution (1 M, 20 μL) and incubated for 1 h in the dark at room temperature. Finally, the mixture was diluted 10-fold with 50 mM ammonium bicarbonate, and incubated at 37°C for 16 h at a protein/
enzyme ratio of 50:1 w/w. After digestion, the solution was acidified by 10 μL formic acid.

2.4 Selective enrichment of phosphopeptides by a TiO₂ column

A 100-μg sample was first loaded onto the TiO₂ column, and then washed with formic acid (pH 3.0) for at least 5 min before the bound peptides were eluted by ammonia (pH 10.8) at a flow rate of 0.05 mL/min. The detection wavelength was 214 nm. A 200-μL TiO₂-eluate was collected according to the eluting peaks, and then acidified and evaporated by vacuum centrifuging. The dried samples were stored at −20°C and mixed with formic acid before μHPLC–ESI-MS/MS analysis.

2.5 Alkaline phosphatase treatment

Multi-phosphopeptide assignments were verified by AP dephosphorylation of the enriched phosphorylated peptides of β-casein digests followed by μHPLC–ESI-MS/MS analysis. A 100-μL volume of alkaline phosphatase solution (1250 μU/μL) was added to 10-μL TiO₂-eluates. After incubation at room temperature for 30 min and 120 min, respectively, the sample was acidified by the addition of 5 μL of 10% v/v formic acid. In the former case, partial dephosphorylation was performed, but in the latter case all phosphate groups of phosphopeptides were removed to give what was defined as completely dephosphorylated product. Meanwhile, samples for parallel experiments were prepared by adding the same solution without AP.

To slow down the dephosphorylation procedure, incomplete dephosphorylation was performed with a low enzyme/substrate ratio at room temperature for indirect detection of multi-phosphopeptides. To investigate the influence of reaction time, 190 μL of alkaline phosphatase solution (125 μU/μL) was added to the lyophilized TiO₂-eluted sample of β-casein digest (100 μg) and incubated for various times. A vial containing 9.5 μL of solution was extracted once over a period of time, and then diluted by formic acid to 50 μL. The same method was used for the treatment of the digests of the milk proteins enriched on the same TiO₂ column. The eluate of 100 μg of milk digest was collected and lyophilized, and then mixed with 250 μL alkaline phosphatase solution (1000 μU/μL), and incubated at room temperature. Every 30 min, 10 μL of solution was taken, and diluted to 1 mg/mL, followed by μHPLC–ESI-MS/MS analysis.

2.6 μHPLC–ESI-MS/MS analyses

For the analysis of the digest of β-casein, 0.1% v/v formic acid and ACN solution containing 0.1% v/v TFA were used as buffers A and B. The volume of sample injected was 2 μL. ESI-MS was performed in a positive ion mode with 2 kV spraying voltage. The heated capillary temperature was 180°C. A total ion chromatogram (TIC) was recorded in the mass range of 500–2000 m/z. The MS/MS collision energy was set at 35%. Protein identification was performed by searching the NCBI bovine.fasta database (www.ncbi.nlm.nih.gov) using BioWorks software with the SEQUEST search program.

3 Results and discussion

3.1 Strategy for the identification of multi-phosphopeptides by μHPLC–ESI-MS/MS

The strategy for the analysis of multi-phosphopeptides by μHPLC–ESI-MS/MS with dephosphorylation treatment is shown in Fig. 1. It can be seen that selective enrichment by TiO₂ permits phosphorylated peptides to be separated from the non-phosphorylated ones, which greatly improves the detection sensitivity of such peptides. Even though, for the multi-phosphopeptides, direct identification by ESI-MS/MS was difficult because of the poor ionization capacity caused by the negative charge suppression of phosphate groups. As demonstrated by MALDI-TOF MS/MS [29], high peak intensity could be obtained for the dephosphorylated forms of multi-phosphopeptides. Therefore, for the separation and identification of phosphorylated peptides by μHPLC–ESI-MS/MS based on the bottom-up strategy, dephosphorylation by AP treatment was adopted. Through comparison of the original, partially dephosphorylated, and completely dephosphorylated results of μHPLC–ESI-MS/MS, more information about the existence and the possible phosphorylation sites of milk proteins, 0.1% v/v TFA and ACN solution containing 0.1% v/v TFA were used as buffers C and D. The volume of sample injected was 2 μL. ESI-MS was performed in a positive ion mode with 2 kV spraying voltage. The heated capillary temperature was 180°C. A total ion chromatogram (TIC) was recorded in the mass range of 500–2000 m/z. The MS/MS collision energy was set at 35%. Protein identification was performed by searching the NCBI bovine.fasta database (www.ncbi.nlm.nih.gov) using BioWorks software with the SEQUEST search program.

Figure 1. Strategy for the characterization of multi-phosphopeptides by μHPLC–ESI-MS/MS with dephosphorylation treatment.
3.2 Complete dephosphorylation treatment for detecting the multi-phosphopeptide of \( \beta \)-casein

In our experiments, bovine \( \beta \)-casein, which has five phosphorylation sites at serine residues, was employed as model phosphorylated protein. Total ion chromatograms of the digest of 100 \( \mu \)g \( \beta \)-casein obtained by \( \mu \)HPLC–ESI-MS/MS are shown in Fig. 2. From Fig. 2A, it can be seen that both phosphorylated and non-phosphorylated peptides were observed in the totally digested products, and the relative intensity of two mono-phosphorylated peptides was not identified due to its low abundance and the negative charge suppression effect of multi-phosphorylated peptides in the ESI procedure.

After enrichment by a TiO\(_2\) column, as shown in Fig. 2B, only mono-phosphopeptides were observed with obviously increased signal intensity, which further demonstrated the selective enrichment capacity of TiO\(_2\) for phosphopeptides. However, the previously reported tetra-phosphopeptide \([8]\) was not identified due to its low abundance and the negative charge suppression effect of multi-phosphorylated peptides in the ESI procedure.

After the addition of 100 \( \mu \)L of 1250 \( \mu \)M AP solution to the 10 \( \mu \)L TiO\(_2\)-eluates of \( \beta \)-casein and incubation at room temperature for 30 and 120 min, respectively, the products were analyzed by \( \mu \)HPLC–ESI-MS/MS. From Fig. 2C and 2D, it can be seen that after AP-treatment the original two mono-phosphopeptide peaks at \( m/z \) 1032.0 and 1279.0 were displaced by 992.0 and 1239.0, respectively, corresponding to the sequences FQSEEQQQTEDELQDK and IEKFQSEEQQQTEDELQDK, respectively. The \(-40 \) Da (+2) shift resulted from the dephosphorylation reaction, reconfirming the existence of mono-phosphopeptides in the digest of \( \beta \)-casein. In addition, after 30 min treatment, as shown in Fig. 2C, two peaks appeared at \( m/z \) 1442.2 and one peak at \( m/z \) 1402.5, respectively corresponding to the mono-phosphorylated and completely dephosphorylated forms of the tetraphosphopeptide, RELEELNPGEIVESLpSpSpSEESITR (\( m/z \) 1562.5). With the further increase of AP-treatment time to 120 min, only the completely dephosphorylated one existed, identified as RELEELNPGEIVESLSSSSEESITR, as shown in Fig. 2D. All these results not only demonstrated that, by complete dephosphorylation treatment, the previously undetectable multi-phosphopeptides information by \( \mu \)HPLC–ESI-MS/MS could be complemented, but also revealed that the dephosphorylation procedure took place step-by-step until all phosphate groups were lost.

3.3 Incomplete dephosphorylation treatment for determining the multi-phosphorylated sites of \( \beta \)-casein

Although complete dephosphorylation treatment by AP was powerful in proving the existence of multi-phosphopeptides in samples, it was hard to deduce the phosphorylation sites on removal of all phosphate groups. The result shown in Fig. 2C reminded us that incomplete dephosphorylation might be a potential method to estimate the locations of phosphorylation sites of multi-phosphopeptides by studying and blasting their partially de-phosphorylated forms, similar to the shotgun method used in DNA sequencing \([33]\). To achieve such a goal, the effects of experimental conditions, such as the substrate-to-enzyme ratio, reaction temperature, and incubation time, were studied. In our experiments, it was found that it was convenient to regulate the degree of dephosphorylation of multi-phosphopeptides by varying the reaction time, rather than the ratio of enzyme/substrate and the reaction temperature. Therefore, the effect of incubation time on the dephosphorylation reaction was studied at room temperature.

3.3.1 Effect of reaction time on incomplete dephosphorylation

Figure 3 shows the total ion chromatogram of the enriched phosphorylated peptides in the digest of \( \beta \)-casein treated with AP at different reaction times. It can be seen that with the increase of AP-treatment time, the dephosphorylated peptides at \( m/z \) 992.0 (a) and 1239.0 (b), the same as those shown in Fig. 2C and 2D, remained almost the same, which further demonstrated that the dephosphorylation of mono-phosphopeptides was easy.
Besides, three new chromatographic peaks (c1, c2, c3) at m/z 1442.5 appeared under each set of reaction conditions, similar to those in Fig. 2C. Meanwhile, the dominant ion peak of neutral loss of H3PO4 (49 Da, +2) in the MS/MS spectrum also confirmed them as phosphopeptides. The different retention times but the same m/z might be due to the different phosphorylation site location. Another peak (d) appearing at m/z 1402.5 corresponded to the completely dephosphorylated form of the tetra-phosphopeptide, as also shown in Fig. 2C and 2D. In addition, it was found that the peak areas of peaks c1, c2, and c3 at m/z 1442.5 and peak d at m/z 1402.5 kept on increasing in the dephosphorylation procedure we studied. Combining these results with those shown in Fig. 2, we could confirm that the multi-phosphopeptides were converted into mono- or di-phosphorylated ones before being completely dephosphorylated.

Our initial motivation was to develop a shot-gun method by using AP dephosphorylation for detecting multi-phosphopeptides, by which partial dephosphopeptides containing different locations of phosphorylation sites could be generated at random before identification by MS/MS. Through the analysis of all partial dephosphopeptides, information about undetectable multi-phosphopeptides could be obtained. However, it was found that, in most cases, only some of the phosphorylation sites could be deduced by this approach, since not all the phosphorylated amino acids of multi-phosphopeptides possessed uniform activities in the dephosphorylation procedure [34], which meant only the comparatively more inert phosphorylation sites would have remained after AP treatment.

### 3.3.2 Estimation of the multi-phosphorylation sites of β-casein

Identification of the phosphorylation sites of partial dephosphopeptides was a difficult task because of the low abundance and the poor sensitivity due to the dominant neutral loss peaks of phosphate.

First, database searching with SEQUEST algorithm was used to deduce the phosphorylation sites. For peptides with the same amino acid sequence but different phosphorylation sites, the result with the highest Xcorr value was considered to be true. According to this principle, information about the potential phosphorylation sites of the above-mentioned partially dephosphorylated peptides generated from the tetra-phosphopeptide of β-casein under different reaction conditions was collected (listed in Table 1), and the statistic study showed that phosphorylation locations of the three mono-phosphopeptides (c1, c2, c3 in Fig. 2) might be at S-17, S-19, and S-18, respectively.

Subsequently, MS/MS spectra were manually validated to assign the phosphorylation sites of the partial dephosphopeptides, which took advantage of the extract mass data exported from MS² spectra. Three criteria were proposed to assess the phosphorylation sites. First, the dominant peak of the MS² spectra should be the neutral loss of

### Table 1. Estimation of the phosphorylation sites of three mono-phosphopeptides (m/z 1442.5) generated from the tetra-phosphopeptide of β-casein by SEQUEST analysis and manual validation.

<table>
<thead>
<tr>
<th>Reaction time (min)</th>
<th>Site of peak 1 (c1)</th>
<th>Site of peak 2 (c2)</th>
<th>Site of peak 3 (c3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>120</td>
<td>S-17</td>
<td>–</td>
<td>S-19</td>
</tr>
<tr>
<td>180</td>
<td>S-15</td>
<td>S-17</td>
<td>S-19</td>
</tr>
<tr>
<td>240</td>
<td>S-18</td>
<td>S-17</td>
<td>S-19</td>
</tr>
<tr>
<td>270</td>
<td>S-17</td>
<td>S-17</td>
<td>S-19</td>
</tr>
<tr>
<td>300</td>
<td>S-15</td>
<td>S-17</td>
<td>S-19</td>
</tr>
<tr>
<td>360</td>
<td>S-17</td>
<td>S-17</td>
<td>S-19</td>
</tr>
<tr>
<td>420</td>
<td>S-17</td>
<td>S-17</td>
<td>S-19</td>
</tr>
<tr>
<td>Possible site</td>
<td>S-17</td>
<td>S-17</td>
<td>S-19</td>
</tr>
</tbody>
</table>

“–” means “not identified”. “S” means “serine”.

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peaks of phosphate. Secondly, the ratio of the relative intensity of any ion peak to that of the dominant peak was not less than 0.1%. (This step was to wipe out the noise infection caused by baseline. The setting in our experiment was low, due to the intensity limit of the ion peaks. If the MS2 spectra were acceptable, this value could be appropriately increased.) Thirdly, at the presumed phosphorylation location, the total intensity of its neighboring y ions, which satisfied the theoretical phosphorylation site conditions (according to the information provided by “Bioworks” software), was higher than that of other possible positions. The candidate phosphopeptides fitting all mentioned conditions would be considered as the “real” ones. By such a method, the potential phosphorylation sites of the three mono-phosphorylated peptides (c1, c2, c3 in Fig. 3) were estimated at S-17, S-19, and S-18, which was consistent with the results produced by the database searching algorithm.

Besides, a di-phosphopeptide generated from the tetra-phosphopeptide of β-casein was found in the SEQUEST result, although it was not observed in the TIC (Fig. 3). To validate the existence of such a peptide by incomplete dephosphorylation, the selective ion scan (SIC) was explored, which could eliminate interference from background ions and provide higher sensitivity than a normal full scan. The ion of m/z 1482.5 was chosen as precursor ion of SIC, and its MS2 spectrum is shown in Fig. 4. Adopting the assessment procedure mentioned above, all the possible phosphorylation sites were speculatively proposed at S-18 and S-19. Then the ion of m/z 1442.5 was also chosen as precursor ion to detect the mono-phosphopeptides from the tetra-phosphopeptide of β-casein, and the results were analyzed by the database searching algorithm and manual validation, respectively. The location of the phosphorylation site of each peptide was found to be the same as previously obtained (data not shown). All these results further demonstrated that, by incomplete dephosphorylation treatment, more information about the phosphorylation sites could be obtained by analyzing the partially dephosphorylated multi-phosphopeptides. In our experiment, the ability to identify phosphopeptides was restricted by the sensitivity of our mass spectrometer. On use of a highly sensitive MS instrument, more information could be obtained.

3.4 Analysis of proteins extracted from bovine milk

To evaluate the applicability of the proposed method for the characterization of multi-phosphopeptides by μHPLC–ESI-MS/MS with alkaline phosphatase treatment, the digests of extracted proteins from bovine milk, abundant in phosphorylated casein proteins [35], were analyzed after enrichment on TiO2. As shown in Table 2, without AP treatment, only four phosphopeptides, including three mono-phosphopeptides and one di-phosphopeptide, were detected in 2 μg of TiO2-enriched milk sample, and no multi-phosphorylated peptides were detected under such experimental conditions.

<table>
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<th>Sequence</th>
<th>MH’</th>
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<th>Protein</th>
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<tbody>
<tr>
<td>FQpSEEQQTDEDELQDK</td>
<td>2062.97</td>
<td>2</td>
<td>beta-casein</td>
</tr>
<tr>
<td>DlGpSEpSTEDQAMEDIK</td>
<td>1928.80</td>
<td>2</td>
<td>casein α-S1</td>
</tr>
<tr>
<td>VPQLEIVPnSAEER</td>
<td>1661.73</td>
<td>2</td>
<td>casein α-S1</td>
</tr>
<tr>
<td>YKVPQLEIVPnSAEER</td>
<td>1953.08</td>
<td>2</td>
<td>casein α-S1</td>
</tr>
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</table>

*p* phosphorylation site.

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Table 2. TiO2-enriched phosphopeptides from the digest of milk proteins without alkaline phosphatase treatment

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Thereafter, in order to examine whether the multi-phosphopeptides existed, the phosphorylated peptides enriched by TiO2 were treated with AP for different reaction times, and then analyzed by μHPLC–ESI-MS/MS. All the concentrated phosphopeptides and their corresponding dephosphorylated forms are listed in Table 3. Two undetected multi-phosphopeptides were validated according to their partially dephosphorylated forms under the same conditions. One was the di-phosphopeptide ELEELNVPGEIVESLSpSpSEESITR generated from the tetra-phosphorylated peptide of β-casein in extracted protein sample of milk, which was the same as that of the standard β-casein digests, except for the lack of arginine (R) caused by the different tryptic cleavage. Its two phosphorylation sites were also identified at S-18 and S-19, corresponding to the above result. The other partial phosphopeptide NANEEYSGSpSpSEEpSAEVATEEVK...
4 Concluding remarks

The strategy of combining TiO$_2$ enrichment and dephosphorylation by AP was proposed for the characterization of multi-phosphopeptides by μHPLC–ESI-MS/MS. Through complete dephosphorylation of the TiO$_2$-eluate, the existence of multi-phosphopeptides could be confirmed by the appearance of additional peaks. In addition, the multi-phosphorylation sites of such peptides could be estimated by analyzing the partially dephosphorylated peptides generated in the procedure of incomplete dephosphorylation. All these results demonstrated that such a method might be of great promise in the study of phosphorylated proteomes by supplying complementary information about the low abundance multi-phosphopeptides, which might be easily lost in shot-gun analysis by μHPLC–ESI-MS/MS.

5 References


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