A novel mesoporous TiO_2-based experimental layout for on-target enrichment and separation of multi- and mono-phosphorylated peptides prior to analysis with matrix assisted laser desorption-ionization mass spectrometry

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A simple method for on-target enrichment and subsequent separation and analysis of phosphorylated peptides is presented. The tryptic digest of a phosphorylated protein, in this case *β*-casein, is loaded onto a spot on a thin stripe made of mesoporous TiO_2 sintered onto a conductive glass surface. After washing

with a salicylic buffer in order to remove the non-phosphorylated peptides, the stripe is placed in an elution chamber containing a phosphate solution. In a way analogous to thin layer chromatography (TLC), the phosphate solution acts as an eluent, clearly separating multi- and monophosphorylated peptides. By performing matrix assisted laser desorption-ionization mass spectrometry (MALDI-MS) along the stripe, the detection of all phosphorylated peptides present in the digest is facilitated, as they are isolated from each other. The method was also tested on commercial drinking milk, achieving successful separation between mono- and multiphosphorylated peptides, as well as a detection limit in the femtomol range. As the enrichment, separation and analysis take place in the same substrate, sample handling and risk of contamination and sample loss is minimized. The results obtained suggest that the method, once optimized, may successfully provide with a complete phosphoproteome.

Introduction

The characterization of phosphorylated proteins is an important[1] but recognized difficult task, and the search for new and improved methods for enrichment and subsequent detection of phosphorylated peptides has been active during the last years. In order to enrich the phosphopeptides most of the new methods make use of the high affinity of the phosphoryl group to metals and metal oxides (e.g., on immobilized metal affinity chromatography (IMAC)[2-5] , metal oxide affinity chromatography (MOAC)[1,6-14], sequential immobilized metal affinity chromatography (SIMAC)[15] and direct on-target enrichment[16-23]). Mass spectrometry (MS) is then commonly used to analyze the peptides. On-target enrichment methods have shown improved results, probably because of the minimization of sample handling. The principle of these methods is to create a matrix assisted laser desorption-ionization mass spectrometry (MALDI-MS) target modified with materials on which the enrichment can be made directly. TiO_2 has proven to be one of the best substrates for specific enrichment of phosphorylated peptides, since it is stable in a wide pH range and the non-specific binding of acidic non-phosphorylated peptides can be avoided by using buffers containing organic acids[6,13,16-18]. However, even though acceptable enrichment efficiencies have been obtained recently, there is still room for improvement and

more remains before a whole phosphoproteome can be determined by means of a simple method. In this study we are taking the on-target enrichment method one step further by combining it with on-target thin layer chromatography (TLC). An on-target TiO_2 stripe through which the phosporylated peptides are eluted is used, leading to the separation of the phosphorylated peptides, both by mass and by degree of phosphorylation.

Experimental Section

Materials

β-casein (from bovine milk, 90%), 2,-5-dihydrobenzoic acid (DHB), salicylic acid (SA), acetonitrile (ACN), trifluoroacetic acid (TFA >99%), ammonium hydroxide solution (25-28%), ammonium dihydrogen phosphate (98%), ammonium bicarbonate, 1,4-dithiothreitiol (DTT) and iodoacetamide (IAA) were obtained from Sigma-Aldrich (Schnelldorf, Germany). Sequence grade trypsin was a product of Roche Diagnostics (Basel, Switzerland). Drinking milk was purchased at a local grocery store. All reagents were used as received. All aqueous solutions were prepared in deionized water (18.4 MΩ cm) obtained from a Milli-Q system (Millipore, Bedford, USA). TiO_2 paste containing nanoparticles with a diameter of about 20 nm and pure anatase crystal structure was purchased from Dyesol (New South Wales, Australia). The paste was further diluted with three parts of terpeniol before use.

Preparation of the modified MALDI target

A TiO_2 stripe was prepared by screen printing on a microscope glass slide (75mm×25mm) coated with a conductive indium-tin oxide (ITO) layer. The stripe was sintered onto the surface following a well defined temperature program (10 min ramp to a final temperature of 180 ^{o}C, hold for 10 min. Second 10 min ramp to a final temperature of 220 ^{o}C, hold for 10 min. Third 10 min ramp to a final temperature of 390 ^{o}C, hold for 10 min. Final 15 minutes ramp to a temperature of 450 ^{o}C, hold for 30 min), and subsequently cooled down before storing in a desiccator. The obtained stripe was ≈ 75 x 1 x $1.25*10^{-3}$ mm in size. The stripe was characterized by a DEKTAK 3 profilometer (Veeco, Mannheim, Germany).

Digestion of β-casein and commercial drinking milk

A portion of 1 mg of *β*-casein was dissolved in 0.5 mL of 100 mM ammonium bicarbonate aqueous solution. A volume of 40 µL of 10 mM DTT was added and the sample was incubated at 50 ^{o}C for 15 min. After cooling down, 40 µL of 20 mM IAA were added and the solution was kept in the dark for 15 min at room temperature. Finally, the protein was digested with trypsin (2.5% w/w) overnight at 37 ^{o}C. Drinking milk was digested with trypsin by following the protocol described by Qiao et al.[16]. A portion of 30 µL of drinking milk with 1.5% fat was diluted with 900 µL of 25 mM aqueous ammonium bicarbonate solution and centrifugated at 15 000 rpm for 20 minutes. The supernatant was removed and denaturated at 100 ^{o}C for 5 minutes, before incubation with 30 µg trypsin at 37 ^{o}C over night. The tryptic digest product was diluted 25 times with a DHB buffer (20 mg/mL DHB in ACN/water/TFA 50/49.9/0.1 (v/v)) before enrichment. According to the protein content given on the label of the milk package, the final protein concentration of the milk digest would be at maximum 4.53 µg/mL. The actual concentration of the digest is probably considerably lower, as not all the protein is collected after centrifugation.

On-target enrichment and separation of phosphopeptides

The tryptic digest of *β*-casein was diluted in DHB buffer to a final concentration of 120 µg/mL (≈ 5 µM). This buffer was used to avoid the non-specific binding of non-phosphorylated acidic peptides[6,23]. A volume of 0.5 µL of the digest solution (≈ 2.5 pmol of protein) was loaded onto the TiO_2 stripe at approximately 1 cm from the edge. To remove the non-phosphorylated peptides, the stripe was washed by immersion in a large volume of SA buffer (18 mg/mL SA in ACN/water/TFA 50/49.9/0.1 (v/v)) with shaking for 30 min. The SA buffer was then removed and replaced by 0.15% TFA in water for another 30 min under constant shaking. The stripe was then let to dry before it was placed in the elution chamber. The required volume of 0.1 M ammonium dihydrogen phosphate aqueous solution (pH = 4.55) was added until the surface of the solution was slightly (~1 mm) beneath the loading site. The sample was then eluted during 15 hours, before it was taken up and left to dry at room atmosphere.

Finally, the stripe was carefully covered with several 0.1 µL drops of 2% (v/v) aqueous TFA solution, and it was left to dry again at room atmosphere. Before the MALDI-MS experiments the stripe was covered with a DHB matrix solution (20 mg/mL DHB in ACN/water/TFA 50/49.9/0.1 (v/v)).

In the case of the drinking milk digest, a portion of 1 µL of the solution (approx. 200 fmol total protein content) was loaded onto a TiO_2 stripe. The same procedure as above was employed to enrich, separate and analyze the phosphopeptides For comparison, the milk digest was also loaded onto two TiO_2 spots, one was left untreated and the other was washed with DHB buffer to remove all non-phosphorylated peptides as described by Eriksson et al.[23]. Before analysis, these spots were treated with 400 mM NH_4OH and let to dry. Then, 2% (v/v) aqueous TFA was added and let to dry. Finally, a matrix solution containing phosphoric acid (20 mg/mL DHB in ACN/water/phosphoric acid 50/49/1 (v/v)) was added.

MALDI-TOF/TOF MS

Mass data was acquired with an Ultraflex II MALDI-TOF/TOF (Bruker Daltonics) in reflector positive mode. A mass range of 700-4000 Da was analyzed. The stripes were divided into 15 numbered sections with a width of about 5 mm each, and all sections were shot randomly with 600 shots/section. For each section a separate spectrum was obtained. The sequences of the detected peptides are cited from the Uniprot Knowledgebase, unless stated otherwise. The sequence numbering is given without considering the signal peptide.

Results and discussion

Enrichment, separation and analysis of β-casein phosphopeptides

Mass data from the MALDI-MS shows that the phosphorylated peptides from the *β*-casein tryptic digest can be found at distances from 0 to 3 cm away from the loading point on the TiO_2 stripe, corresponding to sections 2-8. Figure 1 shows the MALDI mass spectra obtained on each of these sections. A comparison between the spectra from section 2 and section 5 (figure 1a and c) shows clearly that the multi- and mono- phosphorylated *β*-casein peptides have been successfully separated. The

characteristic *β*-casein multiphosphorylated peptide, marked A (*m/z* = 3122.6, *β*(1-25), RELEELNVPGEIVE-pS-L-pS-pS-pS-EESITR) is only visible in section 2, at the same position where the sample was loaded, while the first traces from monophosphorylated peptides, B (*m/z* = 2061.7, *β*(33-48), FQ-pS-EEQQQTEDELQDK) and C (*m/z* = 2556.7, *β*(33-52), FQ-pS-EEQQQTEDELQDKIHPF[15,16,22]), are found in section 5. This implies a separation distance of at least 1 cm for the multi- and the mono- phosphorylated peptides. A comparison between the spectra from sections 5-8 (figure 1c-f) shows also small differences in the retention times of the monophosphorylated *β*-casein peptides. These differences seem to arise from their difference in mass, since the lightest, B (*m/z* = 2061.7), have the highest intensity in section 8 and the heaviest, C (*m/z* = 2556.7), in section 6, while the peptide with intermediate mass, D (*m/z* = 2432.6, *β*(30-48), IEKFQ-pS-EEQQQTEDELQDK) only occurs in section 7. Based on these results, it appears that, when comparing peptides with overlapping sequences, the stripe works in a manner opposite to that of a size exclusion column. Figure 2 shows an illustration of the stripe in real size, with the TiO_2 in gray and the main location of each of the phosphopeptides marked by black spots. However, it is necessary to point out that a certain degree of smearing and, therefore, spreading of the adsorbed phosphopeptides on the stripe is to be expected when adding the 2% TFA solution and the matrix. The contents of a specific section may therefore spread to the neighboring sections, making it difficult to clearly demonstrate the separation. In order to confirm the apparent differences in the retention times according to the mass of the peptides, it appears necessary to work with longer stripes that potentially induce larger separations between different peptides, allowing thus a better detection of individual signals. Such experiments are currently being carried out in order to verify that the proposed separation indeed occurs.

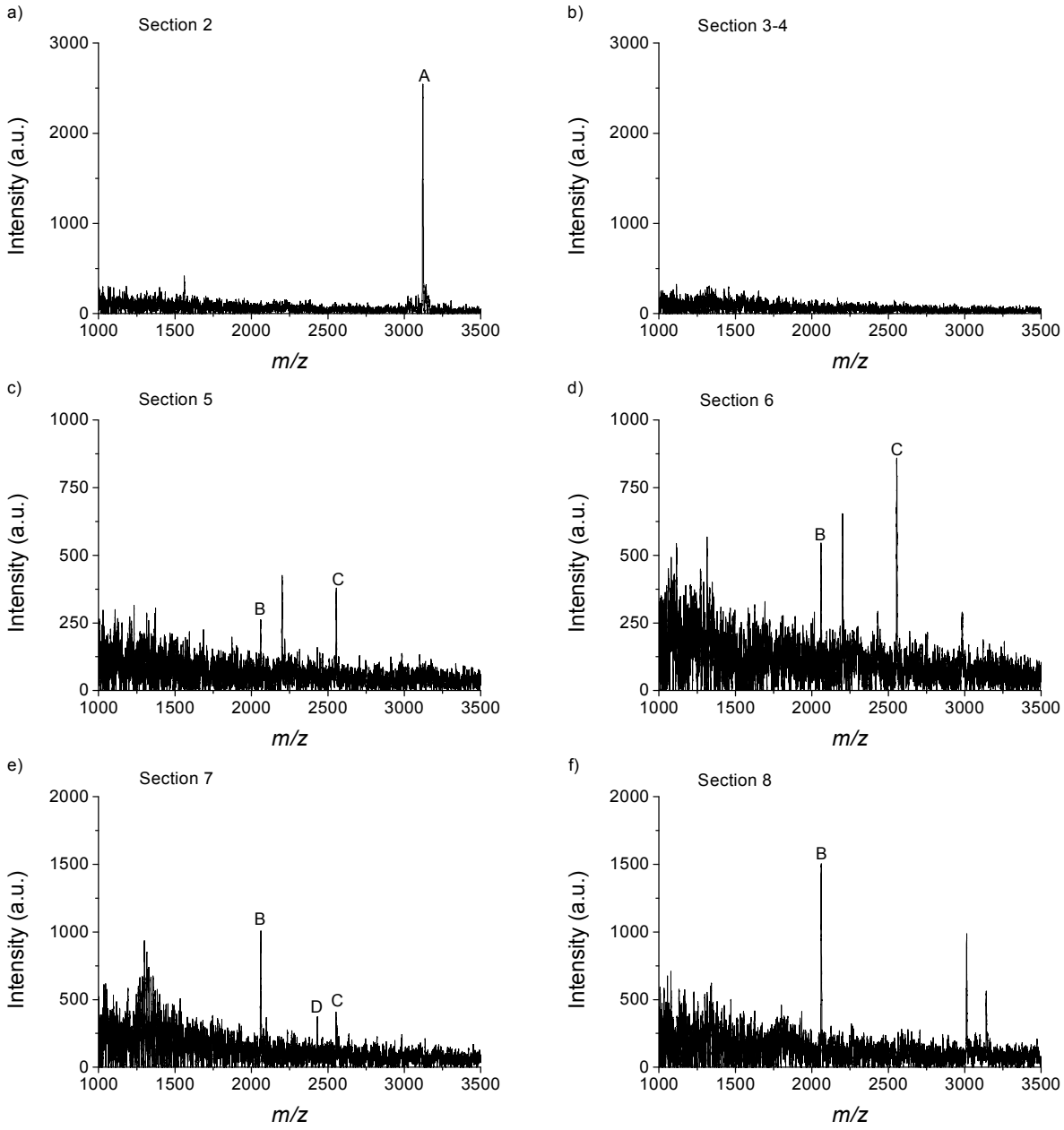

Figure 1. Mass spectra of sections 2-8 of the stripe after the TLC experiment. The *β*-casein phosphopeptide peaks are marked with A-D, where each letter corresponds to a certain peptide as described in the text.

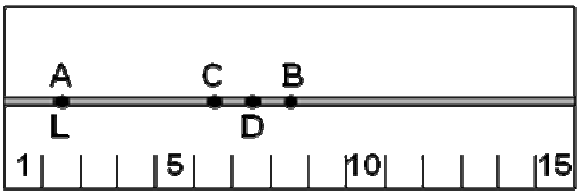

Figure 2. Picture of the modified target in real size with the TiO_2 stripe in gray. The numbered sections are shown. The loading position L, as well as the main positions of the peptides A-D are indicated.

The additional, unmarked, peaks present in figure 1c-f arise from methionine oxidated peptides (*m/z* = 2202, *β*(184-202), D-Mox-PIQAFLLYQEPVLGPVR and *m/z* = 3014, *β*(177-202), AVPYPQRD-Mox-PIQAFLLYQEPVLGPVR). Although previous work has shown that signals from methionine oxidated peptides are commonly observed following enrichment by means of the procedure described above, the reason for their appearance is still not completely understood[23].

In a previous publication[23] from this lab it has been shown that the signal arising from the *β*-casein multiphosphorylated peptide is much smaller than the signals arising from the monophosphorylated peptides, probably due to the difficulty in creating positive ions when several phosphoryl groups are present. By separating the multiphosphorylated peptide from the rest of the mixture, its detection is facilitated, as the present results show. Being able to separate phosphorylated peptides enriched from a complex mixture may prove very valuable when trying to obtain a complete phosphoproteome.

Enrichment, separation and analysis of phosphopeptides in commercial drinking milk

Commercial drinking milk was studied using the protocol described above in order to prove the ability of the described method to separate multi- and mono- phosphorylated peptides from more complex samples at very low concentrations. In total, two multiphosphorylated and four monophosphorylated peptides from *α*-S1- and *β*-casein were identified, as listed in Table 1. Figure 3 shows the mass spectra obtained from the milk digest without enrichment, after on-target phosphopeptide enrichment according to Eriksson et al.[23], and at section 2 after enrichment and elution following the procedure described in this report. Figure 3-c shows clearly that the signals arising from multiphosphorylated peptides are enhanced on the loading section on the TiO_2 stripe. These peptides are not observed on any other section of the stripe. On the other hand, Figure 4 shows how the intensity of the signals arising from monophosphorylated peptides is distributed along the stripe. Clearly, two of the monophosphorylated peptides signals (peptides 1 and 2) reach a maximum at unique sections on the stripe, evidencing different retention times and therefore suggesting separation from each other. The signals from peptides 3 and 6 share the same section for maximum signal intensity, but are clearly separated from the other peptides in the mixture. As in the case of pure *β*-casein, it is observed that peptides with overlapping

sequences and phosphorylation sites at the same relative position (in this case, peptides 1 and 2) have different retention times depending on their mass, with the smallest and lightest (peptide 1) being eluted further into the stripe than the heaviest one (peptide 2).

Table 1. Phosphorylated peptides from a commercial milk sample identified after on target enrichment and separation on a TiO_2 stripe.

	$[M+H]^+$	**Position**	**Peptide sequence**
1	1660.60	α-S1 (121-134)	VPQLEIVPN-pS-AEER
2	1952.08	α-S1 (119-134)	YKVPQLEIVPN-pS-AEER
3	2061.7	*β* (33-48)	FQ-pS-EEQQQTEDELQDK
4	2965.61	*β* (2-25)	ELEELNVPGEIVE-pS-L-pS-pS-pS-EESITR
5	3122.38	*β* (1-25)	RELEELNVPGEIVE-pS-L-pS-pS-pS-EESITR
6	1832.83[+]	α-S1 (104-119)	YLGEYLIVPN-pS-AEER

[+] Caused by alternative splicing, as reported by Larsen et.al.[6]

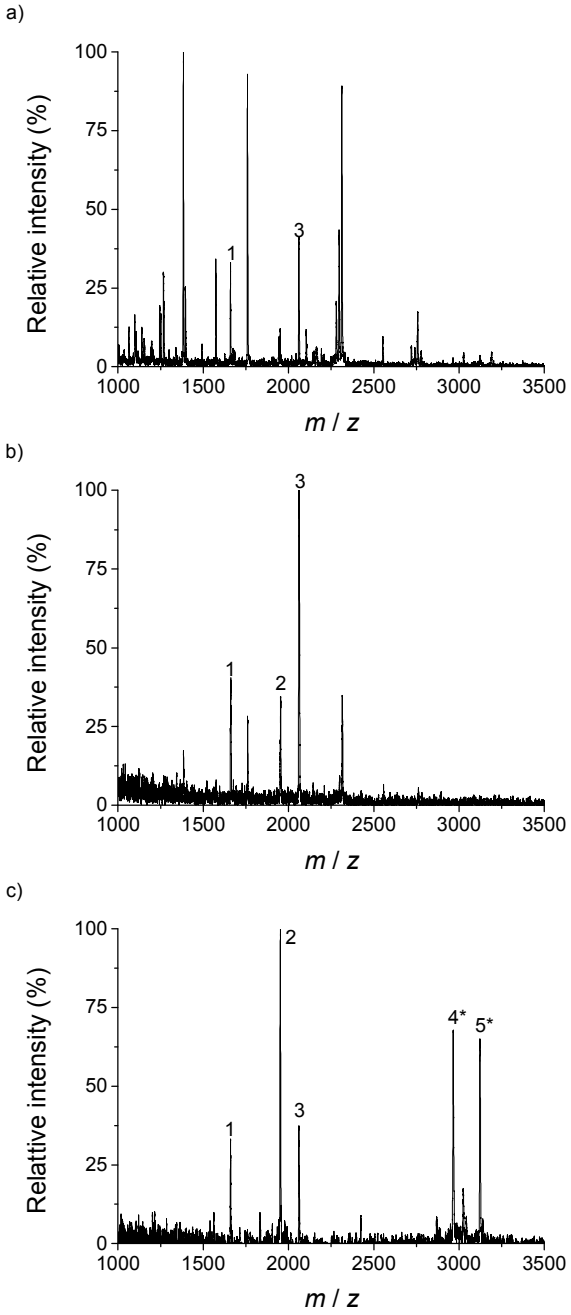

Figure 3. Mass spectra obtained from commercial drinking milk on a TiO_2 modified MALDI target a) without performing any enrichment treatment, b) after optimized on-target phosphopeptide enrichment according to Eriksson et al.[23], and c) at the loading point (section 2) after phosphopeptide enrichment and separation on a TiO_2 stripe. The labeled peaks correspond to the phosphopeptides listed in Table 1. Multiphosphorylated peptides are indicated by an asterisk.

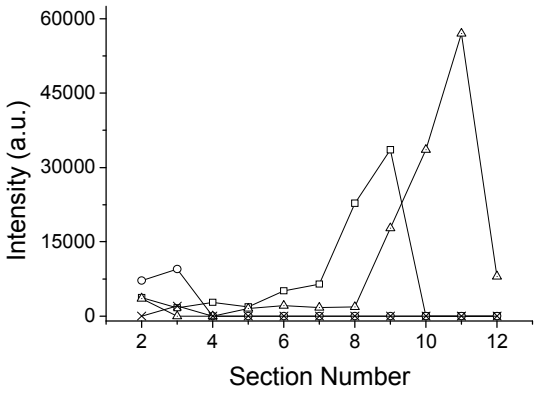

Figure 4. Intensity of the monophosphopeptide signals as a function of the section where the spectrum is obtained. Squares: peptide 1, circles: peptide 2, triangles: peptide 3 and crosses: peptide 6 (peptide numbering according to Table 1).

Noteworthy, one phosphorylated peptide identified from both the pure β-casein and the commercial drinking milk (peptide B in the former and peptide 3 in the latter) is eluted differently in the two cases in spite of the similar experimental conditions (up to section 8 in pure β-casein and up to section 12 with commercial milk). In the case of the more complex mixture, the peptide is eluted further away from the loading point. It is likely that in a complex mixture there is more competition for the adsorption sites on TiO_2 than in a simpler mixture. When working with the drinking milk, the adsorption sites of sections 7-10 are partially covered by peptide 1, and, therefore, their interaction with peptide 3 is diminished and it may elute further into the stripe. Also, small variations between different stripes (e.g., random variations in the height profile) may give rise to slightly different retention times, accounting at least partially for the variance between the two systems.

The separation between multi- and monophosphorylated peptides likely occurs due to the competition between phosphate anions in the eluent and the phosphoryl group on the phosphopeptides for the same adsorption sites on TiO_2. As the eluent reaches the loading point on the stripe some of the enriched phosphopeptides will be displaced by the phosphate and eluted along the stripe. Since the multiphosphorylated peptides are bound to more than one site on the TiO_2, they are harder to displace. In the present case the monophosphorylated peptides where eluted up to 5 cm away from the loading point, while the multiphosphorylated peptides, containing four phosphorylation sites, remained in the loading spot, indicating that they were not desorbed. Although additional experiments with more complex mixtures are needed in order to explore the limits of the described method, it is clear from the presented results that the approach has the potential to become a simple standard method to enrich phosphorylated peptides from a complex mixture and to further separate them according to their degree of phosphorylation. Monophosphorylated peptides also show different retention times and, in the case of peptides with similar sequences, this difference seems to be related to their mass. The successful on-target separation of phosphopeptides will facilitate their identification and allow the detection of phosphopeptides present in low amounts.

Concerning the separation of multiphosphorylated peptides from each other, preliminary research done in this laboratory has shown that, unfortunately, under the conditions described here no separation between multiphosphorylated peptides with different degrees of phosphorylation occurs. In other words, all multiphosphorylated peptides, even those with only two phosphorylations, remain at the loading site and are not eluted (data not shown). Current research aims to find an eluent composition that may achieve also separation of multiphosphorylated peptides. Moreover, the apparent differences on the retention times according to peptide mass are subject to further investigations. Using longer stripes we expect to be able to validate the separation and to learn more about the underlying mechanism.

Conclusions

This study shows the possibility of enrichment and separation of multi- and mono-phosphorylated peptides with a new simple on-target method for MALDI-MS analysis. According to the reported results, the combination of on-target enrichment and TLC using mesoporous TiO_2 as enrichment material as well as stationary phase and a phosphate solution as the mobile phase, leads to a high efficiency enrichment of phosphorylated peptides and to a very clear separation between mono- and multi- phosphorylated peptides, facilitating the detection of individual components of the phosphopeptide mixture. As shown above, the method works even for more complex protein mixtures at low concentrations.

In spite of the long time required to achieve separation (over 10 hours), there are several advantages offered by the method that result in an overall positive balance. As all the steps (enrichment, separation and analysis) are performed on the same substrate, sample loss is minimized and the bias towards the recovery of either mono- or multiphosphorylated peptides is avoided. This is a clear advantage over the common MOAC techniques, which, as has been shown recently, always present certain bias depending on the eluent employed[24].

Moreover, the use of mesoporous anatase titania instead of the more common rutile and brookite mixtures has recently been shown to significantly improve the phosphopeptide enrichment efficiency[22,23,25]. However, in the cited reports, the signals arising from multiphosphorylated peptides

were, depending on the experimental conditions, either absent or very small when compared to those arising from monophosphorylated peptides. The method presented here improves significantly the detection of multiphosphorylated peptides without compromising that of the monophosphorylated ones.

Another clear advantage of the approach described is its simplicity. A thin stripe of mesoporous TiO_2 can be readily prepared on several appropriate substrates and the actual enrichment, washing and separation protocols do not require any special equipment.

Based on these observations, the method, once optimized, has the potential to become a tool for comprehensive phosphoproteome profiling.

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