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Cite this: *Mol. BioSyst.*, 2011, **7**, 1828–1837

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METHOD

Enrichment and sequencing of phosphopeptides on indium tin oxide coated glass slides†

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Received 8th November 2010, Accepted 30th March 2011
DOI: 10.1039/c0mb00269k

Unambiguous identification of phosphorylation sites is of premier importance to biologists, who seek to understand the role of phosphorylation from the perspective of site-specific control of biological phenomena. Despite this widely asked and highly specific information, many methods developed are aimed at analysis of complete proteomes, indeed even phospho-proteomes, surpassing the basic requests of many biologists. We have therefore further developed a simple method that specifically deals with the analysis of multiple phosphorylation sites on singular proteins or small collections of proteins. With this method, the whole purification process, from sample application to MALDI-MS analysis, can be performed on commercially available indium tin oxide (ITO) coated glass slides. We show that fifteen (15) samples can be purified within one hour, and that low femtomole sensitivity can be achieved. This limit of identification is demonstrated by the successful MS/MS-based identification of 6 fmol of monophosphopeptide from β-casein. We demonstrate that the method can be applied for identifying phosphorylation sites from recombinant and cell-derived biological protein samples. Since ITO-coated glass slides are inexpensive and available from several suppliers the method is readily and inexpensively available to other researchers. Taken together, the presented protocols and materials render this method as an extremely fast and sensitive phosphopeptide identification protocol that should aid biologists in discovery and validation of phosphorylation sites.

Introduction

Protein phosphorylation is a key regulator of cellular processes in eukaryotic organisms. The addition of the phosphate group to a target protein has a potential to alter its conformation, shape and molecular size, enzymatic activity and interactions with other proteins.[1] In mass spectrometry based phospho-proteomics the analysis of phosphoproteins and -peptides is challenging because: (1) many phosphoproteins are in relatively low cellular concentrations; (2) the phosphorylation stoichiometry is low and transient; and (3) confirmation is difficult, as typically one peptide is used for confirmation of the phosphopeptide. Consequently, sample purification for phosphopeptides has become an essential step during characterization of protein phosphorylation events, as it addresses some of the problems described above, and it offers a massive reduction in the complexity of the peptide-mixture that the phosphopeptides are normally found in. For this last point purification methods render the analysis of phosphopeptides by virtue of elimination of most other peptides, resulting in a relative abundance of phosphopeptides. Purification methods have now become so successful that recent advances in MS-integrated phosphopeptide purification/analysis methods[2–7] have facilitated phenomenal high numbers (10's of thousands) of site-specific phosphorylation occurrences. Whilst these results are yet to be confirmed by an orthogonal approach, the analytical capacity is impressive. It is also not clear from these studies what component is not analysed, or what the phosphorylation sites are that have been missed. This, we believe, is also a key question. We have therefore been focusing on developing a method that would be simple (few steps), generally applicable (efficient) and addresses low stoichiometry (sensitive), as a platform for analysis of phosphorylation sites within single proteins, not single proteomes.

Enrichment is a key step in this comprehensive approach too, and, when coupled with MALDI the process becomes relatively straightforward and generally applicable. In earlier work we reported that indium tin oxide (ITO) coated glass slides retain phosphopeptides[8] with fixed spatial orientation, and now present further evaluation and application of their use. This evaluation is presented in the context of the time, labor and efficiency and compared with established purification methods, namely immobilized metal affinity chromatography

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† Electronic supplementary information (ESI) available: Tables S1–S3 and images S, 1–7. See DOI: 10.1039/c0mb00269k

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(IMAC),[9] metal oxide affinity chromatography (MOAC), and recently published on-target purification methods.[10–16]

IMAC purification of phosphopeptides is based on the interactions between metal-ions and phosphates bound to peptides. Metal ions ($Fe,^{3+}$ Ga^{3+}, Zr^{4+}, or Al^{3+}) are bound to iminodiacetate (IDA) or nitriloacetate (NTA) forming the IMAC's stationary phase. After sample loading, the resin is washed with acid after which the remaining phosphopeptides are eluted with a high pH.[17] The application of MOAC to enrich phosphopeptides has increased in recent years. The choice of metal oxides (mainly titanium dioxide, TiO_2 and zirconium dioxide, ZrO_2) is explained by their good selectivity, high binding capacity and good recovery of phosphopeptides.[18] Also, metal oxides are more resistant to, so-called, extreme conditions than IMAC, allowing its use in a wider pH-range. The sample is loaded onto a metal oxide affinity column in a low pH and in a high organic solvent to make the environment suitable for exclusive phosphopeptide binding.[18,19] After washing the purified sample is eluted from the column with a high pH solvent, and in many cases this is followed by a reversed phase chromatography step for salt removal.

On-target purification of phosphopeptides has an advantage over traditional chromatography based techniques in that it requires only a few sample-handling steps. This particular feature renders on-target purification to be faster, and smaller sample volumes are typically processed. For these reasons several studies have investigated surfaces for phosphopeptide sample purification.[10–16]

Blacken *et al.*[10,11] modified a MALDI target by carving the MALDI plate and embedding a ZrO_2-coated stainless steel plate into it. They first established the suitability of this new surface for phosphopeptide enrichment,[10] and later performed comparisons with titanium, zirconium and hafnium (IVB metals from the Periodic Table of elements), and evaluated the effect of the surface thickness and preparation.[11] They concluded that from the three tested metals zirconium produced the best results. Hoang *et al.* prepared a functionalized MALDI target plate for phosphopeptide enrichment using immobilized zirconium on a phosphonate self-assembled monolayer (SAM).[14] The plate was structured so that hydrophobic areas outside the sample spotting area enabled concomitant concentration of the sample.[20] The plate enabled larger sample volumes to be used in the method, thereby reducing sample loading errors. High sensitivity was reported with the detection of phosphopeptides from a 1 fmol loading of β-casein. A comparison of IMAC, TiO_2-affinity chromatography and $ZrPO_3$ phosphopeptide purification using 100 fmol of phosphorylated angiotensin II in 1 pmol of BSA digest indicated that the $ZrPO_3$-surface provided the highest specificity.

TiO_2 surfaces have been widely used in various on-target purification approaches. In one study a MALDI target was covered with an array of sintered TiO_2 nanoparticle spots[13] and in two more recent studies a TiO_2 film has been used. Niklew and co-workers[15] described the preparation of a TiO_2 film on top of conductive glass using a sol–gel technique. This study reported 300 fmol sensitivity after their purification steps using the 2062 m/z peak from a digest of β-casein as a standard. In another study, a high sensitivity for phosphopeptide sample purification was shown using a planar surface for which pulsed laser deposition was used to generate a TiO_2 film on top of the MALDI-target.[16] The sensitivity of the method was demonstrated by analyzing 25 fmol of β-casein digest where after washing two phosphopeptides were detected, one of which could be identified using tandem mass spectrometry (MS/MS). Finally, recent reports on a number of new MALDI targets with selective affinity for phosphopeptides have been introduced, but their preparation usually requires very specific expertise and their implementation often involves long incubation times.

In this study we used a commercially available metal oxide coated planar surface for phosphopeptide purification. The surface, a glass slide coated with indium tin oxide, is similar to the conductive glass slides used in imaging mass spectrometry (IMS)[21] to enable IMS and histological analysis of the same tissue section.[22] The glass slides are relatively inexpensive and an array of samples (tens of samples) can be simultaneously purified on the same slide. We report the efficacy of the method by demonstrating its speed in sample purification (15 samples per hour), selectivity and sensitivity (sequence information from 6 fmol of monophosphopeptide from β-casein after purification). Moreover, we show that in addition to commonly used standard β-casein phosphopeptides, this method works well for the identification of phosphorylation sites for *in vitro* phosphorylated transcription factor NFATc1, as well as for samples immunoprecipitated from cells.

Results

Optimization of the sample loading conditions

We have previously shown that ITO coated glass slides can selectively retain phosphopeptides.[8] Preliminary comparison between a planar surface method (using ITO) and chromatographic TiO_2-purification demonstrated the potential of the planar surface purification method using ITO-coated glass slides. In this study, further optimisation of the method was developed using a phosphorylated transcription factor NFATc1. Recombinant NFATc1 was phosphorylated with protein kinase A (PKA), one of the kinases known to phosphorylate NFATc1 on three serine residues and regulate its activity in cells.[23,24] The effects of ammonia, washing solution and loading solutions were systematically tested (Fig. 1). The rationale for these substances was taken from commonly used TiO_2-affinity chromatography methods that involve loading the sample onto a column with high concentration of ACN and TFA, and after washing eluting the phosphopeptides with high pH solution. The ITO-coated glass slide contains two different metal oxides: indium oxide In_2O_3 and tin oxide SnO_3. The order of relative acidity of these three metal oxides is $In_2O_3 < TiO_2 < SnO_3$.[25] Thus, we reasoned that as TiO_2's acidity lies between that of In_2O_3 and SnO_3 we would begin optimisation with the chemicals used in TiO_2 chromatography.

When a tryptic digest of PKA-phosphorylated NFAT was spotted onto an ITO-glass slide, followed by matrix addition and analysis by MS (Fig. 1A), six different phosphopeptides and one oxidized phosphopeptide could be detected from the non-purified sample (1, 1*, 2, 3, 5, 6 and 8; Table S1, ESI†). Next the sample was spotted on ITO, left to dry (at room

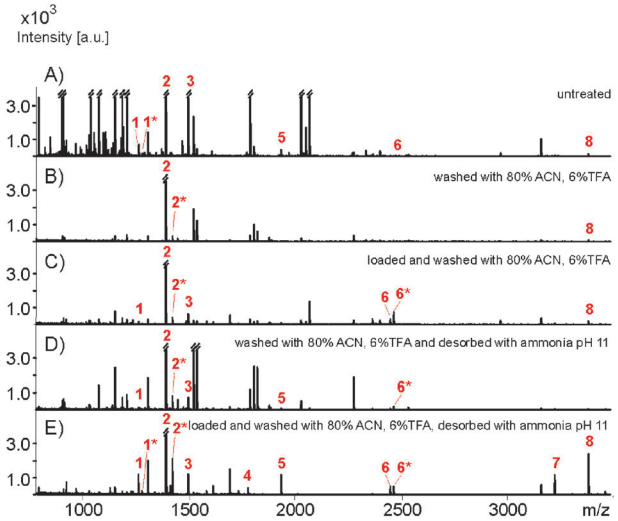

Fig. 1 Different sample loading conditions before the purification step were tested. Spectrum (A) represents the sample without purification. In spectrum (B) the loaded sample has been washed with washing solvent (80% ACN, 6% TFA). In spectrum (C) the sample was loaded on a target with the loading solution (80% ACN, 6% TFA) and in condition (D) ammonia (pH 11) was used to desorb peptides prior to matrix addition, but no loading solution was used. In condition (E) the sample was loaded with the loading solution and peptides were desorbed from the surface using ammonia prior to matrix addition (20 g l^{-1} DHB in 50% ACN, 1% PA, 0.1% TFA). The ammonia was pipetted on top of the purified sample and the matrix was added after 5 min incubation (see Materials and methods). The sample was PKA phosphorylated NFATc1. For peak annotation see Table S1 (ESI†).

temperature), washed (80% ACN, 6% TFA), matrix added and analysed with MS (Fig. 1B). Now the sample complexity was reduced remarkably but this treatment left only two detectable phosphopeptides. When 2 µl of washing solution (80% ACN, 6% TFA) was pipetted on an ITO-glass slide prior to sample loading and used as a loading solution (Fig. 1C) five different phosphopeptides and one oxidized phosphopeptide were detected (1, 2, 3, 6, 6* and 8; Table S1, ESI†). Also, the intensity of non-phosphorylated peptides had reduced. In Fig. 1D, the effect of ammonia is displayed. The preconditioning using the sample loading solution was not performed although prior to matrix addition the sample spot was incubated with an ammonia solution to release the phosphopeptides (pH 11). Five different phosphopeptides were detected, one of which was oxidized (1, 2, 3, 5 and 6*; Table S1, ESI†). However, the spectrum also contained more intense non-phosphorylated peptides (Fig. 1D). Fig. 1E displays the results from preconditioning the ITO slide using the loading solution (80% ACN and 6% TFA) and the sample incubated with ammonia solution prior to matrix addition. Eight different phosphopeptides and three oxidized forms could be detected (1, 1*, 2, 2*, 3, 4, 5, 6, 6*, 7 and 8). These results confirm the usefulness of using loading solution in combination with an ammonia wash prior to the matrix addition and also confirming that similar reagents that are used in TiO_2-affinity chromatography can be used in phosphopeptide purification using ITO coated glass slides.

Optimisation of the TFA percentage in the washing solvent

When a low pH is used in the washing and loading buffers for TiO_2-affinity chromatography, its purpose is to prevent non-specific binding of protonated non-phosphorylated peptides.

In the next experiment we set out to test the effect of TFA concentration in the washing solution. Different concentrations of TFA (1–6%) were used to test its effect on non-phosphorylated and phosphorylated peptides. The effect on phosphorylated peptides was monitored by measuring the radioactivity of the labeled phosphopeptides, before and after the treatment (Fig. 2A). The effect of TFA on non-phosphorylated peptides

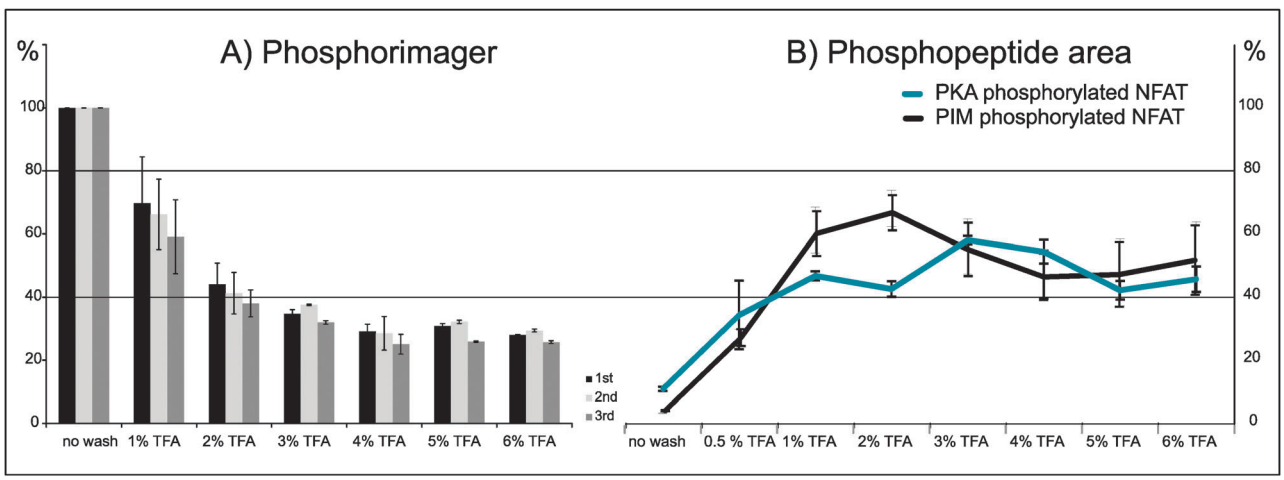

Fig. 2 The effect of TFA percentage on phosphorylated peptides was monitored by measuring the radioactivity of the labeled phosphopeptides derived from Pim-1 phosphorylated NFAT with a PhosphorImager (A) before and after the wash treatment in different percentages of TFA. With each TFA% the sample was washed 3 times and are indicated in the figure as 1st, 2nd and 3rd. The effect on non-phosphorylated peptides was determined by calculating the contribution of phosphopeptide signals (peak area) to the total MS signal (B). The area was calculated from both Pim-1 and PKA phosphorylated NFAT. Phosphopeptides used for the area calculation for PKA and Pim-1 phosphorylated NFAT listed in Tables S1 (PKA) and S2 (Pim-1) (ESI†). Three parallel samples were analyzed (n = 3) and the standard deviation was calculated (error bars).

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(Fig. 2B) was determined by calculating the contribution of phosphopeptide signals to the total MS signal (peak area). Phosphopeptides used in the calculations are listed in Tables S1 and S2, ESI.†

In this TFA optimization experiment we used recombinant, radioactively labeled NFATc1 that was phosphorylated with Pim-1 kinase. PKA and Pim-1 are both basic amino acid-directed kinases and they have very similar phosphorylation target sites.[26,27] We observed that Pim-1 phosphorylated NFATc1 with 50% lower efficiency than PKA (data not shown) and therefore Pim-1 phosphorylated NFATc1 serves as an excellent case-model for optimising the washes for removal of non-phosphorylated peptides. The sample was spotted on an ITO-glass slide and washed with different percentages of TFA in washing solutions (80% ACN + TFA). After washing, photo-stimulated luminescence (PSL) was measured to determine the remaining radioactivity on the ITO-glass slide (Fig. 2). It was observed that the signal decreased as the amount of TFA increased in the washing solvent. After MS analysis the percentage of phosphopeptides from the total peak area was calculated and a maximum could be achieved when the TFA concentration was between one and three percent. Since the higher concentration of TFA in washing solution reduces the recovery of radioactivity (phosphorylated peptides) the percentage of phosphopeptide peak area is at its maximum already at 1%. Therefore 1% TFA was used in the washing solution for subsequent analyses.

Sensitivity test

After optimizing the sample loading and washing conditions we determined the sensitivity of the method (Fig. 3) using a dilution series containing 200, 100, 50, 25, 12 and 6 fmol of tryptic phosphopeptide from β-casein (2062 m/z). We were interested in establishing the limit of identification (LOI) rather than limit of detection (LOD). For each dilution three samples were purified using TiO_2 affinity chromatography and ITO-coated glass slide. Chromatographically purified samples were analyzed on a GroundSteel MALDI target (Bruker Daltonics) and ITO-purified samples were analyzed directly from the ITO coated glass slide.

The TiO_2-purified samples provided positive MS/MS based identification from each replicate when the amount of sample was $\geq$ 100 fmol (Fig. 3A), and in one replicate when 50 fmol was used. The ITO-coated glass slide purification method provided positive identification for all three replicates when the starting amount of phosphopeptide was $\geq$ 12 fmol (Fig. 3B), and one replicate even identified the peptide with just 6 fmol of phosphopeptide.

The comparison was also performed using a mixture of α- and β-casein to determine the limit of detection, LOD (supplementary images 3 and 4, ESI†). The limit of detection for TiO_2-purified samples was 12 fmol (supplementary image 3, ESI†) and for ITO-coated glass slide purification 0.75 fmol (supplementary image 4, ESI†).

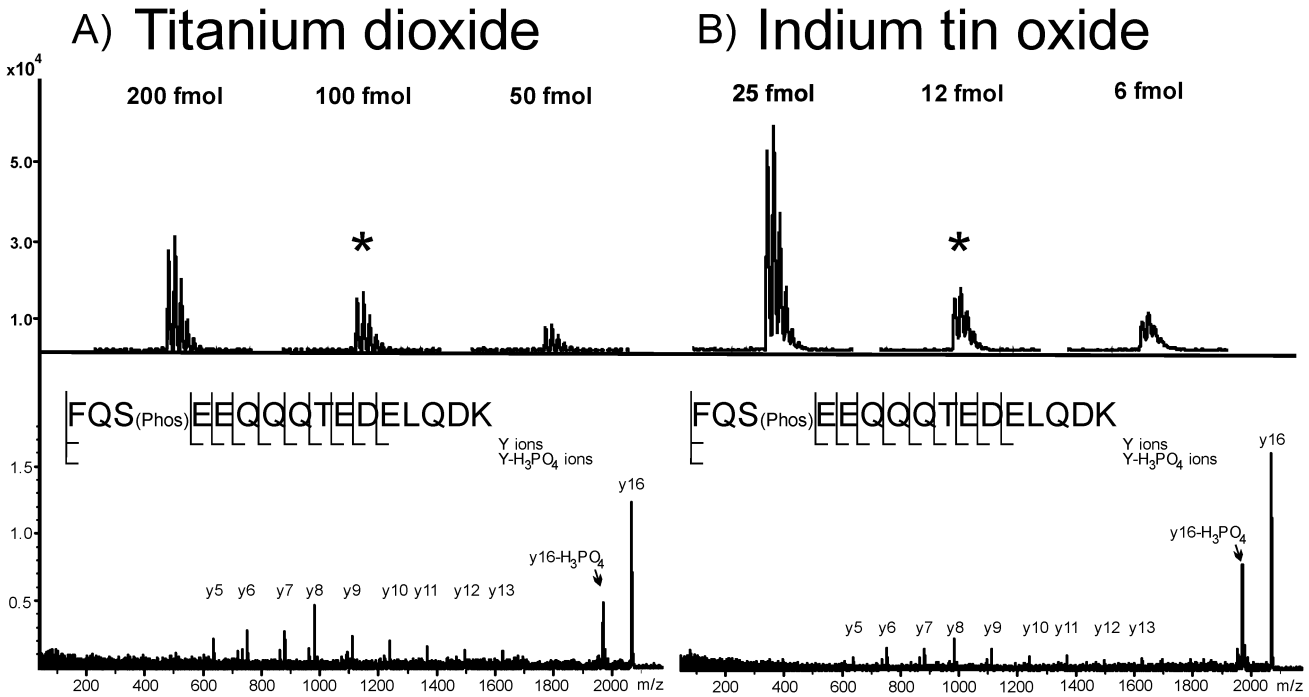

Fig. 3 The sensitivity of the phosphopeptide enrichment method was tested by analyzing a dilution series containing phosphopeptides derived from 200, 100, 50, 25, 12 and 6 fmol β-casein (2062 m/z). TiO_2-purified sample (A) provided positive identification (MS/MS) from three replicates and one replicate was identified when 50 fmol was used. When the samples were purified on a ITO-coated glass slide (B), positive identification was achieved for all three replicates when the starting amount was 12 fmol and one replicate identified the peptide with just 6 fmol of phosphopeptide. The MS/MS spectra from 100 fmol (TiO_2) and 12 fmol identification (ITO) are shown in panels A and B. The TiO_2-purified sample was analyzed using a Ground Steel MALDI target and the ITO-purified sample directly from the ITO-coated glass slide. See supplementary images 6 and 7 (ESI†) for further details and discussion about the laser intensities and resolutions.

Comparison of TiO_2-coated and ITO coated glass slides

We compared the performance of ITO-coated glass slide's capability to retain phosphopeptides to TiO_2-coated glass slides performance reported in our previous study.[8] After the optimization of the method for ITO we were able to detect 18 phosphopeptides from 500 fmol of α- and β-casein mixture using an ITO-coated glass slide whereas in our previous study 16 phosphopeptides were detected (supplementary image 5, Table S3, ESI†).

Identification of NFATc1 phosphorylation sites

After optimisation the ITO developed method was used for the detection of protein phosphorylation in biological samples. In normally growing cells, NFAT proteins are found in the cytoplasm and are phosphorylated on nearly 20 serine residues.[28] We immunoprecipitated NFATc1 from COS7 cells over-expressing the full-length NFATc1 protein. Trypsin digested NFATc1 peptides were dissolved in 10 µl of 10% ACN, 0.1% TFA, and incubated in +37 °C for 15 minutes. One microlitre of sample was deposited onto the ITO-glass slide and purified. All peptide signals were subjected to MS/MS analysis and four different phosphopeptides were identified (Fig. 4 and Table 1). Two peptides had one phosphorylation site (peptides 1 and 4), one was triply phosphorylated (peptide 2) and one had four phosphorylation sites (peptide 3). All together we were able to confirm 5 different phosphorylation sites (SER 233, 245, 278, 282 and 359; Table 1, Fig. 5) and the remaining 4 phosphorylation sites could be potentially attributed to 7 sites (SER 229, 237, 239, 241, 286, 290 and THR 284). One of the confirmed sites was also detected in PKA-phosphorylated NFATc1 (SER 245).

Discussion

Efficiency

The commercially available ITO-coated glass slides offer an efficient and straightforward platform for phosphopeptide purification. The method described here is extremely sensitive and enabled MS/MS identification with 6 fmol of phosphopeptide. Typically, we were able to process 15 samples in one hour using the ITO coated glass slides, whereas the equivalent TiO_2-purifications took about four hours (Scheme 1).

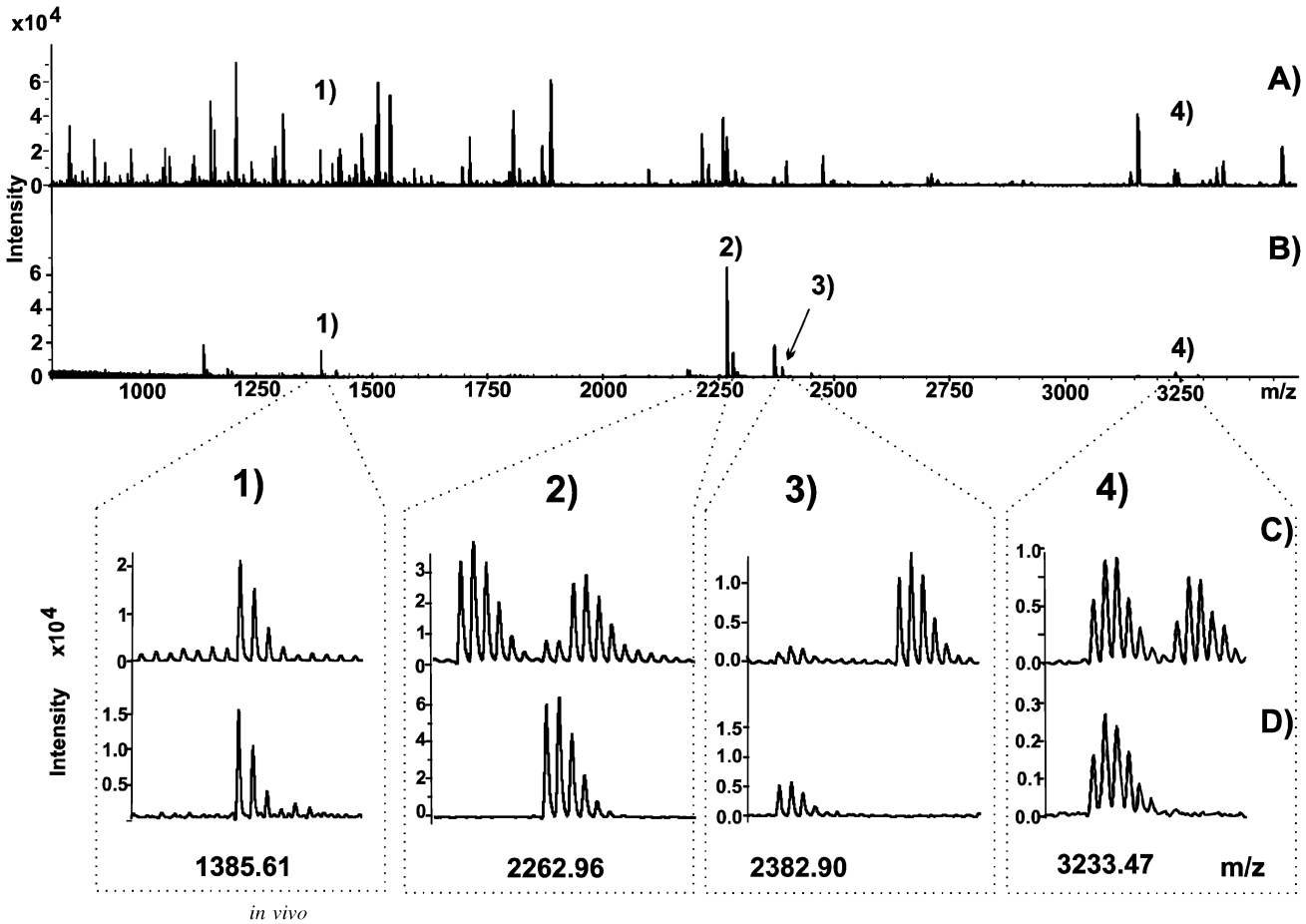

Fig. 4 Tryptic peptides from *in vivo* phosphorylated NFAT (A) were purified according to our optimized method (described in Experimental procedures). The (B) spectrum shows a greatly reduced number of peptides compared to (A), as it almost exclusively contains signals from phosphopeptides. The zoomed spectra (C and D) show the identified phosphopeptides listed in Table 1 and illustrate the problem one faces when non-phosphorylated peptides are not cleared from the plate. Non-phosphorylated peptides are the abundant peptides (C) and for phosphorylated peptides witnessed in (D) little opportunity exists for their automated data-dependent analysis (C2, C3, C4). In this particular case the only successful identification without purification was achieved from peptide 1385 *m*/*z*.

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Table 1 Description of peaks from the *in vivo* phosphorylated NFATc1 -experiment. Sequences for all the numbered peaks from Fig. 4 (No in the table) are presented. Also, the modifications and search engine scores are listed along with the number of detected modifications in each peptide

No	*m/z*	Start	Sequence	End	Modifications	Mascot score	[a]
1	1385.61	244	Ap(S)VTEESWLAGR	255	1xphos@245	76	2.1
2	2262.96	224	GLGACTLLGp(S)PQHSPp(S)Tp(S)PR	243	3xphos@233, 237, 239	31	2.2
					3xphos@229, 233, 241	15	2.3
3	2382.90	274	QPPYp(S)PHHp(S)Pp(T)Pp(S)PHGp(S)PR	292	4xphos@278, 282, 284, 286	26	2.4
					4xphos@278, 282, 284, 290	23	2.5
					4xphos@278, 282, 286, 286	23	2.6
4	3233.47	350	VEPVGEDLGp(S)PPPADFAPEDYSSFGHIR	378	1xphos@359	105	2.7

[a] Supplementary images 2.1 to 2.11. Abbreviations: phos = phosphorylation; carb = carbamidomethylation.

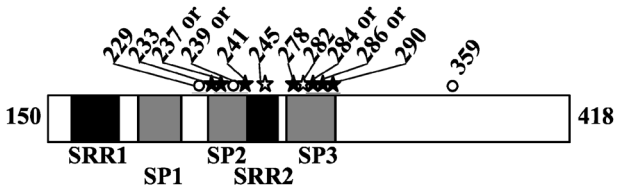
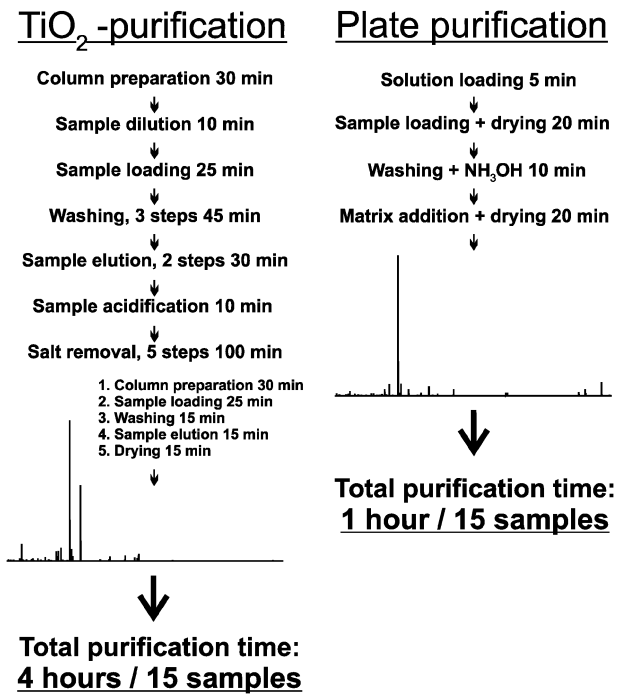


Fig. 5 Phosphorylation of the human NFATc1 regulatory domain in COS 7 cells. Regulatory domain (amino acids 150–418) of NFATc1 is shown, serine rich regions (SRR) and serine-proline motifs (SP) are indicated. Conserved phosphoserines and threonine 282 are indicated with stars, black stars represent sites which are known to be phosphorylated also in NFATc2. Non-conserved serines are marked with circles. Underlined symbols are candidate sites for non-assigned phosphorylation sites.



Scheme 1 Efficiency of phosphopeptide purification using ITO-coated glass slide compared to chromatographic TiO_2-purification.

Notably, with traditional chromatographic purification protocols there are a different number of steps, including column preparation, sample dilution, loading and a number of sample washing and elution steps. After washes phosphopeptides are finally eluted from the TiO_2-column at high pH. Usually the next step prior to MS analysis is reversed phase chromatography (separation or desalting). For the desalting step another column preparation is required and then followed by sample loading, washing, elution and drying.

When using the metal oxide coated planar surface for purification, the loading buffer is pipetted on top of the ITO coated glass slide, after which the sample is added and left to dry in a desiccator. After drying, the sample is washed with a high concentration of ACN and TFA, and subsequently a small volume of ammonia solution is added to release the phosphopeptides from the surface. The matrix solution is then added and the sample left to dry in a desiccator. All together, this approach provides significantly higher throughput with minimal sample handling.

By using a commercially available platform, this surface enrichment method is much more accessible than previously published protocols. For example, the complexities of manufacturing a functionalised MALDI-target for phosphopeptide enrichment was reported by Blacken *et al.*,[10,11] who modified a MALDI target by milling away layers of material to be able to attach their coated stainless steel plate to it. The coating was done by electrospraying a solution of metal oxides onto a stainless steel surface that was freshly plasma-treated with a custom-made plasma reactor. In another study[14] a stainless steel MALDI target was coated in three steps. First, the plate was coated with thiol to form a hydrophobic surface. Then, a 5 × 5 spot array was generated, by applying a mask over the target exposing the plate to strong UV light. Finally, after several washing steps, the plate was incubated overnight in a 0.1 M solution of phosphonate thiol to form a self-assembled monolayer of phosphonate thiol at each spot position. The method described by Qiao *et al.*[13] for the preparation of a MALDI target with TiO_2 nanoparticle spots required a long incubation at high temperature (2–3 hours at 300 °C), separation in mortar (2–3 hours) and sonication (1 hour) for the preparation of a stable suspension of commercial TiO_2 nanoparticles. The suspension was then spotted onto a MALDI target and another high temperature incubation took place (400 °C, 1 hour).

Optimal loading conditions

The sensitivity and efficiency of the ITO surface phosphopeptide purification method was demonstrated with the analysis of NFATc1 phosphorylated with PKA. In addition to

previously known phosphorylation of serine 245[23,24] in peptide 2 (Table S1, ESI†) we detected 3 previously unknown PKA phosphorylation sites in NFATc1. However, we were not able to identify the two previously reported PKA sites at serines 269 and 294, probably because the tryptic peptide containing serine 269 was masked by an unknown compound with identical m/z 917, and serine 294 resides in a large tryptic peptide that was beyond the range for this mass analysis.

The surface of the ITO coated glass possesses surface characteristics that can be significantly different from those expected with a normal glass sample plate. For example, during the sample loading optimisation tests (Fig. 1 and 2) it was found to be crucial to add the ammonia incubation solution (high pH) to the sample in 0.5 μl portions to avoid it spreading across the surface. A similar phenomenon also occurred during addition of the matrix solution: the ammonia droplet was observed to ''move away'' from the pipette tip if the matrix solution was added immediately after the ammonia incubation solution. By waiting for the ammonia incubation solution to evaporate and shrink to approximately half of its original size it was possible to add the matrix in 0.5 μl portions without the ammonia droplet fleeing across the surface. These ''moving away'' phenomena were observed only after washing the samples with a low pH, high organic content solution and were found to be much stronger when higher pH ammonia or higher percentage TFA solutions were used. No improvements in phosphopeptide signals were observed using an ammonia solution with a pH higher than 11 and the sample preparation became extremely challenging. These observations and the results from the PhosphorImager and phosphopeptide area (Fig. 2) led to the decision to use a 1% TFA washing solution and an ammonia solution of pH 11 for sample elution.

The TFA effect

Differences were observed in the TFA optimisation data, when measuring the remaining radioactivity or phosphopeptide peak area. One explanation for this phenomenon may be as follows. Radioactivity can be measured regardless of the sample's complexity whereas the MS-analysis phosphopeptide signal can be affected (suppressed) by non-phosphorylated peptides. When the non-phosphorylated peptides are washed from the MALDI plate more signals from phosphorylated peptides can be detected, although the wash can also decrease the amount of phosphopeptides in the sample.

PhosphorImager data revealed that the loss of radioactivity did not decrease much when sequential washes using the same %TFA solution were performed (Fig. 2). Consequently, we expected that sequential washes might further reduce the load on non-phosphorylated peptides. However it was found that sequential washes did not improve the performance of the phosphopeptide purification, the MS data showed little reduction in spectral complexity.

Sensitivity

The ITO phosphopeptide purification method was able to identify a phosphopeptide from 6 fmol of the sample (Fig. 3). Hoang *et al.*[14] have reported 1 fmol sensitivity for detecting phosphopeptides from caseins. Qiao *et al.*[13] reported the detection of 2062 m/z monophosphorylated peptide from 30 fmol digest and Niklew *et al.*[15] reported 300 fmol sensitivity. Torta *et al.*[16] recently reported a successful MS/MS experiment from 25 fmol of β-casein. Thus the 6 fmol limit of identification and 0.75 fmol limit of detection obtained with the ITO coated glass slides benchmarks the method, by comparison to these similar approaches, as an extremely sensitive method for phosphopeptide enrichment. See supplementary images 6 and 7 (ESI†) for further details and discussion about the laser intensities and resolutions.

NFATc1 phosphorylation sites in cells

In vivo phosphorylation of another NFAT family member NFATc2 has been previously thoroughly characterized in murine T cells,[28] but to our knowledge NFATc1 has not been studied before using mass spectrometry. Altogether, we were able to find five confident and seven potential phosphorylation sites in NFATc1 over expressed in COS7 cells (Table 1; Fig. 5). We could not locate those seven candidate phosphorylation sites, because in Mascot searches they were all equally probable. One could conclude that it is very likely that all are present in the sample and the spectra corresponding to peptides 2 and 3 in Table 1 are mixtures of differentially multiply phosphorylated peptides. Of the previously identified conserved phosphorylation sites in SRR (serine rich repeat) and SP (serine proline) regulatory motifs of NFAT proteins we find evidence for three sites in SP2 motif and four sites in SP3 motif. Interestingly, serine 239 in SP2 motif, which does not have a corresponding residue in NFATc2, was phosphorylated in addition to a conserved serine 237. In SP3 motif of NFATc1 we found 4 phosphorylations in 5 phosphorylation site candidates (peptide 3 in Table 1) but we could not pinpoint the sites except for the serines 278 and 282. In addition to phosphorylation sites in SP motifs we observed a phosphorylation of serine 245 which we also identified from PKA phosphorylated NFATc1 (peptide 1 in Table S1, ESI†). The corresponding conserved serine in NFATc2 was not phosphorylated in the study by Okamura *et al.*[28] This type of minor difference in site occupancy could be due to the availability of regulatory kinases in different cell types. We also identified one phosphorylation in serine 229 located just N-terminal to SP2 motif and one with high confidence in a non-conserved serine 359. We did not detect peptides originating from SRR-1 motif with five conserved putative phosphorylation sites,[28] possibly because of missed tryptic cleavage in the heavily phosphorylated region resulting in a peptide with a m/z over 5000. However, our aim in this study was not to exhaustively characterize NFATc1 *in vivo* phosphorylation but to test the suitability of the ITO phosphopeptide purification method for a typical biological sample containing singly and multiply phosphorylated peptides. More detailed characterisation of NFATc1 phosphorylation in the future will require the use of other proteases in addition to trypsin because of relatively few trypsin cleavage sites in the NFATc1 sequence.

Conclusions

This study provides an optimized protocol for phosphopeptide purification using an ITO-coated glass slide. By using a commercially available platform, this surface enrichment

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method avoids important concerns related to manufacturing a functionalized MALDI-target, including access to the wider public, and QC related to reliability and reproducibility of the targets. Therefore, as the methodology is based on readily available metal oxide coated glass slides, it opens up possibilities for direct application and further development to other researchers with MALDI instruments. The method described here provides researchers with a fast and sensitive phosphopeptide enrichment technology, useful for small sample volumes, and is characterised by minimal sample losses. By minimizing the sample handling steps, we were able to increase throughput by a factor of four, and sensitivity gains were increased approximately by a factor of ten, when compared to chromatographic phosphopeptide enrichment methods. First, we optimized the method using a recombinant human protein, NFATc1, confirmed the high sensitivity with β-casein peptides, and finally demonstrated the efficacy of the method by identifying *in vitro* phosphorylation sites on NFATc1 immunoprecipitated from cells. Application of the method to PKA phosphorylated NFATc1 enabled the identification of 11 different phosphopeptides, with three previously unpublished *in vitro* phosphorylation sites for PKA. Furthermore, with the immunoprecipitated NFATc1 we found evidence for 12 phosphorylation sites many of which were found in multiply phosphorylated peptides. Interestingly, the method appears to be well suited for the purification of triply and quadruply phosphorylated peptides of NFATc1. The method is ideally suited to characterise complex phosphorylation patterns on single proteins or small collections of proteins efficiently, allowing researchers to use this method as a starting point or a validation step for comprehensive phosphorylation analysis. The method should not be considered as the *sine qua non* for phosphorylation. Like other methods, there are cases where the method may not be best for single proteins, but it is too early to define the boundaries in this report. The presented protocols and materials illustrate the method as extremely fast and sensitive for phosphopeptide identification of protocols, which should aid biologists in discovery and validation of phosphorylation sites. As the current method is optimized for manual use, the next step could be to automate sample handling for high throughput phosphoprotein screening.

Experimental procedures

Chemicals

Acetonitrile (ACN; CAS 75-05-8) and phosphoric acid, 85% (PA; CAS 7664-38-2), were purchased from J. T. Baker (Deventer, Holland); ten percent ammonium hydroxide solution (NH_4OH; CAS 1336-21-6), 2,5-dihydroxybenzoic acid (DHB; CAS 490-79-9), trifluoroacetic acid (TFA; CAS: 76-05-1), α-cyano-4-hydroxy-cinnamic acid (CHCA; CAS 28166-41-8), monophosphopeptide from β-casein, α-casein (C6780-250MG) and β-casein (C6905-250MG) were purchased from Sigma (St Louis, USA); formic acid (FA; CAS 64-18-6) from Merck (Darmstad, Germany) and alkaline phosphatase (AP, 20 U μl^{-1}; CAS 9001-78-9) from Roche Diagnostics (Mannheim, Germany). All reagents in the experiments were of analytical grade or better.

GST protein purification and *in vitro* kinase assays

Recombinant Pim-1 and NFATc1 fusion proteins were purified from lysed bacteria using glutathione sepharose beads (GE Healthcare). The bead-bound proteins were then either eluted (NFATc1) with 30 mM glutathione or cleaved (Pim-1) with the PreScission protease (GE Healthcare) according to manufacturer's instructions. The concentrations of purified proteins were estimated with SDS-PAGE and Coomassie staining (PAGE-BLUE, Fermentas). 5 μg of eluted NFATc1 was phosphorylated with 25 units of a protein kinase A catalytic subunit (Sigma) or with 0.5 μg cleaved recombinant Pim-1 per reaction in buffer containing 37.5 mM Tris, pH 8.0, 20 mM $MgCl_2$, and 200 μM ATP for 3 h at 30 °C. For some experiments radioactive ^{32}P γ-ATP (Perkin-Elmer) was added to reach a specific activity of 150 $\mu Ci\ ml^{-1}$ or ~1000 cps $pmol^{-1}$ ATP. Reactions were stopped by boiling in Laemmli sample buffer for 5 min at 95 °C.

Cell culture and immunoprecipitation

COS7 monkey kidney cells were grown in medium supplemented with 10% foetal calf serum, 50 units per ml penicillin, 50 $\mu g\ ml^{-1}$ streptomycin and 2 mM L-glutamine. Cells were transfected with a pBJ5-NFATc1-FLAG vector[29] by electroporation, harvested 72 h later and lysed with buffer containing 50 mM Tris, pH 7.5, 100 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Nonidet P-40, protease inhibitors (Complete EDTA-free; Roche), 50 mM NaF, 20 mM β-glycerophosphate, 2 mM orthovanadate and 250 units per ml of Benzonase nuclease. NFATc1-FLAG expression was confirmed by SDS-PAGE followed by Western blotting using the anti-FLAG antibody (Sigma-Aldrich). For each immunoprecipitation reaction 1 mg of total of protein was mixed with 50 μl of anti-FLAG M2-agarose (Sigma-Aldrich) in 500 μl of RIPA buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1% deoxycholate, 0.1% SDS and above mentioned protease and phosphatase inhibitors).

Gel electrophoresis

Proteins from *in vitro* kinase assays and immunoprecipitations were separated on a Criterion XT Bis-Tris gel using MOPS (3-(*N*-morpholino)propanesulfonic acid) as the running buffer, at 175 V for 60 min and visualised with a colloidal Coomassie staining kit (Invitrogen) according to the manufacturers instructions.

In gel digestion of proteins

Proteins were cut from gel, destained, reduced in 50 mM dithiothreitol at 65 °C for 45 min and alkylated with 20 mM iodoacetamide at RT for 20 min. Digestions were performed overnight at 37 °C in 40 mM NH_4HCO_3, 10% ACN with 0.2 μg of sequencing grade trypsin (Promega) for each digested gel piece containing ~2.5 μg of protein. On the following day peptides were extracted with 50% acetonitrile and 5% formic acid and dried. Before the MS analysis the sample was redissolved in 10% ACN, 0.1% TFA and incubated at +37 °C for 15 min.

Chromatographic TiO_2-purification

TiO_2-purification was done according to previously published protocols[18,30] with minor modifications. In short, approximately 3 mm TiO_2 micro columns (Sachtopore-NP 20 μm, 300 Å; ZirChrom, Anoka, MN) were prepared on top of a 3M C8 Empore Disk, after which the diluted sample (1 : 5 with 80% ACN, 5% TFA) was loaded into the column. The sample was washed once with 10 μl of loading solution, once with 20 μl of washing solution (80% ACN, 1% TFA) and once with 5 μl of MQ-H_2O. The sample was then eluted from the column with 20 μl of ammonium hydroxide solution (NH_4OH, pH 10.5) followed by 2 μl of 30% ACN to elute the phosphopeptides bound to C8-disk. The eluted fraction was acidified with 2.5 μl of 100% formic acid (FA) prior to the desalting step.

Sample desalting

Samples from TiO_2-purification were desalted using C-18 micro columns. C18 Empore Disks from 3M were used as a reversed phase (RP) material. The column was equilibrated with 10 μl of 0.1% TFA and the acidified sample was applied into the column using gentle air pressure. The column was washed with 10 μl of 0.1% TFA and the retained peptides were eluted directly to the MALDI target using 2 μl of 80% ACN, 0.1% TFA. In the case of the casein mixture, the peptides were dried in a SpeedVac and stored at −20 °C for later analysis.

Optimized planar surface purification

2 μl of loading buffer (80% ACN, 1% TFA) was incubated on top of indium tin oxide (ITO) coated glass slides (Bruker Daltonics, Bremen, Germany) prior to sample application. One microlitre of sample was applied on top of an ITO-coated glass slide and mixed with the loading buffer on a target. After sample loading the glass slide was placed into the desiccator to dry. Next the sample was washed with 50 μl of 80% ACN, 1% TFA. 2 μl of NH_4OH (pH 11) were pipetted on top of the sample and 2 μl of MALDI-matrix was added (20 g l^{-1} of DHB in 50% ACN, 1% PA, 0.1% TFA) and left to dry in the desiccator. The matrix was added when the ammonia droplet had reduced in size approximately 50% (roughly 5 min; see discussion below).

PhosphorImager

Peptides from radioactively labelled NFATc1 were spotted on top of ITO-coated glass slides and washed with different concentrations of TFA (1–6% TFA) in 80% ACN. The photo-stimulated luminescence (PSL) was measured using Fujifilm BAS-1800 PhosphorImager before and after the washes and from the difference of these two measurements the percentage recovery of radioactivity was calculated.

MALDI-TOF (TOF) analysis

All MS analyses were performed using Ultraflex II from Bruker Daltonics equipped with a 200 Hz Smartbeam laser system. Data were acquired using FlexControl 3.0 (Bruker Daltonics). The laser settings for acquiring data in MS mode were as follows: offset 64%, range 15% and laser footprint medium. The laser intensity for the sensitivity test was kept at a constant 50%, and for the *in vivo* experiment 44%. In other experiments the laser intensity was adjusted to achieve the best possible result unless otherwise described. Three thousand laser shots were accumulated in the sample loading optimization experiment and for the sensitivity test to determine the limit of identification (LOI) 2000 laser shots for a MS spectrum and 12 000–25 000 for a MS/MS spectrum were acquired. The accumulated laser shots for the *in vivo* experiment were 1000 and 12 000 respectively. The detection range for all MS experiments was 850–3500 m/z.

Peak list generation and spectral processing

Peak lists and spectral processing were done in FlexAnalysis 3.0 (Bruker Daltonics). For generation of the peak lists from the MS spectra the SNAP algorithm was used, the signal-to-noise was set to 3 and the baseline subtraction algorithm was TopHat. For processing the spectra the following parameters were used: the SavitzkyGolay algorithm was used for smoothing with 0.2 m/z width and one cycle, the baseline subtraction algorithm was Median, with flatness values ranging from 0.1–0.3 depending on the baseline level at the low mass area of the spectra, and the MedianLevel values were set to 0.7–0.9 also depending on the spectral quality at the low mass area. Peak list generation and spectral processing for MS/MS data were done using the following settings: the parameters for peak picking were the Snap algorithm with signal to noise threshold 3 and baseline subtraction algorithm TopHat. The processing algorithm was SavitzkyGolay for smoothing with parameters of 0.12 m/z (width) and 4 cycles. Sometimes the MS/MS spectra contained peaks that were missed by the peak-picking algorithm. In these cases manual peak picking was used with the following parameters: centroid peak picking and TopHat baseline subtraction.

Database search

MASCOT version 2.2 (Matrix Science, London, UK) was used for error tolerant database searches[31] and the following settings were used: mass error tolerance for parent 100 ppm and for the fragments 0.6 Da; fixed modification: cysteine carbamidomethylation; variable modifications: methionine oxidation, serine and threonine phosphorylation; number of missed cleavages: two. The data were searched against SwissProt database version 57.13 (514212 sequences) and NCBInr database (10435126 sequences; downloaded 21-02-2010).

Sensitivity test: limit of identification (LOI)

A dilution series of monophosphopeptides from β-casein was prepared containing 200, 100, 50, 25, 12 and 6 fmol μl^{-1} monophosphopeptides in 10% ACN, 0.1% TFA. One microlitre of each dilution was spotted on to an ITO-coated glass slide (×3), purified according to our optimized planar surface enrichment protocol and measured by MS. In parallel, an equivalent sample was subjected to chromatographic TiO_2-purification after which the sample was desalted using a C-18 microcolumn and spotted directly to a GroundSteel MALDI target (Bruker Daltonics).

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Acknowledgements

Sini Eerola is thanked for assistance in laboratory. The authors thank Robert Moulder, Liam McDonnell and David Goodlett for critical reading and commenting the manuscript. Bruker Daltonics and Fanni Virtanen, Raija Andersén, Arttu Heinonen, Anne Rokka, Eliza Ralph and Anni Vehmas from the Turku Proteomics Facility are acknowledged for technical assistance and support. This work was supported by Academy of Finland, University of Turku and Nordforsk.

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