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Short communication

Comparison of IMAC and MOAC for phosphopeptide enrichment by column chromatography

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ABSTRACT

Automated phosphopeptide enrichment prior to MS analysis by means of Immobilized Metal Affinity Chromatography (IMAC) and Metal Oxide Affinity Chromatography (MOAC) has been probed with packed columns. We compared POROS-Fe^{3+} and TiO_2 (respectively IMAC and MOAC media), using a simple mixture of peptides from casein–albumin and a complex mixture of peptides isolated from mouse liver. With theses samples, selectivity of POROS-Fe^{3+} and TiO_2 were pH dependant. In the case of liver extract, selectivity increased from 12–18% to 58–60% when loading buffer contained 0.1 M acetic acid or 0.1 M trifluoroacetic acid, respectively. However, with POROS-Fe^{3+} column, the number of identifications decreased from 356 phosphopeptides with 0.1 M acetic acid to 119 phosphopeptides with 0.1 M TFA. This decrease of binding capacity of POROS-Fe^{3+} was associated with strong Fe^{3+} leaching. Furthermore, repetitive use of IMAC-Fe^{3+} with the 0.5 M NH_4OH solution required for phosphopeptide elution induced Fe_2O_3 accumulation in the column. By comparison, MOAC columns packed with TiO_2 support do not present any problem of stability in the same conditions and provide a reliable solution for packed column phosphopeptide enrichment.

1. Introduction

Protein phosphorylation has been extensively studied since the 1990s and the development of tandem mass spectrometry and affinity media for phosphopeptide enrichment provided effective tools to investigate phosphoproteomes. IMAC was the first widely used affinity medium for phosphopeptide enrichment [1,2]. Later, MOAC chromatography [3,4], essentially with TiO_2 as support, appeared as an interesting alternative and the sequential use of the two methods was proposed to increase the panel of purified phosphopeptides [5,6]. Logically, solutions offered by two-dimensional chromatography with combinations such as SAX-MOAC [7], SCX-IMAC [8], IMAC-HILIC [9], or IMAC-IMAC [10] tend to be preferred for the identification of phosphopeptides from complex peptide mixtures. However, screening experiments aimed at biomarker discovery require the analysis of large sample sets and are not compatible with the multiplication of fractions induced by multidimensional chromatography. Therefore, a single step purification of phosphopeptides still remains a hot topic in phosphoproteomics. From another point of view, repeatability and reproducibility are not the strongest points of phosphopeptide enrichment methods that employed supports packed in pipette-tips or in-vial batch processes. The aim of this work was to develop a method with affinity supports packed in columns and using an HPLC system for process automation and column regeneration. Phosphopeptide enrichment with IMAC and MOAC was tested in this configuration. The method was first evaluated with a tryptic hydrolysate of a bovine casein and albumin mixture. The results obtained with this simple mixture were then validated by the analysis of a complex sample (i.e. a mouse liver extract). Superior results were obtained with MOAC operated at very low pH, and these tests highlighted a specific issue of IMAC column chromatography.

Abbreviations: AA, acetic acid; ACN, acetonitrile; IAA, iodoacetamide; DTT, dithiothreitol; IMAC, immobilized metal ion affinity chromatography; LC–MS/MS, liquid chromatography tandem mass spectrometry; MOAC, Metal Oxide Affinity Chromatography; TEAB, triethylammonium hydrogen carbonate; TFA, trifluoroacetic acid.

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2. Materials and methods

2.1. Chemicals

Acetonitrile (ACN), trifluoroacetic acid (TFA), acetic acid (AA), dithiothreitol (DTT), iodoacetamide (IAA), triethylammonium hydrogen carbonate (TEAB), sodium orthovanadate (Na_3VO_4), sodium fluoride, bovine serum albumin, bovine casein, Glu 1-fibrinopeptide B and TPCK treated trypsin were obtained from Sigma (France). 15.0 Mohms cm water was produced by a Pure Lab Option system (Veolia, France).

2.2. Sample preparation

Liver tissues was processed as previously described [8], with some modification. Briefly 100 mg liver from BALB/c adult mice were homogenized with a piston pellet (Eppendorf, France) in 0.5 ml of buffer containing 0.1 M TEAB pH 9.3, 0.15 M NaCl, 0.1% SDS, 5 mM NaF, 2 mM Na_3VO_4 and 10 mM DTT. The sample was heated 10 min at 100 °C then cooled, and 50 mM IAA was added for 45 min at RT. Soluble proteins (10 mg/100 mg crude extract) were quantified by Bradford assay and digested by trypsin at 37 °C for 18 h (1:100 as E:S ratio). The solution was acidified with TFA and tryptic peptides were purified with a C18 cartridge (washing buffer: 0.1% TFA, elution buffer 60% ACN) then dried with a vacuum centrifuge (Speed Vac, Thermo Scientific, France). Ten mg of dried extract were obtained from 100 mg tissue. Casein mixed with albumin in a 1:10 (w/w) ratio was digested with trypsin, like liver tissue, but with two minor modifications; 0.1% SDS was replaced with 2 M urea and the DTT reduction step was carried out for 1 h at room temperature.

2.3. Chromatographic supports

The IMAC support (POROS 20 MC, Applied Biosystems, France) and the MOAC support (TiO_2, 10 μm, GL Science, Japan) were packed in a 2 mm × 5 cm column and mounted in a HPLC system (Beckman Coulter, France) with a 500 μl sample loop, a column oven set at 35 °C and UV detector set at 280 nm. Sample (1 mg/ml, 400 μl), elution buffer (400 μl) and IMAC buffers (400 μl) were injected with the sample loop. Three different loading buffers were tested with POROS 20 MC and TiO_2, namely buffer A: 0.1 M acetic acid, buffer B: 0.1 M TFA and buffer C: 0.7 M (5%) TFA. Elution buffer was 0.5 M NH_4OH. All buffers were prepared in 20% aqueous ACN. Columns were flushed at 0.2 ml/min for 15 min with 10 mM acetic acid between each runs. IMAC was activated with $FeCl_3$ and regenerated with EDTA as previously described [11].

2.4. LC–MS/MS and data processing

The collected fractions from casein–albumin tryptic digest were analyzed with a capLC system coupled with a Q-ToF Premier (Waters, France). Peptides were eluted from a 300 μm C18 column with a 0.1–30% ACN gradient for 30 min. Glu 1-fibrinopeptide B was added as internal control. Full MS scans were acquired from 350 to 1700 m/z and quantification was carried out with QuanLynx (Waters).

The eluted fractions from liver digests were analyzed with a nanoLC system (Dionex, France) coupled with a LTQ Orbitrap XL (Thermo Scientific). A 75 μm C18 analytical column was used with a 300 μm × 0.5 mm preconcentration cartridge (both from Dionex). A gradient of 5–40% ACN was applied for 105 min. Acquisition was a "TOP 10" method (an Orbitrap MS survey scan followed by ion trap MS/MS scans of the 10 most intense precursor ions). Data processing and peptide identifications were performed with Proteome Discoverer 1.2 (Thermo Scientific); the different thresholds were set to obtain a False Discovery Rate (FDR) < 1%. Excel (Microsoft) was used to remove the redundant peptides and to count the phosphorylated peptides.

3. Results

3.1. Comparison of media and loading buffers with the casein–albumin mixture

The purification methods were evaluated with tryptic peptides from the 1/10 casein–albumin mixture for each column and condition. Three loading buffers were used for POROS-Fe^{3+} and TiO_2 chromatography: 0.1 M acetic acid (pK_a: 4.76), 0.1 M trifluoroacetic acid (pK_a: 0.3) and 0.7 M trifluoroacetic acid. Indeed, the use of 0.1 M acid concentration was mainly reported for IMAC, while TiO_2 has been used with higher concentrations of TFA, up to 0.7 M, in order to decrease non-specific binding [12]. The eluted fractions were analyzed by LC–MS and a recovery yield was determined for the peptides corresponding to three major ions (see Fig. 1). Two of them are monophosphorylated (VPQLEIVPNpSAEER, m/z = 830.88 and FQpSEEQQQTEDELQDK, m/z = 1031.89 from alpha-s1- and beta-casein, respectively) and one is a contaminant peptide from serum albumin (DAIPENLPPLTADFAEDK, m/z = 978.46). With a TiO_2 column, the decrease of pH has no effect on phosphopeptide recovery but decreases the binding of the contaminant peptides. With POROS-Fe^{3+} the decrease of pH lowers the recovery of phosphopeptides too.

3.2. Comparison of media and loading buffers with liver sample

We confirmed the results obtained with peptides from the casein–albumin mixture with a more complex mixture of peptides from mouse liver. A diagram showing the numbers of identified phosphopeptides and non-phosphorylated peptides for the different methods is presented in Fig. 2. It appears clearly that the use of trifluoroacetic acid as loading buffer increased from 18 to 60% and from 12 to 58% the enrichment specificity of IMAC and MOAC, respectively. However, trifluoroacetic acid dramatically decreased the number of identified phosphopeptides purified with the IMAC POROS-Fe^{3+} column, from 356 to 119 phosphopeptides. For this reason, we verified the content of the flow-through fraction by reloading it on the column (i.e. a second pass of the depleted sample). Whatever the affinity support and the buffer, the number of non-phosphorylated peptides was similar in the first and the second pass. However, phosphorylated peptides were mainly obtained in the first pass, excepted with POROS-Fe^{3+}/TFA for which 119 and 107 phosphopeptides were identified in pass 1 and 2, respectively. This clearly pointed out a problem of low binding capacity of POROS-Fe^{3+} IMAC with 0.1 M TFA loading buffer.

3.3. Leaching of Fe^{3+} from IMAC support and rust formation

Upon replacement of 0.1 M acetic acid loading solvent by 0.1 M TFA for the POROS-Fe^{3+} column, the number of identified peptides strongly decreased. Moreover, we identified 50% phosphopeptides in the flow-through. Therefore, we checked a potential Fe^{3+} release from the IMAC support by monitoring UV absorbance (see Fig. 3). When no $FeCl_3$ was used to activate the POROS IMAC support, the flow through produced almost no signal. On the contrary, after activation of POROS with $FeCl_3$, a peak corresponding to Fe^{3+} leaching was produced by the injection of the loading solvents. This peak was moderate with 0.1 M acetic acid (pH 2.9), but saturated the detector when 0.1 M TFA (pH 1.3) was used as loading solvent. When the column was unpacked after 10 separations, we also observed

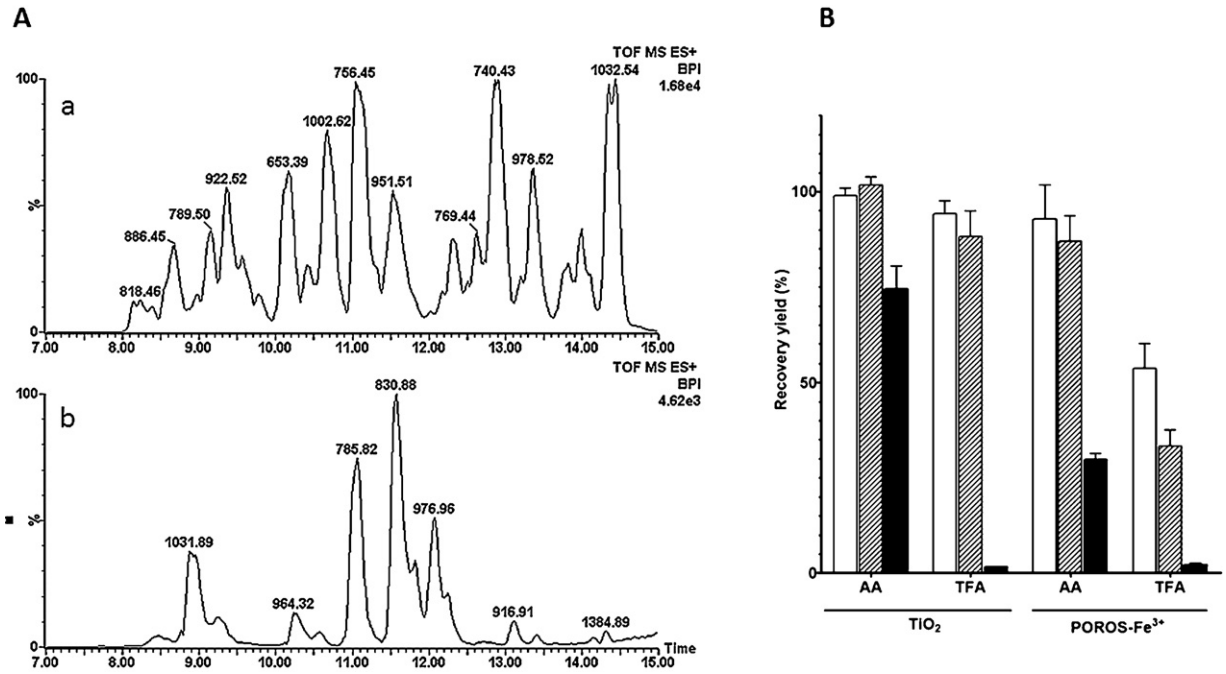

Fig. 1. Enrichment of casein phosphopeptides with POROS-Fe^{3+} and TiO_2 columns. Panel A: base peak chromatogram of casein–albumin sample before enrichment (a) and after enrichment (b) on TiO_2 column (peaks are labeled with m/z values, ion at m/z = 785.84 corresponds to spiked Glu 1-fibrinopeptide B). Panel B: recovery yield of peptides after TiO_2 and POROS-Fe^{3+} enrichment. Peptide VPQLEIVPNpSAEER (m/z = 830.88, white bar), FQpSEEQQQTEDELQDK (m/z = 1031.89, striped bar) are phosphopeptides from alpha-s1-casein and beta-casein, respectively. Peptide DAIPENLPPLTADFAEDK (m/z = 978.49, black bar) is a non-phosphorylated peptide (i.e. a contaminant) from serum albumin. AA and TFA correspond to acetic acid and trifluoroacetic acid used in the loading buffer.

that the POROS support had taken an orange-brown color (see Supplementary Material). An extensive EDTA treatment did not wash the color away, because Fe^{3+} had formed a precipitate. This corresponds to rust formation: the elution buffer (0.5 M NH_4OH) led to the formation of $Fe(OH)_3$ which spontaneously formed a brown precipitate of $Fe_2O_3{\cdot}3H_2O$.

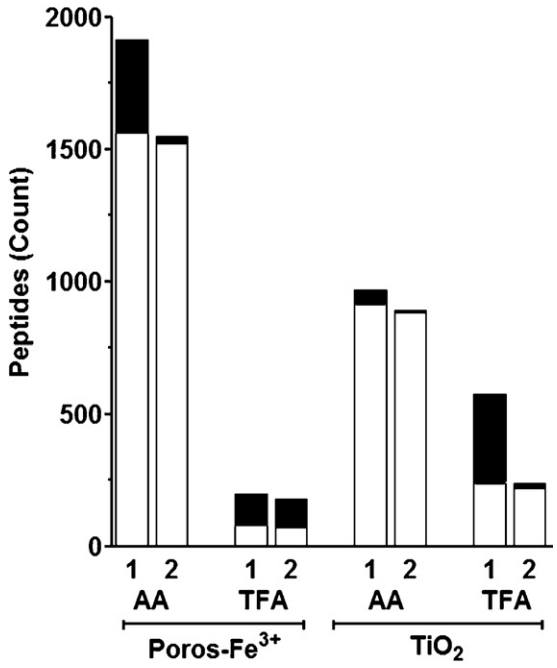

Fig. 2. Number of peptides identified after POROS-Fe^{3+} and TiO_2 purification. (A) Phosphopeptides (striped bar) and unphosphorylated peptides (open bar) are presented for two loading buffers: acetic acid (AA) and trifluoroacetic acid (TFA). Numbers 1 and 2 correspond to the first and the second pass of the sample onto column.

4. Discussion

IMAC-LC and mass spectrometry for phosphopeptides analysis was first reported by Watts et al. [2]. These authors used

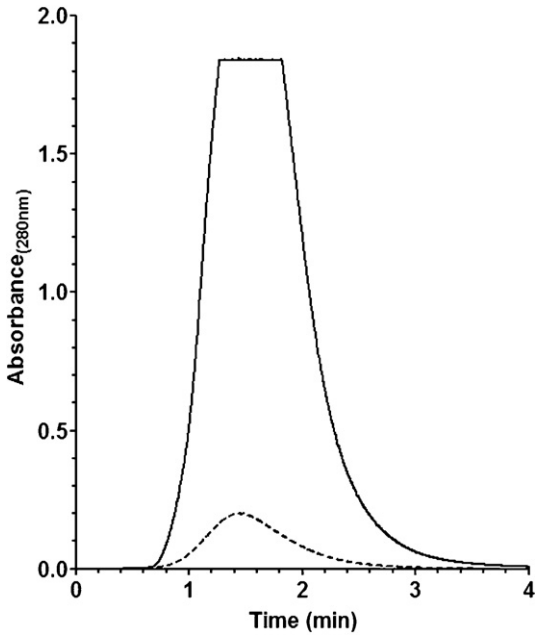

Fig. 3. (A) UV trace (280 nm) for POROS-Fe^{3+} after injection of 0.1 M acetic acid (AA; solid line) and 0.1 M trifluoroacetic acid (TFA; dashed line).

chelating-Sepharose with Fe^{3+} and 0.1 M acetic acid as loading buffer. It quickly appeared that contamination with peptides containing aspartic and glutamic acids was a major weakness of IMAC when it was applied to the purification of phosphopeptides from complex mixtures. Methyl esterification of carboxyl groups has been used to decrease the binding of mono-acidic peptides on IMAC supports [13], but side products (amine esterification) and incomplete esterification are known to increase the complexity of phosphopeptide fractions [14,15]. An alternative consists in lowering the pH of the loading buffer below 3, where D and E are mostly protonated (pK_a: 3.65 and 4.25 respectively) while phosphorylated residues still bear a charge and are able to bind to the IMAC-Fe^{3+} support [16]. However, the high selectivity obtained with a loading buffer at low pH also correlates with a low recovery of phosphopeptides [17,18]. The observation of Fe^{3+} leaching provides an explanation for the balance between IMAC selectivity (low pH) and recovery of phosphopeptides. Indeed, the use of a lower pH increases the salting-out of iron during the loading step of sample onto the column (see Fig. 3). This phenomenon then induces a decrease of phosphopeptide recovery (see Fig. 1), and concomitant occurrence of phosphopeptides in the flow-through fraction (see Fig. 2). Furthermore, we point out an important drawback of the use of Fe^{3+} IMAC enrichment of phosphopeptides by means of a packed, re-usable column: upon reaction with aqueous ammonia, Fe^{3+} precipitates during the elution step of phosphopeptides and accumulates in the column. If re-used, a column packed with IMAC-Fe^{3+} support may contain an unknown amount of Fe^{3+} and Fe_2O_3, and such a "rusty IMAC" support, featuring a mixed IMAC and MOAC mode, may lead to irreproducible binding of phosphopeptides. To our knowledge, this effect has not been described in previous publications, including those involving an online IMAC-Fe^{3+} system [15,18–20]. Thus, it is highly recommendable to use only disposable IMAC columns and to avoid storing Fe^{3+} activated IMAC over a long period. By comparison, MOAC performed with TiO_2 packed columns does not present this drawback, since these columns may be used for tens of runs without loss of affinity even with very acidic loading solvents such as 0.7 M trifluoroacetic acid.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2012.02.028.

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