Enrichment of phosphopeptides at low pH using Phos SpinTrap Fe

Ann-Marie Nissfolk*, Johan Öhman, Gabriella Risberg, Marianne Albenius, Marika Sjödahl and Helena Hedlund


Introduction

Identification of phosphoproteins is essential for understanding the regulation of many biological processes e.g., cell signaling, apoptosis, angiogenesis, and differentiation of stem cells.

Since phosphopeptides in a digested sample are often found in low abundance and ionize poorly, they are difficult to detect by mass spectrometry. Phos SpinTrap™ Fe is designed for phosphopeptide enrichment, which improves identification of phosphopeptides using techniques such as MALDI-TOF and LC-MS/MS.

Binding of phosphopeptides to Phos SpinTrap Fe is pH dependent. In this application, we exploited the advantage of binding the peptide sample at a low pH to obtain highly pure and reproducible enrichment of phosphopeptides.

Conclusions

- Phos SpinTrap Fe utilized at a low pH during peptide binding is an alternative to methyl esterification, providing a simple procedure to enrich phosphopeptides.
- The identification rate was improved from typically 1% of the starting material to 50% after enrichment using Phos SpinTrap Fe at low pH.
Overview

Phos SpinTrap Fe is a new sample preparation product designed for single-use small-scale phosphopeptide enrichment and is based on the affinity of the Fe³⁺ ion for the phosphate group. This product was used in a study to optimize the binding of phosphopeptides at low pH. Subsequently, the most optimal protocol was used to enrich phosphopeptides of two complex samples.

Experimental procedure

1) Buffer screening

Buffers were screened at three different pH (1.5, 2, 2.9) using a model sample: 4 μg monophosphopeptide 0.8% (w/w) from bovine milk (β-casein) added into a background of 480 μg bovine serum albumin (BSA) trypsic fragments. The starting material and the eluates were analyzed by MALDI-TOF MS.

2) Reproducibility experiment

The buffer system at pH 2, with 50% acetonitrile added in the wash/equilibration buffer, was used in a reproducibility experiment with the same model sample described above. The samples were analyzed by MALDI-TOF MS.

3) Experiment with complex samples

Two complex samples were run under the optimized protocol: a trypsin digested sample mix of E. coli extract (strain BL-21) spiked with β-casein 1% (w/w), ovalbumin 0.5% (w/w) and creatin kinase 0.25% (w/w) and a trypsin digested cell extract from yeast (Saccharomyces cerevisiae), 0.2 g/ml. Both samples were diluted twofold with the equilibration/wash buffer, run on Phos SpinTrap Fe and analyzed by LC-MS/MS.

Methods

Protocol

The purification/enrichment of phosphopeptides on Phos SpinTrap Fe columns is a simple, four stage procedure:

- **Equilibration** by adding equilibration/wash buffer
- **Sample application and incubation** for 15–120 minutes with slow, end-over-end mixing or similar gentle agitation.
- **Washing** with equilibration/wash buffer and water
- **Elution** of the target peptides, i.e. phosphopeptides, with elution buffer.

Each step is followed by centrifugation using a standard microcentrifuge.

Recommended buffers

Equilibration/wash buffer:
50 mM glycine-HCl, 50% acetonitrile in water, pH ~2.0

Elution buffer:
1% phosphoric acid, 50% acetonitrile in water, pH 1.5–2.
Identification of phosphopeptides spiked in *E. coli* extract

The digested *E. coli* mix (starting material) and the eluate were analyzed using LC-MS/MS. One phosphorylated peptide and about 1250 nonphosphorylated peptides were detected in the digested sample mix. In the eluate, nine phosphopeptides (representing β-casein and ovalbumin), one *E. coli* protein as well as impurities from the spiked proteins, were identified with high significance (p < 0.001), see Table 1. The identified phosphopeptides in the eluate correspond to 20% of the total amount of identified peptides.

### Results

- The buffer system at pH 2 was the most optimal buffer system in the study and gave highly reproducible results.
- Phosphopeptides were enriched in both the *E. coli* extract (two of the three spiked proteins were found) and in *S. cerevisiae*.

#### Table 1. Phosphopeptides identified in spiked *E. coli* extract after enrichment and elution using Phos SpinTrap Fe. Phosphorylation site is indicated with bold and underlined character.

<table>
<thead>
<tr>
<th>Protein name</th>
<th>Residue</th>
<th>[M+H]+</th>
<th>Sequence</th>
<th>p value</th>
<th>Accession number</th>
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<tr>
<td>Ovalbumin</td>
<td>340-359</td>
<td>2088.91</td>
<td>EVKDAEAGVASGVEETFR</td>
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<tr>
<td>Phosphoglucomutase</td>
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<td>2321.12</td>
<td>GGPLADGIVITPHNPPEDGGIK</td>
<td>6.4e-005</td>
<td>NP_415214*</td>
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<tr>
<td>αS1-casein</td>
<td>121-134</td>
<td>1660.79</td>
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<td>αS1-casein</td>
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* E. coli protein, annotation found in www.phosida.com
× Non-specific tryptic cleavage

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Identification of phosphopeptides in yeast

The digested *S. cerevisiae* extract (starting material) and the eluate were analyzed using LC-MS/MS. In the starting material, only 1% of the detected peptides were phosphorylated (red area in Fig 3A). After enrichment, 50% of the detected peptides were found to be phosphorylated (red area in Fig 3B). Forty-one phosphopeptides encoding 37 proteins were identified after enrichment compared to only three phosphopeptides in the starting material (data not shown).

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Reproducibility experiment

Reproducibility was evaluated by running 10 replicates in parallel where 50% acetonitrile was added to the equilibration/wash buffer.

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Fig 2. Ettan MALDI-TOF MS spectra of (A) starting material (0.8% w/w monophosphopeptide spiked in digested BSA); and (B) overlaid MALDI-TOF MS spectra from 10 replicates using equilibration/wash buffer 50 mM glycine-HCl, 50% acetonitrile pH 2. The β-casein peptide peak of m/z 2062 is enriched in all replicates and both the amplitude of the monophosphopeptide peak as well as its location (m/z) are highly consistent between runs.

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Fig 3. Amount of detected peptides in (A) starting material and (B) in the eluate of yeast. Red represents phosphopeptides and blue represents nonphosphorylated peptides.

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Table 1. Phosphopeptides identified in spiked *E. coli* extract after enrichment and elution using Phos SpinTrap Fe. Phosphorylation site is indicated with bold and underlined character.
## Ordering information

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<th>Product</th>
<th>Quantity</th>
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<table>
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<th>Literature</th>
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<td>Protein and nucleic acid sample prep brochure</td>
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www.gelifesciences.com/sampleprep

GE Healthcare Bio-Sciences AB
Björkgatan 30
751 84 Uppsala
Sweden