OVERVIEW

- A novel phosphopeptide enrichment method has been developed using micro scale solid phase extraction device.
- The enrichment method is selective, robust, and reproducible.
- Sample loading with the displacer (under the trade name Enhancer™) further improves the selectivity.
- The performance of phosphopeptide enrichment is evaluated with phosphopeptide standards and alpha-casein digest.

INTRODUCTION

Phosphorylation is one of the most common post-translational protein modifications in living cells and its investigation is a key interest in proteomics. However, the analysis of phosphorylation in proteins and peptides by mass spectrometry is challenging due to their low abundance and low ionization efficiency. Phosphopeptides are often suppressed in comparison to the unphosphorylated species when measured in complex mixtures. Therefore, it is critical to selectively enrich phosphopeptides prior to mass spectrometry analysis. In these experiments, we show the enrichment method using Waters® MassPREP® Phosphopeptide Enrichment Kit. The robustness and reproducibility of the method is tested using the micro scale solid phase extraction device packed with high affinity metal oxide sorbent. Reduction of non-specific acidic peptides binding is achieved by addition of Enhancer™. This is especially useful for highly complex mixture. The performance of Waters® MassPREP® Phosphopeptide Enrichment Kit is compared to immobilized metal affinity chromatography (IMAC) and titanium dioxide (TiO₂).

EXPERIMENTAL METHOD

1. Sample Preparation
   - Waters MassPREP® Phosphopeptide standard (Table 1) is mixed with MassPREP® Enzyme digestion standard in 1:1, 1:10, 1:100 and 1:1000 mole ratio in loading solution (0.5% TFA in 80% acetonitrile).
   - Tryptic digested alpha-casein derived from bovine milk (Sigma) was prepared in 2µg/µL.

<table>
<thead>
<tr>
<th>Phosphopeptide</th>
<th>Table 1. Amino acid sequence and m/z of the four phosphopeptide standards</th>
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<tbody>
<tr>
<td>α1S2_EQLpSTpSEENSK</td>
<td>1000 (LD+) 455.21 (LD-)</td>
</tr>
<tr>
<td>α5S1 VPQLEIVPNpSAEER</td>
<td>1200 (LD+) 618.11 (LD-)</td>
</tr>
<tr>
<td>α6S1 DIGpSEpSTEDQAMEDIK</td>
<td>1400 (LD+) 732.22 (LD-)</td>
</tr>
<tr>
<td>α8S2_5P NANEEE YpSIGpSpSpSEEpSAEVATEEVK</td>
<td>1600 (LD+) 835.33 (LD-)</td>
</tr>
</tbody>
</table>

2. Instrumentation
   - Waters nanoACQUITY UPLC® and Q-TOP Premier™
   - Waters Alliance 2705 and PDA996 and ZQ™ Single Quadrupole MS
   - Waters MALDI micro MX

3. Methods
   - nanoLC-MS: Analytical column Atlantis dC8, 3.5µm, 75µm×100mm. Flow rate: 0.1µL/min Formic acid in 100% water Mobile phase A: 0.1% Formic acid in 100% water Gradient: 2% - 60% B
   - NanoLC-MS: Waters MALDI micro MX 1% phosphoric acid as a matrix additive

4. Enrichment protocol (the SPE plate is operated using a vacuum manifold):
   - Condition: 200µL water (Vacuum)
   - Load: 200µL of sample in 0.5% TFA in 80% MeCN or in 1% TFA in 80% MeCN (Vacuum, gravity (no vacuum on))
   - Wash 1: 200µL 0.5% TFA in 80% MeCN (Vacuum)
   - Wash 2: 200µL water (Vacuum)

RESULTS

- MALDI on reflectron positive and negative mode
- 1% phosphoric acid as a matrix additive
- Trapping column Symmetry C18, 5µm, 180µm×20mm
- NanoLC-MS: Waters MALDI micro MX
- 100% Formic acid in 100% water
- Gradient: 2% - 60%B
- Injection volume: 2µL, partial loop, Flow rate: 300nL/min.
- Sample loading with the displacer (under the trade name Enhancer™) further improves the selectivity.

CONCLUSIONS

- Waters MassPREP® Phosphopeptide Enrichment Kit offers high selectivity and reproducibility for phosphopeptide isolation.
- The micro scale solid phase extraction device allows fast and robust phosphopeptide enrichment.
- The Enhancer™ chemical compound shows the selectivity towards phosphopeptides in complex mixture by reducing non-specific binding.
- Singly phosphorylated peptides are enriched better using MassPREP® phosphopeptide affinity sorbent than IMAC.
- Higher selectivity is observed using MassPREP® phosphopeptide affinity sorbent than TiO₂.