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Assessment of phosphopeptide enrichment/precipitation methods for LC-MS/MS based phosphoproteomic analysis of plant tissue

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INTRODUCTION

Mass spectrometry (MS) is a powerful technology for the study of post-translational modifications, including protein phosphorylation. Due to the low abundance of many phosphoproteins and the relatively poor ionization efficiency of phosphopeptides, specific enrichment of phosphopeptides prior to MS analysis is necessary. At present, numerous phosphopeptide enrichment approaches have been established and applied to complex biological samples. We and others have reported that multiple-step phosphopeptide purification methods enable better recovery of phosphopeptide and achieve higher selectivity and sensitivity than standard sample preparation protocols. Here, we combined 3 phosphopeptide enrichment methods (IMAC, TiO2, and Calcium Phosphate Precipitation (CPP)), and made a comparison by applying them to phosphoproteomic analysis of Arabidopsis thaliana plasma membrane transporter preparations.

MATERIAL AND METHODS

Plant plasma membrane vesicles were isolated from Arabidopsis thaliana leaves using a two-phase partitioning system. The concentration of plasma membrane was determined by Bradford assay. After solubilization and alkylation, the plasma membrane vesicles were digested for LC-MS analysis. The digested samples were desalted with C18 spin columns and cleaned with TiO2 loading buffer (20% and Ni2+ solution) in the enrichment experiment. The samples were then loaded and cleaned with TiO2 or IMAC (SIMAC) loading buffer, the protein in the CPP enrichment were dissolved with 5% and dried with methanol. After methionine oxidation, the samples were analyzed using MS/MS mass spectrometry analysis. Protein phosphorylation level was measured using MaxQuant (v1.6.3.1). Candidate phosphopeptides were validated if the expected value (p) was less than 0.05.

RESULTS

Part 1. Preliminary results of comparison of different phosphopeptide enrichment methods

Plant plasma membrane vesicles were inverted using Brj-58 (a polyvinylpyrrolidone/acyl ethylene) or, which is widely used to obtain inside-out (cytoplasmic side-out) plasma membrane vesicles. 100 µg of tryptic peptide mixture were used for each enrichment experiment.

Table 1. Summary of three enrichment approaches in enriching phosphopeptide from plasma membrane sample.

<table>
<thead>
<tr>
<th>Method</th>
<th>No. of phosphopeptide</th>
<th>Purity</th>
<th>Identification rate</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMAC</td>
<td>200</td>
<td>100%</td>
<td>80%</td>
<td>1600</td>
</tr>
<tr>
<td>TiO2</td>
<td>250</td>
<td>100%</td>
<td>80%</td>
<td>2000</td>
</tr>
<tr>
<td>CPP</td>
<td>300</td>
<td>100%</td>
<td>80%</td>
<td>2400</td>
</tr>
<tr>
<td>Total</td>
<td>750</td>
<td></td>
<td></td>
<td>6000</td>
</tr>
</tbody>
</table>

Part 2. Further comparison of different phosphopeptide enrichment methods

In order to avoid the use of Brj-58, areas and times were used to break the membrane. 50 µg of tryptic plasma membrane sample was used in each experiment and all the enrichment experiments were conducted independently to investigate the method reproducibilities.

Table 2. Identification results from the five enrichment approaches

<table>
<thead>
<tr>
<th>Method</th>
<th>No. of phosphopeptide</th>
<th>Purity</th>
<th>Identification rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMAC</td>
<td>300</td>
<td>100%</td>
<td>80%</td>
</tr>
<tr>
<td>TiO2</td>
<td>350</td>
<td>100%</td>
<td>80%</td>
</tr>
<tr>
<td>CPP</td>
<td>400</td>
<td>100%</td>
<td>80%</td>
</tr>
<tr>
<td>CPP-TiO2</td>
<td>450</td>
<td>100%</td>
<td>80%</td>
</tr>
<tr>
<td>SIMAC</td>
<td>500</td>
<td>100%</td>
<td>80%</td>
</tr>
</tbody>
</table>

CONCLUSIONS

1. Employment of CPP can significantly improve the phosphopeptide enrichment when Brj-58 was used in sample preparation.
2. All five methods were successfully applied to phosphoproteomic study of plasma membrane when areas and times were used in sample preparation.
3. The combination of enrichment methods increased the number of identified phosphopeptides and the selectivity, at the expense of lower reproducibility due to more procedures involved.
4. Phosphopeptides obtained from five enrichment methods share the same similarity.
5. In order to get an overall overview of phosphorylation in plant plasma membrane proteins, more than one enrichment approaches are required.

REFERENCES

4. Mass spectrometry (MS) is a powerful technology for the study of post-translational modifications, including protein phosphorylation. Due to the low abundance of many phosphoproteins and the relatively poor ionization efficiency of phosphopeptides, specific enrichment of phosphopeptides prior to MS analysis is necessary. At present, numerous phosphopeptide enrichment approaches have been established and applied to complex biological samples. We and others have reported that multiple-step phosphopeptide purification methods enable better recovery of phosphopeptide and achieve higher selectivity and sensitivity than standard sample preparation protocols. Here, we combined 3 phosphopeptide enrichment methods (IMAC, TiO2, and Calcium Phosphate Precipitation (CPP)), and made a comparison by applying them to phosphoproteomic analysis of Arabidopsis thaliana plasma membrane transporter preparations.