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RESEARCH PAPER

A Novel Method for Analysis of Tyrosine Phosphopeptides Based on a Centrifugal Enrichment Device

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Abstract: Protein tyrosine phosphorylation is an important post-translational modification and has become one of the most active areas in proteome research in recent years. Protein tyrosine phosphorylation plays key regulatory roles in numerous signal transduction processes and in occurrence & development of tumor. The study of tyrosine phosphorylation and the activity of it corresponding tyrosine kinases is of great significance for the research of drug targets for cancer treatment. However, tyrosine phosphorylation only represents less than 0.1% of the total cellular protein phosphorylation. Therefore, there is a great challenge to identify tyrosine phosphorylation in real complex samples. In this work, a novel centrifugal device was developed by combination of application of titanium dioxide (TiO_2) and C_{18} reverse phase packing materials for phosphopeptides enrichment and separation, which led to simplified procedure, reduced sample loss and minimized interference. This centrifugal device was made of pipette tips, adapters and eppendorf tubes (1.5 mL). Sample loading, phosphopeptides enrichment, washing, eluting and separation were combined into this device and could be achieved by simple centrifugation. This device was capable of paralleled sample processing with improved analysis throughput. Tandem enrichment by anti-phosphotyrosine antibody resulted in efficient enrichment and large scale identification of phosphotyrosine peptides by mass spectrometry. As a result, a total of 967 phosphotyrosine sites corresponding to 545 proteins were successfully identified from 5 mg of mouse liver proteins, demonstrating the robustness and potential of this new strategy.

Key Words: Phosphotyrosine; Enrichment; Mass spectrometry; Proteome and post-translation modification

1 Introduction

Protein phosphorylation is the most ubiquitous post-translational modification (PTM), playing a pivotal role in several key intracellular processes including cell proliferation, development and differentiation, apoptosis, nerve activity and metabolism[1–3]. Protein tyrosine phosphorylation and protein tyrosine kinase (PTK) are key factors in the process of cell signal transduction and play an important role in cell proliferation, angiogenesis, cell migration, gene transcription and metabolic response mechanisms[4]. Dysregulation of protein tyrosine phosphorylation can disturb the downstream signaling pathway and lead to tumor development[5]. Studies have shown that over-activation of PTK are involved in tumor occurrence, development and prognosis[6,7]. Thus PTK began to attract attention of researchers as an important target for anticancer drug development. So far, the U.S. Food and Drug Administration have approved 28 kinds of small molecule kinase inhibitor as target drugs, more than 80% of them are tyrosine kinase inhibitors[8]. Therefore, it is important for the development and clinical application of antitumor drugs to obtain the information of protein tyrosine kinases and their relevant signaling pathways by the large-scale study of protein tyrosine phosphorylation.

With the advancement of mass spectrometry (MS)

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instrumentation, MS-based post translation modification is becoming the mainstay for high-throughput identification of proteins. However, there are still some significant issues of enrichment of tyrosine phosphoproteomics. Tyrosine phosphorylated proteins are present at very substoichiometric levels (S/T/Y phosphorylation ratios were estimated as 1800:200:1[9]). Thus the detection of tyrosine phosphopeptides by MS can be impaired by signal suppression in the presence of generally more abundant non-phosphotyrosine peptides. It is therefore important for complex samples to develop robust methods that selectively enrich tyrosine-phosphorylated peptides followed by MS/MS. Several proteomic enrichment strategies were developed for tyrosine phosphorylation peptides, such as immunoprecipitation(IP)[10–13], immobilized metal affinity chromatography(IMAC)[14,15], and metal oxide affinity chromatography(MOAC)[16–18]. Antibody-based enrichment was the most widely used method. However, the high complexity of real samples and the limited affinity of anti-tyrosine antibodies severely affect the enrichment selectivity and identification scale. MOAC is often used for enrichment of phosphopeptides, among which titanium dioxide is the most widely applied enrichment materials because of its simple operation, high enrichment selectivity and low cost. However, there are still challenges of this strategy: (i) the large amounts of protein starting material are required; (ii) the complicated and time exhausting experimental procedures will cause the irreproducibility of experiment results; (iii) the unspecific binding of non-tyrosine phosphorylated peptides in highly complex peptide mixtures reduces the identification scale of the method.

In this work, we developed a tandem strategy which combined metal oxide affinity chromatography for phosphopeptides enrichment and immunoprecipitation using antibodies mixture for tyrosine phosphorylation enrichment. In the new strategy, the tyrosine phosphorylation enrichment was performed by a centrifugal enrichment device that combined TiO_2, C_{18} reverse phase packing materials and antibodies. This strategy enriched phosphopeptides from complex samples by the enrichment device, separated them into different fractionations, and then employed anti-tyrosine antibodies for large-scale identification. Unlike the conventional method, this novel enrichment device used a homemade column filled with TiO_2 and C_{18} reverse phase packing materials for the enrichment and separation of phosphopeptides. The device conducted the sample loading, washing, eluting and separating by centrifugation. Thus it simplified the experimental procedures, and reduced sample loss, labor intensity and interference caused by man-made factors in the large-scale analysis of tyrosine phosphopeptide in clinical samples. This strategy was successfully applied in tyrosine phosphoproteomics of mouse liver and identified 849 phosphopeptides corresponding to 967 phosphotyrosine sites and 545 proteins, which provided robust evidence for physiological function research of tyrosine phosphorylation and targets screening for cancer treatment.

2 Experimental

2.1 Instruments and reagents

Orbitrap Fusion MS, Easy-nLC, Thermo Heraeus Fresco 17, Thermo Scientific Savant RVT5105 Refrigerated Vapor Traps (Thermo Fisher Scientific Company, USA), BP211d Electronic Balance (Sartorius, Germany), SBH200D Dry heater (Stuart, UK), JY92-II Ultrasonic Cell Grinder (Ningbo Scientz Biotechnology, China), and MILLI-Q (Millipore Millipore, USA) were used in this study.

Complete Tablets (> 99.9%) and PhosSTOP Tablets (> 99.9%) were purchased from Roche, Germany. Sequencing grade modified trypsin (> 99.9%), dithiothreitol (> 99%), and idoacetamide (> 98%) were purchased from Promega, USA. Urea (> 99.0%), acetonitrile for LC-MS (> 99.9%), ammonium hydroxide solution (> 61.7%) were purchased from Sigma-Aldrich, USA. Trifluoroacetic acid (> 99%) was purchased from Acros, Belgium. HEPES (> 99%) was purchased from Amresco, USA. TiO_2 beads were purchased from GL Sciences, Japan. C_8 disk, C_{18}disk were purchased from 3M, USA. C_{18} reverse phase packing materials (i.d. 3 μm) was purchased from Agela Technologies, China. PTM-703 Pan Anti-phosphotyrosine antibody agarose conjugated beads (Regular) was purchased from PTM-Biolabs, China. P-Tyr-100Phospho-tyrosine monoclonal antibody was purchased from Cell Signaling Technology, USA.

2.2 Peptide preparation of mouse liver

Livers of 10-week C57BL/6 mice were extracted and homogenized in lysis (9 M urea, 20 mM HEPES, pH 8.0, phosphatase inhibitor tablets and complete protease inhibitor tablets). After homogenized by a tissue grinder, the lysates were sonicated, and followed by a centrifugation. The clarified supernatant was transferred into a new tube.

Approximately 5 mg of lysate supernatant proteins was diluted with lysis. The sample was reduced in 4.5 mM (final concentration) DTT at 55 ºC for 30 min, cooled to room temperature, and then alkylated in 10 mM (final concentration) freshly made IAA at room temperature in the dark for 30 min. The sample was then diluted 4 times with 20 mM HEPES and digested overnight at 37 ºC in the presence of trypsin (trypsin-protein, 1:100, *m/m*). The supernatant was applied to a prewashed SepPak C_{18} column, lyophilized and stored at –80 ºC.

2.3 Enrichment of phosphopeptides

In the experiment, a 200-μL pipette tip filled with 4 mg of TiO_2 beads and one layer of C_8 film was used as TiO_2