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RESEARCH PAPER

High Concentration Trypsin Assisted Fast In-Gel Digestion for Phosphoproteome Analysis

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Abstract: Polyacrylamide gel electrophoresis (PAGE) is a powerful protein separation technology. Combined with mass spectrometry, it could identify thousands of proteins in proteomics analysis. However, the time-consuming procedure restricts its broad applications in proteomics study. In this work, it was found that high concentration of trypsin did not compromise subsequent phosphopeptide enrichment after in-gel digestion, but could promote the in-gel digestion. Hence, a new, fast and robust digestion method was established. Firstly, 50 μg of HeLa cell protein was separated into 5 fractions by SDS-PAGE, and then the proteins were digested with high concentration trypsin for 30 min. Finally, the phosphopeptides were enriched by Ti-IMAC Tip. After liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis, about 2000 phosphorylation sites were identified in the experimental group, while less than 1500 phosphorylation sites were identified in control group. With the aid of high concentration of trypsin, in-gel digestion could be completed within only 30 min, and more phosphorylation site identifications and lower percentage of missed cleavages were acquired than those in control experiments. The experiment results demonstrated that high concentration of trypsin could not only accelerate the in-gel digestion, but also improve the phosphoproteome coverage.

Key Words: High concentration trypsin; In-gel digestion; Phosphoproteome analysis

1 Introduction

Polyacrylamide gel electrophoresis (PAGE), as a high resolution protein separation technology, has been implemented to fractionate complex protein mixture for in-depth proteomics analysis[1]. In combination with mass spectrometry, thousands of protein dots can be successfully identified by in-gel digestion, which has been an indispensable part of bottom-up proteomics research. Compared with multidimensional liquid chromatography-tandem mass spectrometry (LC-MS/MS) method, gel-based approach offers distinct advantages. Firstly, molecule weight based pre-separation greatly reduces the sample complexity, thus the dynamic range of proteome analysis is greatly increased. Secondly, the high tolerance to chaotropic agent, surfactant and denaturing buffer, which are totally detrimental to subsequent mass spectrometer detector, also makes it a brilliant choice for researchers[2,3]. However, the time-consuming, labor-intensive procedure and low digestion efficiency of in-gel digestion greatly inhibit its further application in proteomics[4]. The in-gel digestion usually involves multi-steps of shaking, spinning, shrinking, as well as overnight proteolysis, which directly increase the analysis time and lead to low throughput of sample analysis. Moreover, limited accessibility of the protease to substrate proteins arising from the depressed diffusion mobility is the main reason for the poor tryptic proteolysis compared to in-solution digestion. Hence, many researchers have been devoted to developing fast and robust in-gel digestion methods to facilitate the sample preparation of gel-based process.

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To promote the digestion efficiency, Havlis *et al*[5] studied the kinetics of in-gel digestion of proteins. They investigated the effect of reaction temperature, enzyme concentration, digestion time and surface area of gel pieces on the yield of digestion products. Based on the kinetic data, they found that with a relatively higher concentration of the modified trypsin (1.5 μM) and at a higher temperature (58 °C), complete digestion of protein substrate could be achieved within only 30 min. Moreover, the specificity and the efficiency were not affected at all, which was mainly attributed to the reductive methylation of trypsin. The modified trypsin exhibited lower autolysis and was tolerant to high reaction temperature without losing its stringent substrate specificity. However, it has not been widely applied to proteomics study probably because of its high cost. On this basis, Dycka *et al*[6] investigated the acceleration of the ultrasonic treatment and infra red light on in-gel digestion. Both of the two approaches can shorten the digestion time from overnight to only a few minutes, thus making it possible to digest proteolytic fast and efficiently. Furthermore, microwave-enhanced enzyme reaction is another strategy usually used to accelerate in-gel digestion, but it is still difficult to be applied in analyzing the complex protein mixture[7–9]. Surfactant provides a viable alternative for in-gel protein digestion because it can promote the efficiency of protein digestion by solubilizing and denaturing proteins. Saveliev *et al*[3] found such a MS compatible detergent, sodium 3-((1-(furan-2-yl)undecyloxy) carbonylamino) propane-1-sulfonate. They systematically studied the impact of this surfactant on key steps of in-gel digestion and then established an optimized protocol, in which protein digestion and peptide extraction were combined in a single step and the experiments of digestion and extraction were completed within an hour with a better peptide recovery in comparison with conventional method. Nonetheless, the effect of the degradation products of surfactant on MS analysis remains to be further studied.

As we all know, the higher concentration of enzyme used for protein hydrolysis will benefit the proteolysis[10], which is a straightforward approach to speed up the protein digestion. But the trypsin autolysis is aggravated as well. High intensity autolysis peak would do nothing but seriously hinder the scanning of the analyzed peptides. That's the reason why trypsin-to-protein ratio used is usually no more than 1:25 for proteomics analysis. In this study, it was found that autolysis of high concentration trypsin would not affect the LC-MS/MS analysis of phosphoproteome due to the extremely high specific phosphopeptides enriching pretreatment, in which only phosphopeptides could be enriched from other peptides. In this work, this strategy was applied to phosphoproteome analysis. Aided by the high concentration of trypsin, the in-gel digestion reaction was completed within only 30 min, however, it needs as long as about 16 h for the in-gel digestion by conventional method. For phosphoproteomics analysis, almost 2000 phosphorylation sites were identified in only 50 μg of HeLa cell protein mixture by centrifugation tips packed with Ti(IV)-IMAC beads. Compared with conventional method, 40% more sites were identified, and lower missed cleavage rate was acquired. It proved that high concentration of trypsin assisted in-gel digestion could not only speed up the proteolysis reaction, but also increase the digestion efficiency. Moreover, this new approach also exhibited advantages in identifying proteins with high molecular weight or in low abundance, demonstrating its favorable complementation with conventional methods.

2 Experimental

2.1 Instruments and reagents

LTQ Orbitrap Velos mass spectrometer (Thermo, San Jose, CA, USA) equipped with a nano-flow electrospray ion source and a six-way valve was used in this work. Accela 600 HPLC system for separation consisted of a degasser and a quaternary survey MS pump (Thermo, San Jose, CA).

HeLa cells were obtained from the Institute of Cell Bank, Chinese Academy of Sciences (Shanghai, China). RPMI-1640 cell culturing medium was purchased from Gibco Invitrogen Corporation (Carlsbad, CA, USA). Formic acid (FA), trifluoroacetic acid (TFA), ethylene diamine tetraacetic acid (EDTA), ethylene glycol tetraacetic acid (EGTA), protease inhibitor Cocktail (for use with mammalian cell and tissue extracts), phosphatase inhibitor, TPCK treated trypsin, iodacetamide (IAA), 1,4-dithiothreitol (DTT), N,N,N',N'-tetramethylethylenediamine (TEMED), and ammonium persulfate (AP) were obtained from Sigma (St. Louis, MO, USA). 40% Acrylamide/Bis solution (29:1) and Coomassie Brilliant Blue R-250 were purchased from Bio-Rad (CA, USA). PageRuler Prestained Protein Ladder and Sample Loading Buffer were obtained from Thermo (Rockford, USA). Acetonitrile (ACN, HPLC grade) and 25% ammonia solution were purchased from Merck (Darmstadt, Germany). GELoader tips (20 μL) were purchased from Eppendorf (Hamburg, Germany). Deionized water used in all experiments was purified with a Milli-Q system (Millipore, Milford, MA, USA). Other chemicals were all of analytical grade.

2.2 Cell Lysis and sample preparation

Human HeLa cells were cultured in RPMI 1640 medium supplemented with 10% newborn bovine serum and penicillin/streptomycin at 37 °C in 5% CO_2 atmosphere. When the cells were grown to about 90% density, each plate of cells was harvested and rinsed twice with cold PBS. Protein extraction was conducted as described in previous work[11]. The harvested HeLa cell pellets were homogenized by an