SUPPORTING INFORMATION

Integration of Cell Lysis, Protein Extraction and Digestion into One Step for Ultrafast Sample Preparation for Phosphoproteome Analysis

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Materials. RPMI-1640 cell culturing medium were purchased from Gibco Invitrogen Corporation (Carlsbad, CA). NP-40 was from New England BioLabs (Ipswich, MA). Formic acid (FA) was obtained from Fluka (Buches, Germany). Trifluoroacetic acid (TFA), ethylenediaminetetraacetic 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), and 1,4-dithiothreitol (DTT) were obtained from Sigma (St. Louis, MO). Acetonitrile (ACN, HPLC grade) and 25% ammonia solution were purchased from Merck (Darmstadt, Germany). GELoader tips (20 μL) were purchased from Eppendorf (Hamburg, Germany). Aspire RP30 desalting tips was obtained from Thermo Scientific (San Jose, CA). Deionized water used in all experiments was purified with a Milli-Q system from Millipore (Milford, MA). Other chemicals were all of analytical grade.

Cell Culture and Cell Counting

The human HeLa cells and HepG-2 cells were cultured in RPMI 1640 medium, supplemented with 10% new born bovine serum and 100 U/mL of penicillin and streptomycin at 37℃ in 5% CO_2 atmosphere. When the cells were grown to about 80% density, each plate of cells were harvested and rinsed twice with cold PBS. After the last rinse, cells were dispersed completely and counting was performed three times in parallel. Aliquots of 100 000 cells were stored at -80 ℃ for usage.

Preparation of Ti (IV) - IMAC Tips

The Ti^{4+}-IMAC microspheres were prepared as previously reported in our lab, and the GELoader spin tips were packed with the Ti (IV)-IMAC beads referred to the protocol described by Zhou et al.[1-3] Firstly, fill a piece of cotton into the tip as a plug. Then re-disperse 1.0 mg Ti-IMAC beads with loading buffer (80% ACN, 6% TFA) and pipette out the slurry into the tip. Remove the solvent by centrifugation in an adaptor.

Finally, equilibrate the tip twice with the loading buffer.

Phosphopeptide Enrichment by Ti (IV) -IMAC Tips

Enrichment of the phosphopeptides followed the protocol reported by Zhou et al.[2] Briefly, the digest was blended with identical volume of loading buffer (80% ACN, 6% TFA). The resulted solution was then loaded onto a Ti (IV)-IMAC tip by centrifuging at about 1000 g for 30 min. The tip was firstly washed by washing buffer 1 (50% ACN, 6% TFA, 200 mM NaCl) and washing buffer 2 (30% ACN, 0.1% TFA) at 1500 g for 15 min, respectively to remove the non-specifically bound peptides. The specifically bound phosphopeptides were thoroughly eluted by 100 μL 10% ammonia-water twice and then dried down in a Speed-Vac.

Influence of Large Amount of Free Trypsin on Phosphopeptide Enrichment

HeLa cell proteins (1.0 mg), which have been reduced and alkylated, were equally divided into four aliquots. For each aliquot, different amounts of trypsin were added to make the ratios of trypsin to protein (w/w) 1:25, 1:1, 5:1 and 10:1, respectively. Protein digestion was performed overnight at 37 °C. The phosphopeptides in the mixture were then isolated by Ti (IV)-IMAC beads as conventional protocol described by Zhou et al.[1] The phosphopeptides were identified by LC-MS/MS analysis.

Quantitative Analysis by Stable Isotopic Labeling

Because the incompatibility of the buffers to dimethyl labeling, 50 mM Tris-HCl buffer (pH 8.2) in the three protocols was changed into 100 mM TEAB buffer (pH 8.2). Two aliquots of HeLa cells (~100 000 cells) were processed in parallel according to one-step protocol. After the cells were digested into peptides, the two samples were labeled separately with light and heavy stable isotope dimethyl labels, and were combined together. The labeled phosphopeptides were then enriched by Ti (IV)-IMAC Tip.[4] LC-MS/MS analysis was performed in duplicate, and the acquired

MS data was searched by software MaxQuant 1.3. The same samples were also processed by Protocol A and Protocol B followed with labelling and quantification in the same way.

For quantitative comparison of the phosphopeptides generated by the three procotols, the samples were processed as following. Three aliquots of HeLa cells were digested into peptides by the one-step method, Protocol A and Protocol B, respectively. And the resulting peptides were labeled separately with heavy, intermediate and light stable isotope dimethyl labels. And then these labeled peptides were combined together for phosphopeptide enrichment.[4] LC-MS/MS analysis was performed in triplicate.

Mass Spectrometry Analysis

Dried peptides were redissolved by 0.1% FA and loaded onto the autosampler for mass spectrometry analysis performed on a LTQ Orbitrap Velos (Thermo, San Jose, CA) with an Accela 600 HPLC system for separation (Thermo, San Jose, CA). The automatic analysis system included a 4 cm capillary trap column (200 μm i.d.) and a 12 cm capillary analysis column (75 μm i.d.) both packed with C_{18} AQ beads (5 μm, 120 Å). Flow rate of the LC-MS/MS system was adjusted to about 200 nL/min, and the RP gradient was set as follows: from 0% to 2% mobile phase B (ACN/0.1% FA) for 2 min; 2% to 25% B for 90 min; 25% to 35% B for 5 min; 35% to 80% B for 3 min; 80% B for 10 min and finally equilibration with mobile phase A (H_2O / 0.1% FA) for 20 min.

The mass spectrometry was performed according to the following settings: a spray voltage 2.2 kV; ion transfer capillary 250 °C; full mass scan m/z 400-2000 at a resolution of 60000; one full scan acquired by 20 MS/MS scans at a minimum signal intensity 300; MS/MS fragmentation mode CID. And the dynamic exclusion function was set as follows: repeat count 1; duration 30 s; exclusion list size 500; exclusion duration 90 s.

Database Searching

All the MS raw data was transformed to files of *.mgf format by software Proteome Discoverer 1.4. Then searched them using Mascot Daemon version 2.3 against a database Uniprot human, which owned 88473 sequences and was downloaded from website of www.uniprot.org. The parameters were set as follows: Trypsin restriction two missed cleavages; parent ions mass tolerance 10 ppm; fragment ions mass tolerance 0.8 Da; dynamic modifications, Met (+15.9949 Da), Ser, Thr and Tyr (+79.96633 Da). Cys (+57.0215 Da) was set as static modification for control groups, while no static modification was set for experimental group. The obtained data was optimized according to significance threshold $p < 0.01$ and ions score > 25; meanwhile the false discovery rate (FDR) was controlled to < 1% at peptide level. The statistics data of phosphorylation sites was obtained using our in-house software.

Quantification by MaxQuant

Quantification of phosphopeptides was performed by searching all the RAW files with software MaxQuant version 1.3 (http: //www. maxquant.org/).[5] The database used was a UNIPROT database of human, which has 88473 sequences and was downloaded from website of www.uniprot.org. For MaxQuant searching, oxidation of methionine (+15.9949 Da), phosphorylation of serine, threonine and tyrosine (+79.96633 Da) was set as variable modification. Carbamidomethylation on cysteine (+57.0215 Da) was set as a fixed modification for the two conventional protocols, while no fixed modification was set for the new protocol. Peptide mass tolerance was set to 10 ppm and fragment mass tolerance was set to 0.5 Da. The false discovery rates (FDRs) for both peptide and protein identifications were set to 0.01. Peptides were searched with fully tryptic cleavage constraints, and up to two missed cleavage sites were allowed. For quantification, lysine and peptide N-termini in light dimethylation (+28.0313 Da), intermediate dimethylation (+32.0564 Da) and heavy dimethylation (+36.0757 Da) were set as light, intermediate and heavy labels. The other settings were the same as the conventional search.

Trysin Activity Assay

The activity was determined by monitoring the enzymolysis of trypsin substrate, *N*-*α*-benzoyl-$_L$-arginine ethyl ester hydrochloride (BAEE) to ultraviolet-absorption *N*-*α*-benzoyl-$_L$-arginine (BA) at 253 nm by a UV spectrophotometer.[6-8] The BAEE hydrolysis is a zero-order kinetics reaction; hence the increase of absorbance with time at 253 nm is linear, and the slope is linear to the trypsin activity, as well. BAEE (0.2 mM) was prepared in 50 mM NH_4HCO_3 buffer (pH 7.8) and spiked with 0.5 μg, 1.0 μg, 2.0 μg, 5.0 μg, 10.0 μg, 20.0 μg of trypsin, respectively. UV absorbance was recorded from zero time in 1 min intervals and four measurements were averaged for every time point.

References

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Supplementary Figures

Figure S-1. Investigating the influence of huge amount of trypsin on phosphopeptide enrichment. X axis showed different amounts of trypsin added. Y1 axis was average values of three times MS identification of unique phosphorylation sites from a quarter of 250 μg HeLa cell proteins. Y2 axis was average values of the enrichment specificities. The error bar was standard deviation.

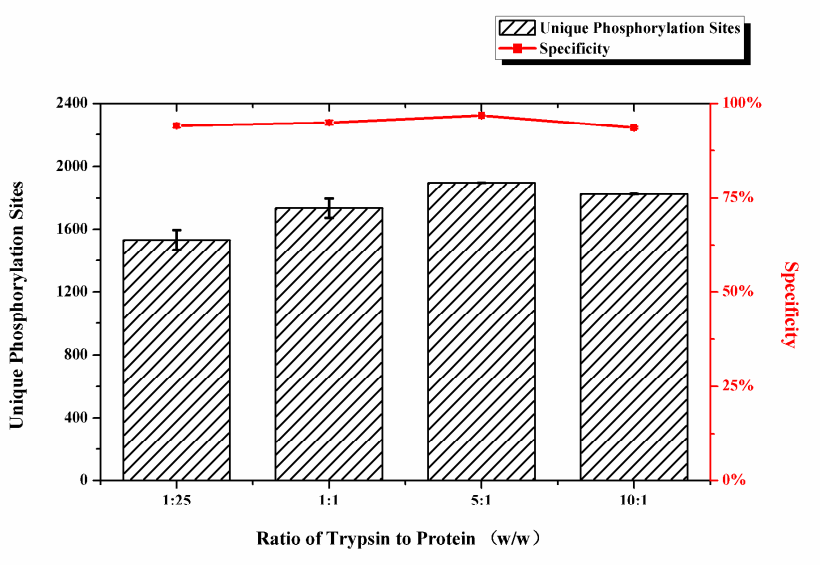

Figure S-2. Identification results of three sample preparation experiments for the one-step method and two conventional methods. (A) Overlaps of phosphorylation sites among three sample preparation experiments for the three methods. The same amount of HeLa cells (10^5 cells) was processed by the three methods and three sample preparation experiments were performed for each method. (B) Overlaps between every two methods. For each method, the sites were combined by the three experiments.

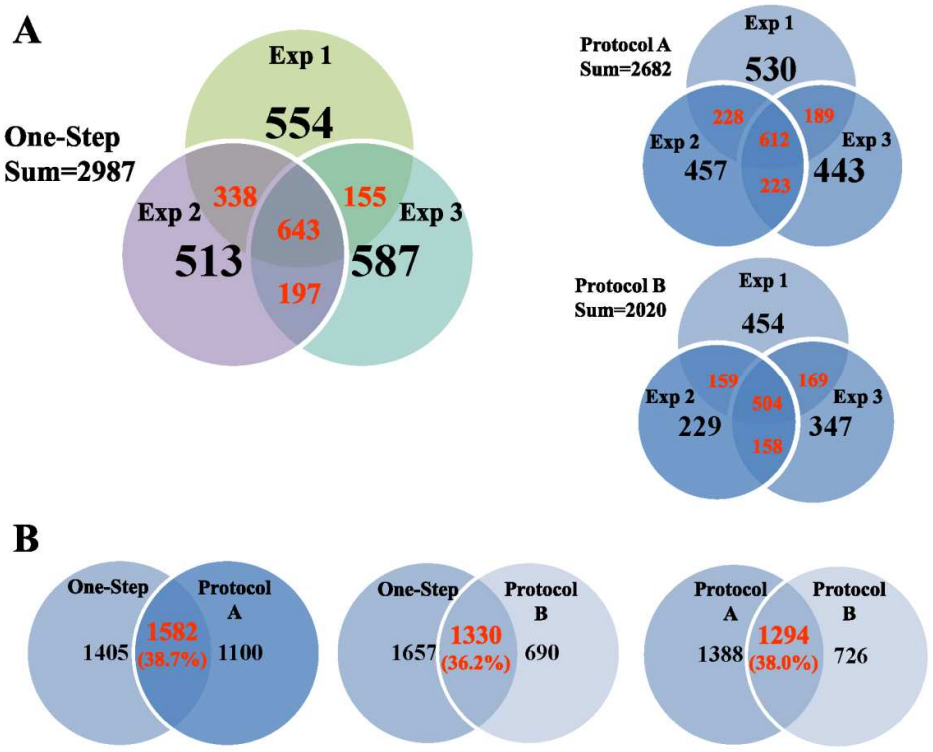

Figure S-3. Comparing the recovery of phosphopeptides by three methods. The peptides of one-step method, Protocol A and Protocol B were labeled separately with heavy, intermediate and light stable isotope dimethyl labels and pooled together for quantitative phosphoproteomics analysis.

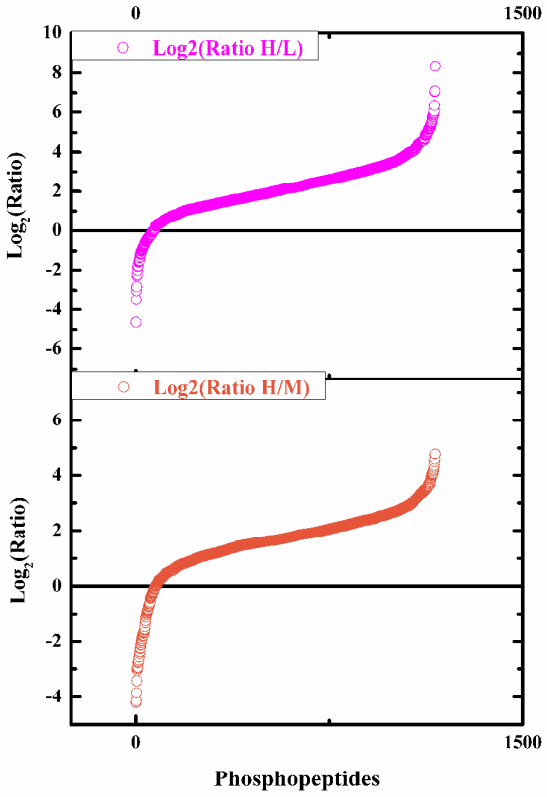

Figure S-4. Comparing the performances of the three methods for quantitative phosphoproteomics analysis. Two aliquots of the same sample were processed by each method and labeled separately with light and heavy stable isotope dimethyl labels. After being combined in 1:1, the labeled sample was subjected to quantitative phosphoproteomics analysis.

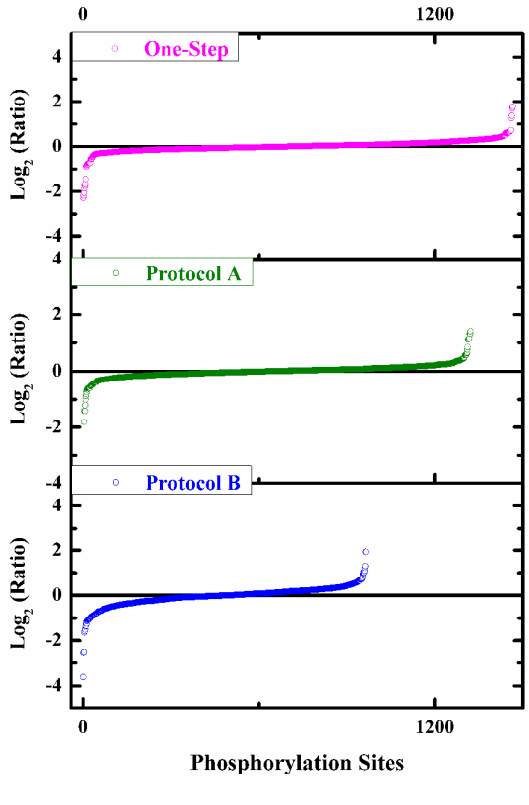

Figure S-5. The dependence of hydrolysis rate on the amount of added trypsin. The hydrolysis rate, represented by the slope of the UV absorbance change at $A_{253\ nm}$ with the reaction time, increased significantly with the increase of trypsin amount. When the amount of typsin increased to 40-fold of the initial amount, the hydrolysis of the product could be nearly completed within 1 min.