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Sequential enrichment of singly- and multiply-phosphorylated peptides with zwitterionic hydrophilic interaction chromatography material

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ABSTRACT

An interesting and novel method for the selective and sequential enrichment of singly- and multiply-phosphorylated peptides with a zwitterionic material "Click TE-Cys" is presented. Retention mechanisms between phosphopeptides and Click TE-Cys are systematically investigated by checking the influence of acetonitrile content, pH value, and buffer concentration on the retention of phosphopeptides. Both hydrophilic interaction and electrostatic interaction are involved in retention between phosphopeptides and Click TE-Cys. Based on these results, an optimized method is established for selective enrichment of phosphopeptides using Click TE-Cys. This method not only exhibits high selectivity for phosphopeptides, but also fractionates singly- and multiply-phosphorylated peptides into two fractions. This method was evaluated using relatively complex samples, including peptide mixtures of α-casein and bovine serum albumin (BSA) at a molar ratio of 1:10 and skim milk. This efficient and optimized protocol has great potential for enriching multiply-phosphorylated peptides and could be a valuable tool for specific enrichment of phosphopeptides in phosphoproteome analysis.

1. Introduction

Reversible protein phosphorylation controls enzyme activity and regulates vital bioprocesses, such as cell signal transduction, proliferation, differentiation, and apoptosis [1]. Mass spectrometry (MS) is commonly used in phosphoproteome analysis, allowing characterization of phosphorylated peptide sequence and phosphorylation sites [2,3]. However, the phosphopeptide-detection strategy remains challenging due to difficulties in phosphopeptide MS analysis. Phosphopeptides are of low-abundance in peptide mixtures and their signals are easily suppressed by those of co-eluting and strongly ionizing non-phosphopeptides [4]. Moreover, the identification of multiply-phosphorylated peptides (MPPs) is more challenging given their lower abundance than that of singly-phosphorylated peptides (SPPs) and greater vulnerability to suppression [5]. Therefore, to enhance the relative abundance of phosphopeptides, especially MPPs, and reduce interference by non-phosphopeptides, it is necessary to develop selective materials and establish corresponding methods for phosphopeptide enrichment prior to MS analysis [6,7].

Among phosphopeptide enrichment methods, immobilized metal-ion affinity chromatography (IMAC) [8] and metal-oxide affinity chromatography (MOAC) [9,10] are widely used in phosphoproteome analysis. In the last decade, hydrophilic interaction chromatography (HILIC) has become increasingly popular for phosphopeptide enrichment. Because amino acids with phosphate groups are more hydrophilic than other common amino acids, phosphopeptides are more strongly retained on HILIC materials as compared to their counterparts [11]. Besides, ion exchange chromatography (IEC) including strong cation exchange chromatography (SCX) and strong anion exchange chromatography

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(SAX) separates phosphopeptides from non-phosphopeptides according to their different solution charge states [12–14]. Later, electrostatic repulsion–hydrophilic interaction chromatography (ERLIC), a combination of ion exchange and hydrophilic interaction, was developed for phosphopeptide isolation [15–17]. When ERLIC was used to separate phosphopeptides, hydrophilic interaction and electrostatic repulsion played primary and secondary roles in solute retention, respectively. Selective enrichment of phosphopeptides was obtained by adjusting solution pH and acetonitrile (ACN) concentration [18].

In this study, a novel zwitterionic HILIC material "Click TE-Cys" [19,20] is facilely synthesized based on the "thiol-ene" click reaction between cysteine and vinyl silica. The improved hydrophilicity, selectivity, and column efficiency of Click TE-Cys [19,20] is important for chromatography separation, which has exhibited great potential in the separation of oligosaccharides, basic compounds, and glycopeptide enrichment [21–24]. Different from typical zwitterionic stationary phases, Click TE-Cys features a uniform distribution of both positive and negative charges parallel to the silica gel surface. The surface charge of Click TE-Cys can be altered by adjusting the solution pH. As a mixed-mode chromatographic material, Click TE-Cys exhibits both hydrophilic-interaction and ion-exchange characteristics. Taking advantages of the hydrophilicity and electrostatic attraction provided by Click TE-Cys, separation of phosphopeptides and non-phosphopeptides can be easily performed, as well as fractionation of SPPs and MPPs. Here, we explored phosphopeptide retention on Click TE-Cys though establishment of system methodology. To understand the retention mechanism, we carried out investigations to study the influence of ACN concentration, solution pH, and salt concentration on phosphorylated peptide retention. Based on the above retention mechanism, selective phosphopeptide enrichment methods were established and validated using complex sample systems and practical sample systems.

2. Experimental

2.1. Materials

Bovine Serum Albumin (BSA), α-casein, dithiothreitol (DTT), iodoacetic acid (IAA) and ammonium bicarbonate (NH_4HCO_3) were from Sigma Aldrich (St. Louis, MO, USA). Ammonium formate (NH_4FA), ammonium acetate (NH_4Ac), trifluoroacetic acid (TFA) and formic acid (FA) were from Acros (Fair Lawn, NJ, USA). Sequencing grade modified trypsin was from Promega (Madison, WI, USA). Ammonium hydroxide ($NH_3{\cdot}H_2O$) was from Fluka (Buchs, Switzerland). Acetonitrile (ACN) was from Merck (Darmstadt, Germany). GELoader tips were from Eppendorf (Hamburg, Germany). Water used in experiments was purified using a Milli-Q system (Millipore, Bedford, MA, USA). The zwitterionic stationary phase Click TE-Cys [19,20] (spherical silica particle size: 5 μm; pore size: 100 Å; surface area: 300 $m^2\,g^{-1}$) was prepared in-house. TiO_2 (particle size: 5 μm) was from GL Sciences (Tokyo, Japan). C18 was from Sunchrom (Friedrichsdorf, Germany).

2.2. Apparatus

Mass spectrometry (MS) was carried out on a quadrupole time-of-flight (Q-TOF) electronic spray ion (ESI) mass spectrometer (Waters, Manchester, UK) coupling with a nanoACQUITY^{TM} Ultra-Performance LC (Waters, Milford, MA, USA).

2.3. Preparation of Click TE-Cys

The synthesis procedure of Click TE-Cys materials was facilely achieved in two steps followed the published article [19,20]. The vinyl silica was prepared *via* the horizontal polymerization of vinyl trichlorosilane on silica (particle size: 5 μm; pore size: 100 Å; surface area: 300 $m^2\,g^{-1}$). Then, a "thiol ene" click reaction between vinyl silica and cysteine was carried out in the presence of azodiisobutyronitrile (AIBN) to obtain the functionalized stationary phase (designated Click TE-Cys).

2.4. Enrichment of phosphopeptides

2.4.1. Preparation of tryptic digests

Digestion of standard protein was as previously described with minor modification [25]. α-casein (1 mg) was dissolved in NH_4HCO_3 buffer (50 mM, 1 mL) and digested by trypsin for 17 h at 37 °C with a 1:30 (w/w) enzyme/substrate ratio. BSA (0.5 mg) was dissolved in guanidine hydrochloride (0.5 M) in NH_4HCO_3 buffer (50 mM, 10 μL) for 3 h. The denatured protein samples were reduced with DTT (50 mM, 4 μL) for 2 h at 37 °C. Then, IAA (50 mM, 5 μL) was added for alkylation and the resulting solution was incubated in the dark for 30 min at ambient temperature. After being diluted tenfold with NH_4HCO_3 buffer (50 mM), the solution was mixed with trypsin at an enzyme/substrate ratio of 1:30 (w/w) and incubated for 17 h at 37 °C. Frozen skim milk (1 mL) was mixed with 4 ml ACN and sonicated, then the mixture was centrifuged and the supernatant discarded. The rest of the protein, about 3.4 mg, was dissolved in urea (8 M) in NH_4HCO_3 buffer (50 mM, 1040 μL) for 3 h. The denatured protein samples were reduced with DTT (50 mM, 17 μL) for 2 h at 37 °C. Then, IAA (50 mM, 68 μL) was added for alkylation and the resulting solution was incubated in the dark for 30 min at ambient temperature. After being diluted tenfold with NH_4HCO_3 buffer (50 mM), the solution was mixed with trypsin at an enzyme/substrate ratio of 1:30 (w/w) and incubated for 17 h at 37 °C.

2.4.2. Retention mechanism investigation

For investigating phosphopeptide retention affected by ACN content, 2 mg of Click TE-Cys microspheres in ACN were packed into GELoader tips. Then the microspheres were washed with ACN/H_2O (10:90 (v/v), 30 μL) and equilibrated with ACN/H_2O/FA (80:20:0.01 (v/v), 30 μL). α-casein tryptic digest (4 μL) was re-dissolved in ACN/H_2O/FA (80:20:0.01 (v/v), 26 μL), and loaded into the microcolumn. The microcolumn was subsequently washed with ACN/H_2O/FA (80:20:0.01 (v/v), 30 μL), ACN/H_2O/FA (70:30:0.01 (v/v), 30 μL), ACN/H_2O/FA (60:40:0.01 (v/v), 30 μL), ACN/H_2O/FA (50:50:0.01 (v/v), 30 μL) and H_2O/FA (100:0.01 (v/v), 30 μL), respectively. To each fraction was analyzed by MS.

For investigating phosphopeptide retention affected by the solution pH, the aforementioned ACN step-gradient were followed besides changing 0.01%FA to 1%FA and water.

For investigating phosphopeptide retention affected by the different buffer concentrations, the aforementioned ACN step-gradient were followed besides changing 0.01%FA to 10 mM NH_4Ac and 100 mM NH_4Ac.

2.4.3. The method for phosphopeptides segmented enrichment

2 mg of Click TE-Cys microspheres in ACN were packed into GELoader tips. Then the microspheres were washed with ACN/H_2O (10:90 (v/v), 30 μL) and equilibrated with ACN/H_2O (80:20 (v/v), 30 μL). α-Casein tryptic digest (2 μL) was re-dissolved in ACN/H_2O (80:20 (v/v), 28 μL), and loaded into the microcolumn. The microcolumn was firstly washed with ACN/H_2O (80:20 (v/v), 30 μL), then washed twice with 100 mM NH_4Ac in ACN/H_2O (80:20 (v/v), 30 μL) and quadruply with 100 mM NH_4Ac in ACN/H_2O (70:30 (v/v), 30 μL). The microsphere were then equilibrated with ACN (30 μL) and washed with ACN/H_2O/FA (70:29:1 (v/v), 30 μL). Finally, the microcolumn was equilibrated with ACN/H_2O (70:30 (v/v), 30 μL) and washed with 0.1% $NH_3{\cdot}H_2O$ (30 μL). Corresponding fractions