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C_{60}-fullerene bound silica for the preconcentration and the fractionation of multiphosphorylated peptides

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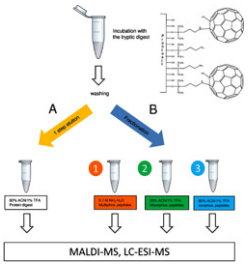
HIGHLIGHTS

- Phosphopeptides can be selectively fractionated using C_{60}-aminopropylsilica.
- Selective isolation of mono- and multiphosphorylated peptides was performed according to their pI values.
- Elution was carried out using pH gradient in presence of different concentration of acetonitrile.
- The binding and fractionation can be attributed to amino groups together with the hydrophobic fullerene moieties.

GRAPHICAL ABSTRACT



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ABSTRACT

Phosphorylation of proteins is an important cellular regulatory process. The analysis of protein phosphorylation is challenging due to the high dynamic range and low abundance natures of phosphorylated species. Mass spectrometry (MS) of phosphopeptides obtained from tryptic protein digests is the method-of-choice for characterization of phosphorylated proteins. However, determination of phosphopeptides by MS represents a major challenge, especially in the presence of unmodified peptides. Due to lower ionization efficiency of phosphopeptides, as well as the fact that the stoichiometry of phosphorylation is often present at low relative abundance, efficient enrichment of the phosphorylated peptides prior to MS analysis is therefore of high demand. In addition, successful identification of peptides with different phosphorylation grades still remains challenging.

This work presents a new strategy for enrichment and subsequent selective elution of multi-, mono- and nonphosphorylated peptides based on their difference in pI by using pH gradient elution in presence of different concentration of acetonitrile prior to matrix-assisted laser desorption/ionization time-of-flight mass spectrometric analysis (MALDI-MS). The developed protocol was successfully applied for α-casein tryptic digest and bovine serum albumin digest spiked with 9 synthetic phosphopeptides. Further selectivity for phosphopeptides was demonstrated by fractionation of peptides from a milk digest.

1. Introduction

Protein phosphorylation is one of the most common mechanisms for covalent modification of proteins and is found in as many as one-third of eukaryotic gene products [1,2]. Reversible

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phosphorylation of proteins plays a crucial role in a large number of biological processes, such as signal transduction, cell proliferation, differentiation and apoptosis [3–5]. In order to understand the molecular basis of these regulatory mechanisms, various methods have been developed to identify the specific sites of phosphorylation [6,7]. Although the number of cellular phosphoproteins is relatively high, the phosphorylated residues themselves are generally of low abundance due to the sub-stoichiometric nature of this modification [8]. The detection and sequencing of tryptic phosphopeptides derived from such proteins has become an important aspect of biological and biomedical research. However, the prevalence of nonphosphorylated peptides in protein digests has made it necessary to develop efficient separation and enrichment methods for phosphopeptide. Up to now, mass spectrometry, especially matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and electrospray ionization mass spectrometry combined with liquid chromatography separation techniques (LC–ESI-MS) have been key technologies in characterizing protein phosphorylation and each approach presented strengths and limitations [9–13]. However, because of the low stoichiometry and reversibility of phosphorylation on proteins [14,15], as well as the low ionization efficiency of phosphopeptides, it remains challenging to thoroughly characterize phosphorylation sites using mass spectrometry. Thus, the enrichment of phosphoproteins and phosphopeptides prior to MS analysis is mandatory [16,17].

A variety of enrichment methods such as immunoprecipitation [18,19], chemical-modification strategies [20], strong cation exchange chromatography [21], strong anion exchange chromatography [22], and affinity chromatography [23,24], have been developed to enrich phosphopeptides out of proteolytic peptide mixtures. The former of these techniques involves beta-elimination of the phosphate group from SerP and ThrP residues under alkaline conditions that specifically label the resulting dehydroalanine derivative by the addition of various nucleophilic groups [20,25,26]. However, the harsh alkaline treatment often causes unwanted side reactions in protein/peptide samples [27]. Among the above-mentioned methods, affinity chromatography, including immobilized metal affinity chromatography (IMAC) and metal oxide affinity chromatography (MOAC) have been widely employed.

Immobilized metal-ion affinity chromatography (IMAC) has been commonly utilized for the selective enrichment of phosphopeptides [28–32]. The negatively charged phosphopeptides are retained by iminodiacetic acid (IDA) or nitrilotriacetic acid (NTA) resins through their affinity to metal ions (Ga^{3+} or Fe^{3+}) [33,34]. However, this method is prone to low recoveries and/or nonspecific binding of nonphosphorylated peptides [35]. Recently, the phosphate group was used to replace IDA and NTA as chelator and utilizing Zr^{4+} as a metal ion [36,37].

Recently, metal oxide affinity chromatography (MOAC) has been widely used in large-scale phosphoproteome analysis due to the specific and reversible affinity of phosphate groups to the amphoteric surface of metal oxides via bidentate binding [38,39]. Microparticles of titanium dioxide (TiO_2) [40–42], zirconium dioxide (ZrO_2) [43–45], aluminum hydroxide ($Al(OH)_3$) [46] and other metal oxides [47] have been explored in phosphopeptide isolation and exhibit higher specificity for trapping phosphate groups compared to the IMAC approach. According to established protocols, peptide mixtures are loaded onto the column under acidic conditions, washed with the appropriate solution and the bound phosphopeptides eluted with basic solutions. Because of the stability of the metal oxides over a wide pH range, acidic buffers can be employed to avoid binding of nonphosphorylated peptides to the active surface [48].

Whereas the characterization of singly and doubly phosphorylated peptides has almost become routine, identifying phosphorylation events at multiple residues within a small region of a protein is still problematic. The presence of mono- or nonphosphorylated peptides in samples for MS suppresses the ionization of multiple phosphorylated peptides and thereby decreases the chance to detect them. A number of strategies have been developed to enhance the detection of multiphosphorylated peptides. Larsen et al. developed a strategy, referred to as SIMAC (sequential elution from IMAC), based on sequential separation of monophosphorylated peptides and multiply phosphorylated peptides from highly complex biological samples. This allows individual analysis of both two pools of phosphorylated peptides using mass spectrometric parameters differentially optimized for their unique properties [49]. Chang et al. developed polyarginine-coated diamond nanoparticles for selective extraction and enrichment of multiphosphorylated peptides through multiple arginine-phosphate interactions [50]. Hydroxyapatite has been applied for the selective separation and fractionation of phosphorylated peptides employing phosphate buffer gradient for stepwise elution of peptides. The developed protocol permitted selective isolation of mono- and multiphosphorylated peptides [51].

An important area of research in modern material nanoscience concerns carbon-based materials, among which fullerenes take one of the first places. The potential utility of surface-linked fullerene materials [52] has resulted in the development of synthetic methodologies to covalently incorporate C_{60}-fullerene into inorganic or organic matrices [53–55]. This can be achieved either by chemical modification of a matrix with a reagent able to add to an appropriate fullerene or by preliminary introduction of a reactive function onto the C_{60}-fullerene, followed by a bond-forming step between the modified fullerene and the solid support. In our laboratory, epoxy fullerene was covalently bound to amino derivatized silica microparticles and successfully applied for desalting peptides and proteins [56].

In the presented study, the authors report a novel affinity procedure to enrich phosphorylated peptides out of complex mixtures using C_{60}-aminopropylsilica. Stepwise elution conditions allow a straightforward and selective isolation of mono- and multiphosphorylated peptides according to their pI values. Tryptic digest of a model phosphoprotein (α-casein) was analyzed for the efficiency of selective isolation of multiphosphorylated peptides by MALDI-TOF-MS. Furthermore, the developed protocol was employed for the analysis of bovine serum albumin tryptic digest spiked with synthetic phosphopeptides carrying different numbers of phosphate groups. Phosphopeptides derived from tryptic milk digest were selectively fractionated using C_{60}-aminopropylsilica.

2. Experimental

2.1. Chemicals and materials

C_{60}-fullerene (≥99.5%) was purchased from SES RESEARCH (Houston, TX, USA). (3-Aminopropyl)triethoxysilane (99.0%); 2,5-dihydroxybenzoic acid (DHB) (puriss, p.a.); 3-chloroperbenzoic acid (≤77%); trifluoroacetic acid (TFA) (≥99.5); toluene (puriss, p.a.); methanol (≥99%); ethanol (99%); ammonium hydroxide (25.0%); orthophosphoric acid (85.0%); acetonitrile (ACN) (G Chromasolv for HPLC); water (Chromasolv plus, for HPLC); hydrochloric acid (ACS reagent, 37%); dithiothreitol (≥99.0%); *n*-octylglucopyranoside (≥99.0%); 4-toluenesulfonic acid (>99%); iodoacetamide (≥98.0%); ammonium bicarbonate (≥99.5%), α-casein and serum albumin from bovine lyophilized powders (≥96%) were purchased from Sigma–Aldrich (Vienna, Austria). ProntoSil 300-3-Si (300 Å pore size, 3 μm particle size) and ProntoSil 300-5-Si (300 Å pore size, 5 μm particle size) were purchased from