Journal of Chromatography A, 1457 (2016) 76–87

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Mimicking nature: Phosphopeptide enrichment using combinatorial libraries of affinity ligands

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ARTICLE INFO

Article history:
Received 3 March 2016
Received in revised form 8 June 2016
Accepted 9 June 2016
Available online 11 June 2016

Keywords:
Phosphopeptide enrichment
Phosphoproteomics
Synthetic ligands
Affinity
Magnetic nanoparticles
Iron oxide

ABSTRACT

Phosphorylation is a reversible post-translational modification of proteins that controls a plethora of cellular processes and triggers specific physiological responses, for which there is a need to develop tools to characterize phosphorylated targets efficiently. Here, a combinatorial library of triazine-based synthetic ligands comprising 64 small molecules has been rationally designed, synthesized and screened for the enrichment of phosphorylated peptides. The lead candidate (coined A_8A_3), composed of histidine and phenylalanine mimetic components, showed high binding capacity and selectivity for binding mono- and multi-phosphorylated peptides at pH 3. Ligand A_8A_3 was coupled onto both cross-linked agarose and magnetic nanoparticles, presenting higher binding capacities (100-fold higher) when immobilized on the magnetic support. The magnetic adsorbent was further screened against a tryptic digest of two phosphorylated proteins (α- and β-caseins) and one non-phosphorylated protein (bovine serum albumin, BSA). The MALDI-TOF mass spectra of the eluted peptides allowed the identification of nine phosphopeptides, comprising both mono- and multi-phosphorylated peptides.

1. Introduction

Phosphorylated proteins are involved in complex signaling networks, by establishing specific protein–protein interactions, and are implicated in a wide range of human diseases [1–4]. It is vital to unveil the interaction landscape of these phosphorylated proteins and their biological binders. The identification of phosphorylated proteins and peptides by mass spectrometry (MS) is particularly challenging due to their low relative abundance in biological samples and to the dynamic character of protein phosphorylation. It is therefore necessary to enrich the samples prior to MS analysis [5–7].

In recent decades, a plethora of affinity reagents and affinity-based materials has been developed for the specific capture of phosphorylated proteins and peptides, with antibodies still being the most commonly used affinity binders. However, as the phosphate group presents low immunogenicity and is susceptible to cleavage during the immunization process, the generation of high-quality phosphorylation-state specific antibodies is difficult [8]. Other common strategies for phosphopeptide enrichment rely on metal coordination of the phosphate moiety, using for example immobilized-metal affinity chromatography (IMAC) [9] and metal oxide affinity chromatography (MOAC) [10], or through the use of small chemical ligands (e.g. Phos-tag) [11]. Both IMAC and MOAC (mainly TiO_2) are by far the most commonly used techniques, as they reportedly bind both mono- and multi-phosphorylated peptides. However, multi-phosphorylated peptides present high binding affinity to MOAC resins, which makes their elution difficult for further analysis. Therefore, mono-phosphorylated peptides are more easily detectable using MOAC. IMAC resins are good options to profile multi-phosphorylated peptides, due to the strong avidity interactions between these peptides and IMAC supports. The main disadvantage of both these metal coordination methods is the non-specific binding of acidic peptides. Nonetheless, these methodologies have been used and optimized for decades, and implemented in large-scale phosphoproteomics studies [6,12].

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http://dx.doi.org/10.1016/j.chroma.2016.06.032
0021-9673/

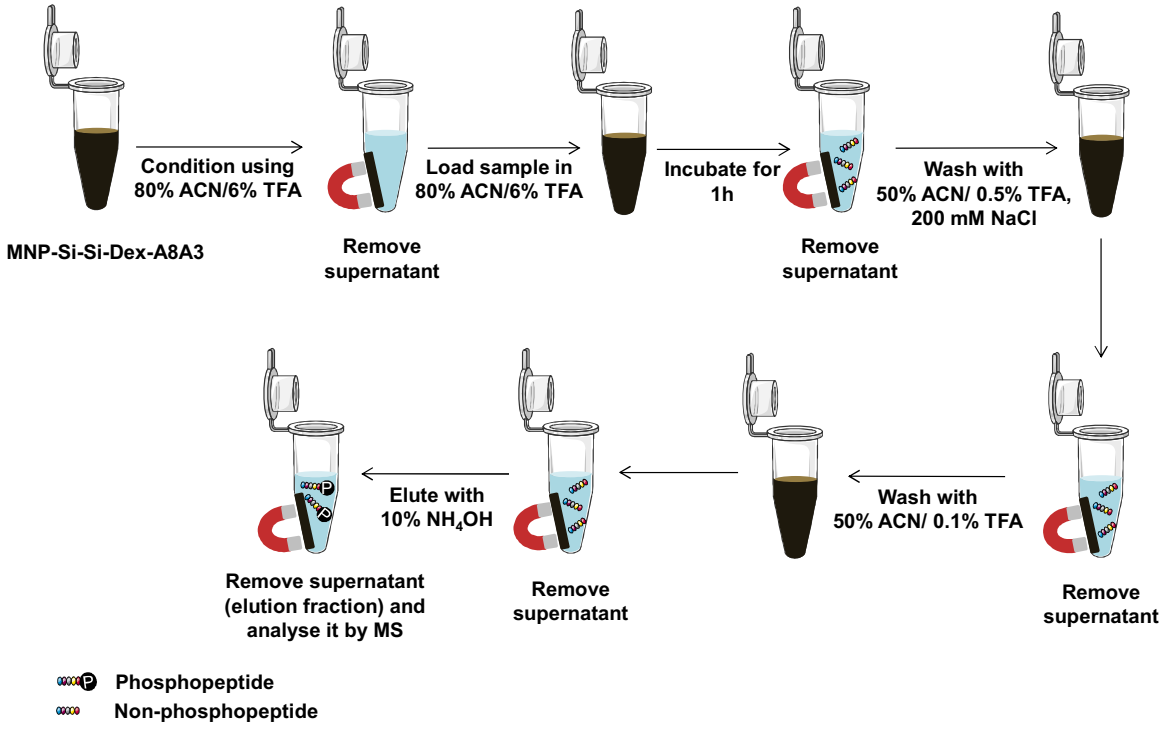

Fig. 1. Phosphopeptide enrichment using MNP-Si-Si-Dex-A_8A_3.

Non-affinity methods, such as ion exchange chromatography and hydrophilic interaction chromatography, have also shown promise towards global phosphopeptide identification [13,14]. However, considering the complexity of the human phosphoproteome, all these methodologies and materials do not provide sufficient selectivity and specificity. There is a real need to develop new methods and materials that can complement each other and promote the identification of a larger number of phosphorylated peptides.

Biomimetics are a class of chemically defined and characterized affinity ligands, which can be engineered to bind particular target biomolecules. Among them, the triazine nucleus emerged as a suitable scaffold to introduce molecular diversity [15,16]. Triazine-based ligands have been explored as affinity reagents to capture a variety of biological targets, with the advantages of presenting affinity and selectivity, high robustness and fast development at low cost [17–19]. Our group has explored the combination of these synthetic ligands with a variety of stimuli-responsive materials, namely magnetic nanoparticles (MNPs), to create innovative platforms for the enrichment and purification of biologics. Examples include the use of antibody selective synthetic ligands [20] coupled to magnetic particles [21–24] and magnetic monoliths [25]. In fact, superparamagnetic nanoparticles, as those based on iron oxide, are well suited for bioseparations due to their easy synthesis, surface modification and manipulation under an external magnet, as well as their compatibility with biological samples [26,27].

Considering the complexity of the interactions between phosphoproteins and natural binding domains, triazine-based affinity reagents designed towards phosphorylated targets and mimicking naturally observed interactions, emerge as valuable tools to overcome the limited selectivity of current phosphoprotein enrichment methods. In this context, this work explored the development of tailor-made biomimetic affinity reagents for phosphorylated peptides immobilized onto both cross-linked agarose and magnetic particles, and the application of these materials for the enrichment of phosphorylated species.

2. Materials and methods

2.1. Materials

All reagents were of the highest purity available and the solvents were pro-analysis. 2-Propanol was purchased from Roth. Acetone and dimethylformamide (DMF) were acquired from VWR. Acetonitrile (ACN), ethanol absolute, hydrochloric acid (HCl), sodium acetate, sodium chloride (NaCl) and sodium hydroxide (NaOH) were obtained from Panreac. 2,5-Dihydroxybenzoic acid (2,5-DHB), 3-aminopropyltriethoxy silane (APTES), agmatine sulfate salt (A_7, Fig. 2b), amino-2-propanol (A_2, Fig. 2b), ammonium bicarbonate (NH_4HCO_3), ammonium hydroxide solution (NH_4OH), cadaverine dihydrochloride (A_5, Fig. 2b), cyanuric chloride, dextran from *Leuconostoc mesenteroides* (average mol wt 150,000), DL-dithiothreitol (DTT), epichlorohydrin, histamine (A_8, Fig. 2b), iron(II) chloride tetrahydrate, iron (III) chloride hexahydrate, iodoacetamide (IAA), *N,N*-dimethyl-4,4′-azodianiline (A_1, Fig. 2b), phenethylamine (A_3, Fig. 2b), phosphoric acid (H_3PO_4), sodium bicarbonate ($NaHCO_3$), sodium metasilicate pentahydrate, sodium thiosulfate, tris(hydroxymethyl)aminomethane (Tris), tryptamine (A_6, Fig. 2b), tetraethyl orthosilicate (TEOS), trifluoroacetic acid (TFA), and tyramine (A_4, Fig. 2b) were acquired from Sigma–Aldrich. Sepharose^{TM} CL-6B was acquired from GE Healthcare, glycine from Acros Organics and acetic acid glacial from Pronalab. Urea was acquired from Merck. Nitrogen gas was provided by Air Liquide. Ser-Gln-Val-Phe-Pro-Trp (SW6), pSer-Gln-Val-Phe-Pro-Trp (SW6-P), Thr-GlnVal-Asp-Ala-Trp (TW6), pThr-Gln-Val-Asp-Ala-Trp (TW6-P), Tyr-Glu-Glu-Ile-Pro-Trp (YW6), pTyr-GluGlu-Ile-Pro-Trp (YW6-P), Tyr-Ala-Gly-Ser-Thr-Asp-Glu-Asn-Thr-Asp-Ser-Glu-Trp (YW13), and Tyr-AlaGly-pSer-pThr-Asp-Glu-Asn-pThr-Asp-Ser-Glu-Trp (YW13-P) peptides were > 98% pure and were purchased from Genecust and Caslo. Trypsin Gold, Mass Spectrometry Grade was purchased from Promega. α-Casein from bovine milk (Cat. No. C6780), β-Casein from bovine milk (Cat. No. C6905),