Journal of Chromatography A, 1428 (2016) 202–211

Contents lists available at ScienceDirect

Journal of Chromatography A

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

Characterization of post-translationally modified peptides by hydrophilic interaction and reverse phase liquid chromatography coupled to quadrupole-time-of-flight mass spectrometry

Oswaldo Hernandez-Hernandez[a], Jesus Eduardo Quintanilla-Lopez[b], Rosa Lebron-Aguilar[b], Maria Luz Sanz[a], F. Javier Moreno[c,*]

[a] Instituto de Química Orgánica General, Consejo Superior de Investigaciones Científicas (CSIC), C/Juan de la Cierva 3, 28006 Madrid, Spain
[b] Instituto de Química-Física "Rocasolano", Consejo Superior de Investigaciones Científicas (CSIC), C/Serrano 119, 28006 Madrid, Spain
[c] Instituto de Investigación en Ciencias de la Alimentación (CIAL), Consejo Superior de Investigaciones Científicas-Universidad Autónoma de Madrid (CSIC-UAM), C/Nicolás Cabrera 9, Campus de Cantoblanco, 28049 Madrid, Spain

ARTICLE INFO

Article history:
Received 13 May 2015
Received in revised form 8 July 2015
Accepted 24 July 2015
Available online 29 July 2015

Keywords:
Phosphopeptides
O-Glycopeptides
Caseinomacropeptide
Post-translational modifications
HILIC
Tandem mass spectrometry

ABSTRACT

This work explores the use of both hydrophilic interaction liquid chromatography (HILIC) and reverse phase liquid chromatography (RPLC) for the separation and subsequent characterization of bovine caseinomacropeptide (CMP) phosphopeptides and *O*-glycopeptides using a quadrupole-time-of-flight (QTOF) mass spectrometer with electrospray ionization. Two neutral, ethylene bridged hybrid (BEH) amide and polyhydroxyethyl aspartamide (PHEA), and a zwitterionic, sulfobetaine (ZIC), stationary phases were used for the HILIC mode, whilst an octadecylsilane (C_{18}) stationary phase was employed for the RPLC separation. Overall, developed HILIC-QTOF method using the ZIC or BEH amide stationary phases resulted to be the most efficient methods to separate and characterize post-translationally modified (PTM) peptides without the need of any previous fractionation or derivatization step. The separation of phosphopeptides and differently sialylated *O*-glycopeptides in the ZIC stationary phase was dominated by an electrostatic repulsion interaction mechanism between the negatively charged phosphate groups or sialic acid moieties and the negatively charged terminal sulfonate group of the stationary phase, whereas the separation of either non-modified peptides or neutral *O*-glycopeptides both free of basic amino acids was based on a partitioning mechanism. In neutral amide columns, the separation was mainly dominated by hydrophilic partitioning, leading to a higher retention of the post-translationally modified peptides than the unmodified counterparts due to the hydrophilicity provided by the phosphate groups and/or *O*-glycans. As a consequence, HILIC-ESI-QTOF MS operating in the positive ion mode is a powerful tool for the characterization of underivatized *O*-glycopeptides and phosphopeptides.

1. Introduction

Post-translational modifications (PTMs) of proteins are involved in key processes that govern the regulation of cellular processes, like cell division, growth, and differentiation [1]. About 5% of the genomes of higher eukaryotes can be dedicated to enzymes that carry out PTMs of the proteomes [2], leading to an important protein microheterogeneity and increasing the functional diversity of the proteome. Among the wide number of PTMs of proteins, the most common are phosphorylation and glycosylation both playing a significant role in defining the physicochemical and biological properties of such proteins. Bearing in mind that PTMs are normally present in sub-stoichiometric amounts and their ionization is often suppressed by unmodified peptides of high abundance, their elucidation remains as a major analytical challenge [3]. To facilitate this task, enrichment strategies of glycopeptides and phosphopeptides and/or highly sensitive and selective analytical methods have been developed over the last years.

Characterization of the PTM sites and the glycan or phosphate microheterogeneity is normally performed by LC–MS after digestion of the proteins, since MS spectra of PTM peptides provide more robust data deconvolution than intact proteins [4]. Reverse phase liquid chromatography (RPLC) has traditionally been the dominant

* Corresponding author. Tel.: +34 910017948; fax: +34 910017905.
E-mail addresses: ohernandez@natac.es (O. Hernandez-Hernandez), je.quintanilla@iqfr.csic.es (J.E. Quintanilla-Lopez), rlebron@iqfr.csic.es (R. Lebron-Aguilar), mlsanz@iqog.csic.es (M.L. Sanz), javier.moreno@csic.es (F.J. Moreno).

http://dx.doi.org/10.1016/j.chroma.2015.07.096
0021-9673/

technique for the analysis of protein digests, however, the low retention and poor selectivity for polar analytes, such as glyco- and phosphopeptides, is a recognised major drawback. In contrast, hydrophilic interaction liquid chromatography (HILIC) in combination with electrospray-tandem mass spectrometry (ESI-MS^2) has evolved during the last decade as a powerful analytical tool in structural glycoproteomics [5–15]. To date, phosphoproteome analysis by HILIC has received less attention, although electrostatic repulsion-hydrophilic interaction liquid chromatography (ERLIC), a subset of HILIC separation which employs charged interactions, has shown to be very efficient for the selective separation of phosphopeptides [3,16–18]. In addition, McNulty and Annan [19] and Singer et al. [20] used amide, and cross-linked diol or polyamine HILIC stationary phases, respectively, for the efficient separation of phosphopeptides.

Considering that there is a wide variety of HILIC stationary phase surface chemistries commercially available, and that the retention mechanism of HILIC is still under debate [21,22], a comparison study of different HILIC columns addressed to the characterization of phosphopeptides and glycopeptides could contribute to gain a better understanding of the relationship between the separation mechanism of PTM peptides and the physicochemical characteristics of the used HILIC stationary phases. Therefore, in this work three different HILIC stationary phases (i.e., ethylene bridged hybrid (BEH) amide, polyhydroxyethyl aspartamide (PHEA), and zwitterionic sulfobetaine (ZIC)) and a reverse phase (octadecylsilane (C_{18})) stationary phase have been employed to comparatively study the separation of phosphopeptides and *O*-glycopeptides derived from proteolytically digested bovine caseinomacropeptide (CMP). CMP has been chosen because it is a multifunctional 7 kDa peptide, consisting of a heterogeneous group of acidic peptides due to extensive PTMs, with important food and pharmaceutical applications [23]. As a consequence, a comprehensive characterization of PTM peptides without any previous derivatization or enrichment step has been attempted with the four studied LC columns by electrospray ionization-tandem mass spectrometry using a quadrupole time-of-flight analyzer (LC-ESI-QTOF).

2. Materials and methods

2.1. Materials

All chemicals were of analytical grade unless otherwise stated. Ultrapure water (18.2 MΩ cm, with levels of 1–5 ng mL^{-1} total organic carbon and <0.001 EU mL^{-1} pyrogen) produced in-house with a laboratory water purification system (Milli-Q Synthesis A10, Millipore, Billerica, MA, USA) was used throughout. Acetonitrile of LC–MS CHROMASOLV® grade, formic acid and ammonium hydroxide were from Sigma–Aldrich (St. Louis, MO). Ammonium acetate was from Panreac (Barcelona, Spain).

2.2. Preparation of hydrolyzed CMP

Mixture of CMP A and B variants (Davisco Foods International, Le Sueur, MN, USA) was subjected to a combined trypsin/chymotrypsin proteolysis (overnight at 37 °C and pH 7) at 1:0.05:0.025 CMP:trypsin:chymotrypsin ratios (w:w:w) [11]. Enzymatic activities of porcine pancreas trypsin (EC 3.4.21.4, type IX-S) and bovine pancreas α-chymotrypsin (EC 3.4.21.1, Type I-S) were 13,000–20,000 U and ≥40 U/mg of protein, respectively.

2.3. Preparation of model glycosylated peptides

Aliquots of a solution consisting of 0.25 mg mL^{-1} of three different model peptides, that is acetyl-Ser-Asp-Lys-Pro (Sigma), acetyl-Lys-Ala-Ala (Sigma) and Met-Gly-Met-Met (Sigma), and 0.25 mg mL^{-1} of the following and individual carbohydrates: (i) galactose, (ii) lactose, (iii) maltopentaose, (iv) Neu5Acα2-3Galβ1-4Glc, v) Neu5Acα2-6Galβ1-4Glc, and (vi) Neu5Acα2-6Galβ1-4GlcNAcβ1-3Galβ1-4Glc in 10 mM ammonium acetate pH 6.8, were lyophilized. These were kept under vacuum in a desiccator at 40 °C for 1 day (for galactose) and 3 days (for the rest of carbohydrates) at water activity of 0.44 achieved with a saturated K_2CO_3 solution [11].

2.4. Chromatographic separation by LC-ESI-QTOF

Chromatographic analysis were carried out using four different columns: (i) Ethylene bridge hybrid with trifunctionally-bonded amide phase (XBridge amide column; 150 mm × 4.6 mm; 3.5 μm particle size, 135 Å pore size, Waters, Hertfordshire, UK); (ii) Polyhydroxyethyl aspartamide stationary phase (PolyHydroxyethyl-A (PHEA) column; 100 mm × 2.1 mm; 3 μm particle size, 300 Å pore size, The Nest Group, Inc., Southborough, MA); (iii) Sulfoalkylbetaine zwitterionic stationary phase (ZIC®-HILIC column; 150 mm × 2.1 mm, 3.5 μm particle size, 200 Å pore size, SeQuant™, Umea, Sweden) and (iv) octadecylsilane stationary phase (Hypersil HyPurity C_{18} column; 100 mm × 2.1 mm, 3 μm particle size, 190 Å pore size, Thermo Fisher Scientific).

Samples (20 μL) were analyzed using an Agilent 1200 Series LC system (equipped with a binary pump, an autosampler and a column oven) coupled to a 6520 quadrupole time-of-flight mass spectrometer (QTOF) using an ESI interface working in the positive ion mode. All instruments were from Agilent Technologies (Santa Clara, CA). The electrospray source parameters were adjusted as follows: spray voltage 4.5 kV, drying gas temperature 300 °C, drying gas flow rate 6 L min^{-1}, nebulizer gas pressure 30 psi, and fragmentor voltage 150 V. Nitrogen (99.5% purity) was used as drying and nebulizer gases, while nitrogen of a higher purity (99.999%) was used as the collision gas. MS^2 spectra were obtained by CID, selecting the target masses and applying collision energies ranging between 30 and 80 eV.

The optimization of the different linear binary gradient programmes of water and acetonitrile with different modifiers (that is, formic acid, ammonium acetate or ammonium hydroxide) at different concentrations is described in Section 3.1 and summarized in Table 1.

Determination of the pH of the hydro-organic solutions ($^{s}_{w}$pH) used as mobile phase was carried out using a pH meter (S40 Seven Multi, Mettler-Toledo International Inc, USA) equipped with a combined glass Ag/AgCl electrode (Mettler-Toledo International Inc, USA), after calibration with 4.01, 7.00 and 9.00 aqueous buffer solutions.

2.5. Data treatment

Data acquisition and processing were done using Agilent Mass Hunter Workstation Acquisition v. B.02.00 software. Initial assignment of observed ions to the corresponding amino acid sequences were based on the known sequence of κ-casein by using the protein database Swiss-Prot and TrEMBL and the tools Peptide Mass and FindPept available at www.expasy.org. Parameters for the search were the following: (i) monoisotopic peptide masses were indicated as $[M+H]^+$ with cysteines treated with nothing; (ii) as enzymes, trypsin/chymotrypsin were chosen; (iii) two missed cleavages were allowed; (iv) peptides with a mass larger than 500 u were displayed; (v) the mass tolerance was kept at 0.5 u; (vi) potential phosphorylation on serine and threonine residues was considered.

In order to confirm the sequence of these assigned peptides, their experimental MS^2 spectra were compared with the