Microchemical Journal 118 (2015) 238–241

Contents lists available at ScienceDirect

Microchemical Journal

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

Peptide labeling with lanthanide-NHS-ester-DOTA investigated by nano-HPLC☆

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ARTICLE INFO

Article history:
Received 31 October 2013
Received in revised form 24 June 2014
Accepted 2 July 2014
Available online 2 August 2014

Keywords:
Lanthanides
Peptide labeling
NHS-DOTA
Nano-IP–RP–HPLC–UV
MALDI–MS

ABSTRACT

Labeling of peptides and proteins using chelators binding metal ions has become a novel approach for quantitative proteomics in recent years. The aim of this work was the optimization of a new method for peptide derivatization with lanthanide labels (holmium, lutetium, and thulium) followed by nano high performance liquid chromatography (nano-HPLC) separation with UV detection. Matrix-assisted laser desorption ionization–mass spectrometry (MALDI MS) was used to confirm the derivatization and to identify the derivatized peptides. Peptides were labeled with the three different lanthanide metals using a bifunctional DOTA-based (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) reagent. The results demonstrate that the derivatization reaction using the chelating reagent DOTA-NHS-ester was effective for single peptides and peptide mixtures. Furthermore, an efficient pre-cleaning method was applied by nano-HPLC using a C-18 trap column for elimination of the excess of labeling reagent. The application of the optimized method to label peptides in a Cytochrome C digest delivered comparable results to those obtained with model peptides.

1. Introduction

The proteome of an organism, a cell or a body fluid, is defined as the protein population present at a certain time and under defined conditions [1]. In contrast to the genome, the proteome is highly dynamic, since proteins are constantly synthesized and degraded in the biological environment. Therefore, the composition of a given proteome can be altered depending on the biological state of the organism [2]. Thus, protein identification as well as relative or absolute quantification of a given protein in different biological conditions, i.e. healthy and diseased states [3], and the identification of post-translational changes are the basis for understanding the fundamental processes that occur in living organisms [4].

The development of new analytical–chemical strategies for protein analysis is important for progress in proteomics [5]. Besides protein identification, their relative or absolute quantification is in the center of interest. Quantification methods have been developed to enable large scale protein analysis and the investigation of relative protein expression by applying radioactive isotopes [6,7], fluorescent tags [8] and light/heavy stable isotopes in protein labeling. Techniques, such as SILAC, ICAT, ICPL and isobaric labeling, have been widely used to compare protein abundance levels. This allows, for example, the relative quantification of a single protein in differently labeled samples. Spiking unlabeled samples with known concentrations of isotopically labeled synthetic peptides was used with success for absolute peptide quantifications [9–11]. In general, the chemical derivatization of functional groups in biomolecules by different kind of labels (e.g. radioactive and stable isotopes, fluorescent tags or metals) is a widely used approach in bioanalysis in order to allow the sensitive and specific detection of the target analytes [12,13].

A recent labeling approach employs metals, especially lanthanides for peptide and protein quantification. An advantage is that in addition to conventional LC-MS methods, LC-ICPMS can be employed for specific metal detection and quantification [14]. In this context, lanthanides show high ionization efficiency and low background signals and thus high sensitivity in ICPMS. Due to the impossibility of direct covalent bonding between proteins and lanthanides, complexes with metal chelating agents, i.e., DTPA or DOTA derivatives, are used as a link to protein functional groups like the amine or sulfhydryl groups in peptides and proteins [15–17].

Lanthanide-DOTA labels have been recently applied to the absolute quantification of proteins by flow injection (FIA)–ICPMS [18]. HPLC–MALDI MS and HPLC–ICPMS were used to analyze lanthanide labeled peptides [19]. However, it is important to optimize the labeling procedure and HPLC separation prior to mass spectrometry, including the removal of the excess labeling reagent [19]. HPLC with UV detection

☆ Paper presented at the Brazilian Congress on Analytical Chemistry.
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http://dx.doi.org/10.1016/j.microc.2014.07.002
0026-265X/

is an excellent alternative avoiding the use of the high costly ICPMS and MALDI MS instruments during method development and optimization. Moreover, the use of miniaturized LC systems such as nano-HPLC enables the analyses of small sample volumes on the low microliter level and thus the consumption of the high costly lanthanide labels is considerably reduced.

The aim of this work was the optimization of a new method for peptide derivatization with lanthanide labels (holmium, lutetium, and thulium) followed by nano high performance liquid chromatography (nano-HPLC) separation with UV detection. Matrix-assisted laser desorption ionization–mass spectrometry (MALDI MS) was used to confirm the derivatization and to identify the derivatized peptides. Peptides were labeled with the three different lanthanide metals using a bifunctional DOTA-based (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) reagent. Holmium, lutetium, and thulium were chosen because they form thermodynamically and kinetically stable complexes with DOTA. Moreover, they are monoisotopic, with the exception of Lu, that has only two isotopes (^{175}Lu/^{176}Lu, natural abundance of 97.4 and 2.6%, respectively), which simplifies the isotopic pattern of the labeled peptides in MALDI MS [20].

2. Experimental

2.1. Chemicals

The chelating agent DOTA-NHS-ester was obtained from CheMatech (Dijon, France). Model peptides were synthesized by standard Fmoc solid-phase peptide synthesis (R. Pipkorn, DKFZ, Heidelberg, Germany). Sequences included peptide T1: VKCFNCGK, S34: CCTKPESER, S35: VLASSAR, and S36: GLACLLPK. All standard proteins, tris(2-carboxyethyl)phosphine (TCEP), triethylammonium bicarbonate buffer (TEAB), triethylammonium acetate buffer (TEAA), trifluoracetic acid (TFA), S-methyl methanethiosulfonate (MMTS), dimethyl sulfoxide (DMSO), acetonitrile (ACN) and the lanthanide(III) chloride hexahydrate salts (holmium, lutetium, thulium), Glu1-fibrinopeptide B and α-cyano-4-hydroxycinnamic acid (CHCA) were purchased from Sigma-Aldrich (Taufkirchen, Germany). Deionized water (18.2 MΩ cm) was prepared with a Millipore Milli-Q water purification system Advantage A10 (Merck, Molsheim, France). Cytochrome C was obtained from Dionex (Amsterdam, The Netherlands).

2.2. Sample preparation

Derivatization of the standard peptides and their mixture labeled with DOTA was carried out according to the procedure proposed by Gregorius et al. [21]. In order to demonstrate the applicability of the metal-labeling strategy and peptide identification, we also tested the procedure on a Cytochrome C digest.

2.2.1. Labeling procedure

Prior to derivatization, a reducing step for 1 h at 60 °C using a TCEP molar excess of 3 times per cysteine residue was performed on all samples [21]. After this, free cysteine residues were alkylated with a 5 times molar excess of MMTS per SH group.

All samples were derivatized for 1 h in 75% ACN, 25% 100 mM HEPES at pH 7.5 with a 100 times molar excess of DOTA-NHS-ester (dissolved in a 10 mM DMSO water free solution) for each free amino group. This means that, besides the N-terminus, all the peptides were also derivatized at the e-amino groups of lysine (K). Subsequently, 10 mM stock solutions of holmium, thulium, and lutetium were diluted in 100 mM TEAA buffer (pH 5) to final concentrations of 100 nM. Complexation of the DOTA-NHS labeled peptides with lanthanides was performed by addition of a 10 times molar excess of the particular lanthanide metal ion compared to the DOTA-NHS-ester. Complexation was performed for 1 h at room temperature. The final concentration of each peptide standard solution and the peptide mixture were 5 μmol L^{-1} and 15 μmol L^{-1}, respectively. All treatments performed for peptides were also performed for the Cytochrome C digest.

2.3. Peptide separation by nano-Ion Pair–Reversed Phase–HPLC (nano-IP–RP–HPLC)

The high excess of labeling reagent and metal ions used for derivatization and complexation delivers an unacceptable high background for subsequent analysis. Therefore, the excess of unbound labeling reagents and metal ions was eliminated by loading the labeled peptides onto a C-18 trap column (Acclaim PepMap100 C18; 5 mm, 0.3 × 10 mm; Dionex, Idstein, Germany) before being eluted onto the analytical column [19]. At this stage, different washing times were tested in order to achieve optimal sample cleanup prior to analysis while possible sample loss was simultaneously minimized. Six minutes was found to be optimal by prior tests performed on the nano-LC [19]. This washing time of the trap column refers to the time that the sample remains on the trap column, while washed with the mobile phase (3% ACN, 0.1% TFA) at a flow rate of 30 μL min^{-1}. After washing procedure, the valve was switched to elute the analytes onto the analytical column. Analytical separation was carried out using an Acclaim PepMap100 C18 separation column (5 μm 75 μm, 150 mm, Dionex, Idstein, Germany). The separation was performed at a flow rate of 0.3 mL min^{-1} in A (0.05% aqueous TFA) and B (80% ACN, 0.04% TFA, 20% deionized water (v/v/v)). A linear gradient of 5% B to 70% B during 20 min was used for separation. After separation, 35 minute wash runs (95% B during 10 min followed by 5% B during 25 min) were conducted between sample injections. UV detection was performed at 214 nm.

2.4. Peptide analysis by MALDI–TOF–MS

The mixture of the four standard peptides (T1, S34, S35, and S36) was separated and spotted onto a MALDI target using a U3000 nano-HPLC system (Dionex, Idstein, Germany) directly coupled to a Probot microfaction collector (LC Packings, Amsterdam, The Netherlands). This step was performed following a previously published protocol [21]. After elution, the peptides were directly mixed with the matrix solution (CHCA, 3 mg mL^{-1} in 70% ACN, 0.1% TFA, 5 nM Glu^1-fibrinopeptide B) at a 1:3 ratio (v/v) and spotted each 20s over a range of 4–65 min. All measurements were performed on a SCIEX AB TOF/TOF 5800 (Applied Biosystems, Darmstadt, Germany), in reflectron positive ion mode.

3. Results and discussion

The efficiency of the derivatization reaction was monitored by analyzing the different forms of the model peptides by MALDI MS after LC separation of a mixture of the four labeled peptides. The monoisotopoic m/z values and the labeling positions in the amino acid sequence of the peptides are presented in Table 1. Further, this Table shows the theoretical and experimental m/z values for the unmodified (FP, free cysteines and Cys modified with MMTS), NHS-DOTA labeled (LP) and metal-labeled peptides (MLP).

Cys residues were protected by modification with MMTS leading to a mass increase of 45.99 Da. Derivatization with DOTA led to the formation of covalent bonds (carboxamide bonds) of the NHS-activated DOTA carboxyl groups with peptide amino groups, transforming FPs into LPs resulting in an increase in mass of 386.19 Da per free amino residue. In a second step, the metal was complexed by coordinative bonds with the ring system present in DOTA, resulting in MLPs.

Non-derivatized peptides (without DOTA) delivered only none or very weak signals (low S/N ratios), indicating for an almost complete derivatization of the peptides with MMTS (derivatization of Cys) and of amino groups with DOTA (Table 1). Further, only very weak signals were observed for metal free DOTA-derivatized peptides, indicating the previously reported high affinity of DOTA derivatized peptides for the lanthanide ions [21].