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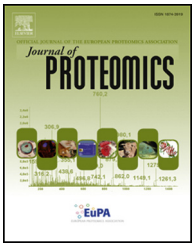

A synthesis of new, bi-labeled peptides for quantitative proteomics

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ABSTRACT

Isotopically labeled peptides are often used in proteomics as internal reference allowing quantification of peptides by isotopic dilution method. Although the synthesis of peptides labeled with stable isotopes is relatively simple, there are several factors limiting application of these standards in proteomic research: cost of labeled derivatives of amino acids, time needed to obtain labeled peptide and problems with quantification of the standard. To solve these problems we developed a method of synthesis of peptides labeled with heavy oxygen and with a dabsyl moiety. The chromophoric group facilitates the determination of peptide concentration while sequence of peptide allows enzymatic cleavage of fragment containing dabsyl from peptide leaving "natural" sequence with incorporated ^{18}O atoms. The approach proposed herein is based on the "analytical construct" concept. The experiments performed on model peptides demonstrated that response factors in HPLC analysis of labeled peptides does not depend on the sequence and tryptic hydrolysis of obtained conjugates is completed in minutes producing labeled standards useful in quantitative proteomics.

Biological significance
The reported method allows for a cheap and efficient synthesis of peptides labeled with heavy isotopes, and for their precise quantification. Peptides of our design are stable, and the isotopic label, which is a part of the peptide backbone, is stable as well. Moreover, they can be quickly quantified in solution at any time, so the possible decomposition of standard or a non-uniform distribution of the peptide in lyophylisate does not pose a problem. Therefore, we deem our synthesis to be useful for a broad range of quantitative proteomics methods. In addition, the procedure described herein allows direct application of crude peptides as the analytical standards. The elimination of expensive and time-consuming chromatographic purification reduces the cost of AQUA peptides and gives the possibility of a rapid preparation of large libraries of proteolytic fragments.

Abbreviations: Dab, dabsyl moiety; TCTU, *O*-(6-chlorobenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate, Fmoc, fluorenylomethyloxycarbonyl group; DIEA, diisopropylethylamine; SPPS, solid-phase peptide synthesis.

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1. Introduction

Stable isotope labeling of proteins and peptides is useful in quantitative proteomics [1]. Such peptides can be used as internal references when assessing the concentration of a sample by mass spectrometry, for example, using multiple reaction monitoring (MRM) method [2,3]. The following stable isotopes: ^{2}H, ^{13}C, ^{15}N, and ^{18}O are often used for this purpose [1,4,5]. Labeling with ^{18}O has several significant advantages over the other isotopes. Every introduced atom increases the mass by 2 and incorporation of ^{18}O in vitro is easier than ^{13}C or ^{15}N. On the other hand, unlike deuterium, heavy oxygen does not induce a change in the retention time [6]. Several protocols for ^{18}O labeling in vitro have been published. They can be divided into two main categories: enzymatic [7,8] and acid-catalyzed [9–11]. Isotopically labeled peptides may be also obtained using commercially available amino acid derivatives containing ^{13}C or ^{15}N atoms. Therefore, from synthetic point of view, the preparation of labeled peptides does not pose a problem.

However, there still remains the problem of precise determination of the quantity of the standard especially if only a limited amount of sample is available. In case of peptides, it is not possible to determine the amount of the peptide by simply weighting it, as the lyophylisate contains counter ions and possibly also a large amount of solvent [12]. Moreover, the peptides in question usually need to be purified, thus increasing the cost of the analysis, and their concentration can change over time due to decomposition, introducing an additional source of error [13]. Even if the concentration in the dry product is determined, sometimes complete dissolution of the peptide might not be possible, and therefore the concentration in solution does not correspond to that calculated for the amount of peptide used.

A quantification of peptides is often performed by the method of amino acid analysis, which has been described as the “golden standard” [12]. However, the error of this method sometimes exceeds 10% [14]. In addition, amino acid analysis requires a relatively large sample of pure compound. The synthesis of labeled substances at a large scale is expensive, so the quantification of the standards by amino acid analysis can be prohibitively expensive. On the other hand, milligram quantities of labeled peptide may be sufficient to perform thousands of analyzes. It is clearly visible that a new analytical method for absolute quantification of peptides in solution is desired.

We have, therefore, decided to develop an approach based on the concept of analytical construct [15,16]. This term was introduced by Geysen et al. [17]. The strategy relies on two orthogonal linkers within the synthesized compound: one links the proper compound to an “analytical enhancer,” the other one links the whole compound to the resin. Such constructs have found their use mainly in the analysis combinatorial libraries when each of many compounds was synthesized in a very small amount. Therefore, the construct contained an analytical enhancer, for example, a chromophore group for UV detection, or a quaternary ammonium salt for MS analysis. Depending on the cleavage conditions, either the whole construct, containing the enhancer, could be cleaved, or just the desired compound without the enhancer.

The general structure of analytical constructs described herein is depicted in Fig. 1. They are composed of the following elements: the sequence corresponding to the tryptic peptide, cleavable linker and chromophore. The desired peptide, which has the same sequence as a peptide derived from a protein of interest during tryptic digestion, is attached directly to a Wang resin. This part is labeled with heavy oxygen. Then at the N-terminus of this part, a linker containing a cleavage site for trypsin is attached, followed by dabsyl moiety at its N-terminus. The idea of the experiment is also shown in Fig. 1. Briefly, the synthesized peptide, labeled with ^{18}O, is quantified by HPLC, mixed with the protein solution and subjected to enzymatic hydrolysis. As dabsyl moiety is cleaved together with the linker, a shorter isotopically labeled peptide, the analytical standard, is liberated. The whole mixture is then subjected to LC-MS analysis.

2. Experimental section

2.1. Materials and reagents

1,4-Dioxane was purchased from POCh (Gliwice, Poland), concentrated sulfuric acid (96% H_2SO_4) from Stanlab (Lublin, Poland), sodium chloride from Chempur (Piekary Śląskie, Poland), TFA from Merck (Darmstadt, Germany) and Fmoc-protected amino acids and preloaded Wang resins (0.54–0.77 mmol/g) from NovaBiochem (Merck, Darmstadt, Germany). All the remaining chemicals, including ^{18}O labeled water (97% ^{18}O), were purchased from Sigma-Aldrich (St. Louis, MO, USA). Water was purified using a Hydrolab purification system (Hydrolab, Poland).

2.2. Peptide synthesis

Safety warning: as coupling reagents are toxic and potentially carcinogenic, gloves and protective glasses must be worn for peptide synthesis. Peptides were synthesized by standard SPPS Fmoc strategy on Wang resin, as described [18]. The synthesis was manually performed in polypropylene syringe reactors (Intavis AG) equipped with polyethylene filters. The coupling reactions were performed in DMF, with TCTU (3 Eq) in presence of DIEA (6 Eq). The Fmoc protecting groups were removed with 25% piperidine solution in DMF. The sequences chosen were model peptides basing on sequences of tryptic peptides of human serum albumin (HSA) and fibrinogen.

2.3. Fmoc-amino acid labeling procedure

The conditions for these reactions have been published previously [19]. Briefly, 5 mg of the Fmoc-protected amino acid derivatives were dissolved in 2 M HCl in dioxane, with 5% $H_2^{18}O$ and either left overnight, or treated with microwaves (110 °C) for 15 min. The solvent was then evaporated in a stream of dry nitrogen. Then the protected amino acids were used directly for regular solid-phase peptide synthesis with no purification.

2.4. Chromophore tagging

Dabsyl: 2 Eq of dabsyl chloride was dissolved in 2 ml of DMF, and 4 Eq of DIEA was added. The mixture was added to a