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Microwave-assisted ^{18}O labeling of Fmoc-protected amino acids

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Recently, there has been an increased interest in isotopical labeling of peptides. Although there are several techniques allowing for a complete labeling of all carboxyl groups in peptides, regioselective labeling would be beneficial in many situations. Such labeling requires the use of ^{18}O-labeled Fmoc amino acids. We have designed a method for such labeling that is an improvement on a technique proposed earlier. The new procedure is suitable for microscale synthesis and could be used in peptide and proteomics laboratories. Although for the majority of tested amino acids our method gives good labeling efficiency, it is time consuming. Therefore, we have decided to use microwave-assisted procedure. This approach resulted in reduction of reaction time to 15 min and increased reaction efficiency. Copyright © 2014 European Peptide Society and John Wiley & Sons, Ltd.

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Keywords: ^{18}O labeling; quantitative proteomics; peptide synthesis; stable isotopes; microwave-assisted labeling

Introduction

Peptides labeled with stable isotopes are a useful research tool in biochemistry, molecular biology, and analytical chemistry. They can be used as internal standards for quantitative mass spectrometry (MS) analysis of peptides that makes them useful in proteomics [1–3]. The labeling of peptides also influences the nuclear magnetic resonance, MS, and infrared spectra, which helps in studies on peptide structure, interactions (both intermolecular and intramolecular), and fragmentation pathways [4–7]. Such approach can be also relevant to protein analysis. The labeling with ^{18}O is convenient and often used in proteomic research because the heavy oxygen atom can be directly incorporated into a peptide molecule [8]. Currently, there are many protocols for labeling peptides with ^{18}O, both by acid catalysis and by treatment with proteolytic enzymes in $H_2^{18}O$, as well as for labeling of carboxylic acids, for example, iodoacetic acid used for tagging cysteine containing peptides [9–13]. Some of those methods require harsh conditions or long reaction times; therefore, they are of limited use as standard laboratory procedure. Moreover, they offer only limited selectivity in distribution of the heavy oxygen. A direct peptide labeling can affect either the C-terminal carboxyl group or side chain carboxyl groups or all of them. A method of synthesis of labeled building blocks for selective incorporation of ^{18}O during peptide synthesis has been developed by Marecek *et al.* [14]. It involves the use of HCl in $H_2^{18}O$:1,4-dioxane solution, with 20 ml of labeled water for 3–5 g of Fmoc amino acid. The synthesis is carried out under reflux, and the products are isolated by evaporation of the reaction medium. This procedure requires significant reaction times and in some cases repetition of equilibration, particularly for isoleucine (34 h for each equilibration). However, for solid-phase synthesis of labeled peptide standards for MS, a quick small-scale procedure for preparation of protected amino acids might be useful.

Microwave-assisted synthesis is a well-known way of increasing the rates of organic reactions. It is frequently used in peptide synthesis both for coupling reactions and deprotection and cleavage [15,16].

Taking this into account, we have designed a new method suitable for preparation of small portions of labeled Fmoc amino acids, which can then be easily incorporated into peptides according to the standard Fmoc strategy. The application of a microwave synthesizer reduced the reaction time to 15 min. To the best of our knowledge, this is the first efficient microwave-assisted method of ^{18}O labeling, applicable for milligram quantities of Fmoc derivatives, consuming 20 μl of $H_2^{18}O$ per 5 mg of material.

Materials and Methods

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). $H_2^{18}O$ (97% ^{18}O), DABSYL chloride, LCMS grade acetonitrile, and metallic sodium were bought from Sigma-Aldrich (St Louis, MO, USA). TFA was bought from Merck (Darmstadt, Germany) and Fmoc-protected amino acids and preloaded Wang resins from NovaBiochem (Merck, Darmstadt, Germany). Water was purified using a Hydrolab purification system (Hydrolab, Poland).

Fmoc Amino Acid Labeling

Fmoc amino acid samples (5 mg) were placed in 2 ml ampoules. An amount of 2 M HCl in dioxane (380 μl, prepared earlier by saturating

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1,4-dioxane with dry HCl) was added, followed by $H_2^{18}O$ (97% ^{18}O) (20 μl). The ampoules were flame sealed and left rotating for 24 h at room temperature. The solvent was then evaporated in a stream of dry nitrogen. The product was analyzed by high-performance liquid chromatography (HPLC) and MS (data presented in supporting material) and used for peptide synthesis without further purification.

Microwave-assisted Labeling

An ampoule, prepared as described in Results and Discussion, was placed in a microwave vial partially filled with water acting as sheath liquid. The CEM Discover synthesizer (Matthews, NC, USA) equipped with gas cooling system and magnetic stirring was operated in standard mode, in which the temperature and time of irradiation are set, and the synthesizer adjusts the power to maintain the temperature. The temperature was set to 110 °C, and the reaction times were set to 15, 30, and 60 min. The sample was then evaporated to dryness in a stream of dry nitrogen and analyzed by HPLC and MS (data presented in supporting material).

Peptide Synthesis

The synthesis was carried out in syringe reactors (Intavis, Koeln, Germany) according to standard Fmoc strategy [17]. For each synthesis, 5 mg of Wang resin loaded with corresponding Fmoc amino acid (0.55–0.65 mmol/g) was used. In each coupling step, four equivalents of unlabeled Fmoc amino acids were used with TCTU as coupling agent. In the case of ^{18}O-labeled derivatives, only two equivalents (from 2 to 3 mg) were used to reduce the cost of synthesis. The peptides were then cleaved with TFA:H_2O mixture (95 : 5, v:v), lyophylised, and analyzed by HPLC and MS (exemplary data presented in supporting material).

MS Analysis

Mass spectra were recorded on a Bruker micrOTOF-Q mass spectrometer and apex ultra 7 T FT-ICR mass spectrometer (Bremen, Germany) equipped with electrospray ion source in the Laboratory of Mass Spectrometry at the Faculty of Chemistry, University of Wrocław. The samples were dissolved in a 50 : 50 acetonitrile–water mixture containing 0.1% HCOOH

HPLC Analysis

The analysis was performed on Dionex chromatography system, using a Jupiter Proteo column 90A, 250 × 4.6 mm, 4 μm (Phenomenex, Torrance, CA, USA). The eluents were as follows: (A) 0.1% TFA in water and (B) 0.1% TFA in acetonitrile:water (80 : 20, v:v). The gradient was from 0% to 80% B in A in 40 min, flow rate 1 ml/min. Dionex variable wavelength detector operating at 210, 254, and 277 nm was used. In the case of DABSYL-tagged samples, 210, 254, and 473 nm absorption were analyzed. In order to observe all the possible products, the data registration was extended to include the column regeneration and re-equilibration.

Results and Discussion

We studied the ^{18}O incorporation to standard Fmoc amino acid derivatives using a solution of HCl in dioxane to catalyze the isotope exchange reaction. These reaction conditions excluded from our experiments the derivatives containing acid-labile side chain protecting groups (Boc and Trt). For this reason, we have chosen the following amino acids: glycine, alanine, phenylalanine, valine, leucine, isoleucine, proline, and methionine. Other amino acids, if necessary, can be labeled by our method by using different protecting groups or introducing the acid-labile protecting group after labeling.

The labeling procedure without microwave assistance required 24 h at room temperature. Some of Fmoc derivatives (notably glycine) did not dissolve completely despite vigorous mixing, which could have impaired the labeling efficiency. After 24 h, the ampoules were opened, and their contents evaporated in a stream of dry nitrogen and subsequently analyzed by HPLC and MS. No degradation of Fmoc derivatives was observed; the obtained products were practically pure according to HPLC chromatograms. We also verified that no deprotection occurred using the ninhydrin reaction. However, the efficiency of labeling – determined by comparing the intensities of the peaks corresponding to unlabeled, monolabeled, and bilabeled species in the mass spectra – was not uniform. The 24 h incubation at room temperature resulted in 85–90% labeling for some of the amino acids (alanine, phenylalanine, and leucine); whereas for valine and isoleucine, the exchange was 45% and 27%, respectively. The difficulty of isoleucine labeling has been reported in literature [14] and attributed to steric hindrance. The results are summarized in Table 1.

In order to improve the efficiency and shorten the reaction time, we have decided to use a CEM microwave synthesizer. As the volume of our sample was low (less than 0.5 ml), we had to use a sheath liquid to fill the vial and act as thermal buffer for better control of microwave energy. In standard operation mode, the CEM synthesizer adjusts the microwave energy to maintain the specified temperature. A small volume of the reaction medium in a typical 10 ml reaction vessel may lead to complete evaporation of the sample; therefore, we developed a method of vial insertion [7] Water was chosen as the sheath liquid because of its high specific heat and boiling point similar to that of dioxane (the picture of reaction vessel containing sealed ampoule and water is presented in supplementary material). As expected, reaction in a microwave reactor proceeded rapidly. The maximum efficiency (over 85% for all examined Fmoc amino acids) was obtained in 15 min – extension of the reaction time (to 30 and 60 min) did not affect the labeling yield. With the exception of methionine, the protected amino acids did not undergo any noticeable decomposition, as verified by HPLC and negative ninhydrin reaction. Moreover, no amino acid repetitions were observed during peptide synthesis. Only in the case of methionine that a significant

Table 1. Labeling efficiency of obtained Fmoc-protected amino acids calculated by comparing the intensity of the peaks corresponding with unlabeled, monolabeled, and bilabeled species in mass spectra

Amino acid	Labeling efficiency – regular method (%)	Labeling efficiency – microwave-assisted method (%)
Ala	90	90
Phe	86	91
Gly	65	92
Ile	27	88
Leu	88	92
Val	45	88
Pro	70	92
Met	84	86[a]

[a]Partial decomposition observed.

decomposition was revealed as two chromatographic peaks of roughly equal intensities. However, methionine is known to decompose under acidic peptide hydrolysis conditions [18]. The results are summarized in Table 1, and representative results for valine are shown in Figure 1.

We have also synthesized a series of peptides using the labeled amino acids directly after the exchange procedure. The sequences of model peptides were selected from proteolytic digests of various proteins, investigated in our research team [19]. Fmoc-AKAAF is a model peptide, containing phenylalanine to facilitate ultraviolet (UV) detection, whereas Fmoc-VGSK and Fmoc-QIK are tryptic fragments of human serum albumin (HSA), dabsyl-AKADLAK, prepared for UV–visible studies, contains ADLAK – a tryptic fragment of HSA; Fmoc-GVFR and Fmoc-IETMR are tryptic fragments of HSA propeptide and of bovine serum albumin, respectively. To increase the sensitivity of UV–visible detection, the peptides were treated with dabsyl chloride or left with N-terminal Fmoc group. The synthesis was carried out on solid support, according to a standard Fmoc protocol, with four equivalents of regular Fmoc derivatives and only two equivalents of the labeled derivatives, in order to reduce the cost of the synthesis. Each labeled amino acid used in peptide synthesis brings a two units shift to the final peptide mass, as could be seen in Figures 2 and 3. According to MS data, the incorporation of the ^{18}O label into the peptides was nearly quantitative. The location of the heavy oxygen was verified by MS/MS method. It showed unambiguously that the ^{18}O stays in the carbonyl group of the labeled amino acid.

To summarize, we have developed an improved scale-down procedure of Fmoc amino acid labeling protocol, which gives good results for almost all tested Fmoc-protected amino acids. Our method is suitable for microscale synthesis (3–5 mg of amino acid derivative) using commercially available reagents. We have used 20 μl of $H_2^{18}O$ to obtain 5 mg of labeled amino acid, which is comparable with the consumption described by Marecek *et al*. [14]; however, our method is much faster and practically quantitative, which is especially important in case of small amounts of special Fmoc derivatives. Moreover, our method does not require a reflux setup, so the whole apparatus is less complex and less prone to contamination with atmospheric water. We have also highlighted that microwaves significantly accelerate and increase the yield of the reaction. The method is effective for all the examined amino acids except for methionine, which undergoes decomposition. Although the

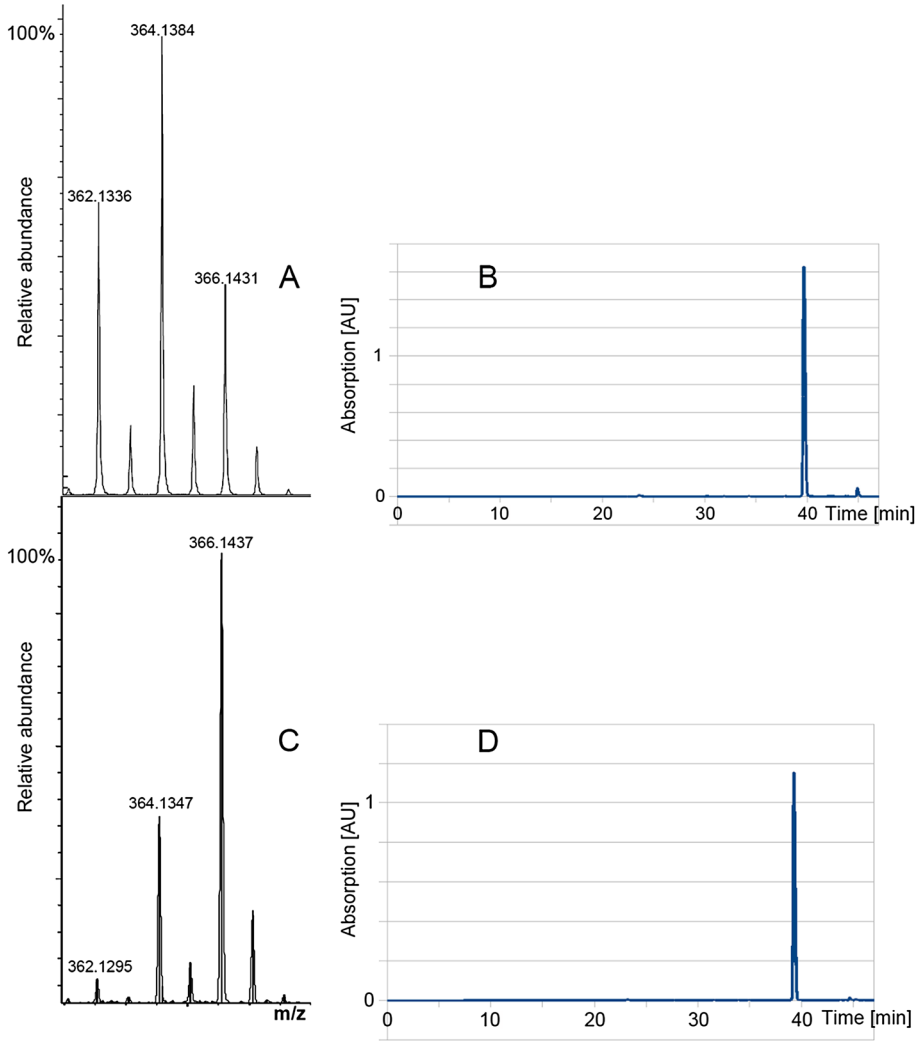

Figure 1. Fmoc valine-labeled without (panels A and B) and with microwave assistance (panels C and D). MS spectra (panels A and C) and chromatograms registered at 254 nm (panels B and D). The peaks in panel A at 362.1336, 364.1384, and 366.1431 correspond to $[M + Na]^+$ ions with no, 1 and 2 heavy oxygen atoms, respectively. In panel B, peaks at 362.1295, 364.1347, and 366.1437 correspond to $[M + Na]^+$ ions with no, 1 and 2 heavy oxygen atoms, respectively.

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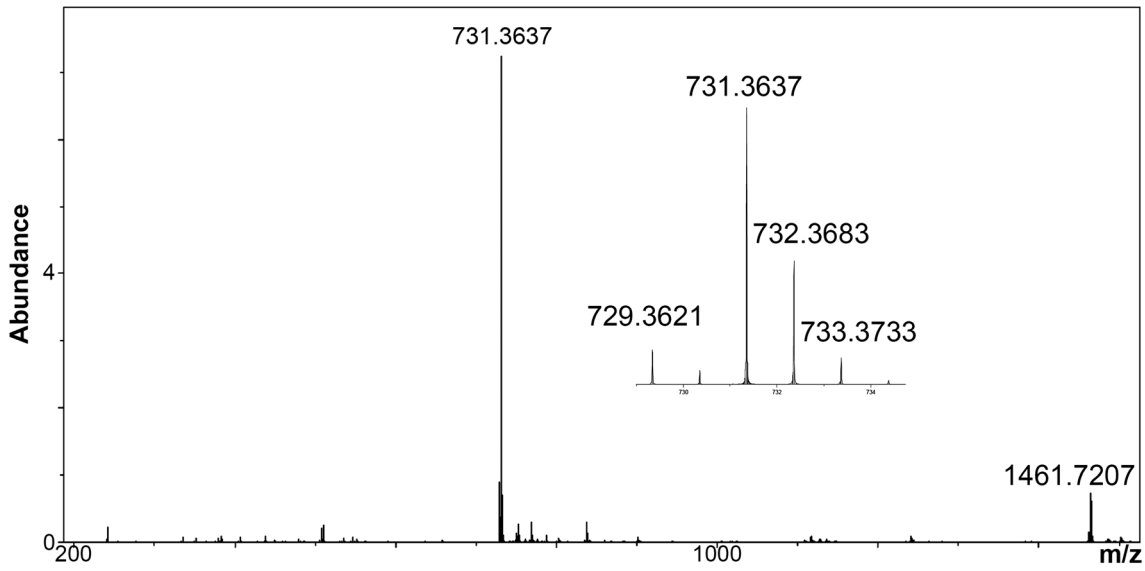

Figure 2. MS spectrum of Fmoc-AKAAF synthesized with one alanine labeled without microwave assistance. The region of peak at 731.3673 (corresponding to $[M+H]^+$ ion) was enlarged to show the isotopic pattern. The peak at 1461.7207 corresponds to a noncovalent dimer.

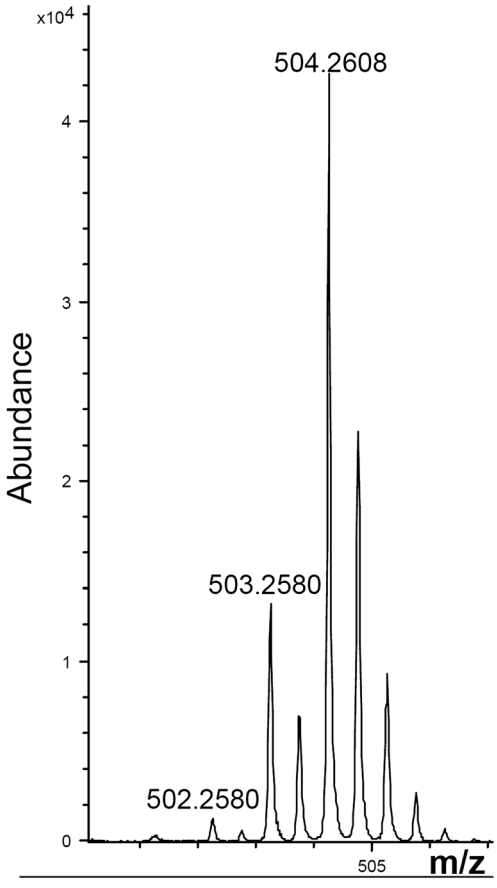

Figure 3. Mass spectrum of DABSYL-AKADLAK synthesized with two alanines labeled with microwave assistance. The peaks at 502.2580, 503.2581, and 504.2608 correspond $[M+2H]^{+2}$ ions with to no, 1 and 2 heavy oxygen atoms, respectively.

microwave-assisted procedure is fast and efficient, a more time-consuming microwave-free approach is also available for most of the tested amino acid derivatives. The proposed methods enable a cost-efficient synthesis of peptides permanently labeled with stable isotope as standards for quantitative proteomics.

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