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Development of continuous microwave-assisted protein digestion with immobilized enzyme

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ABSTRACT

In this study, an easy and efficiency protein digestion method called continuous microwave-assisted protein digestion (cMAED) with immobilized enzyme was developed and applied for proteome analysis by LC–MS^n. Continuous microwave power outputting was specially designed and applied. Trypsin and bromelain were immobilized onto magnetic micropheres. To evaluate the method of cMAED, bovine serum albumin (BSA) and protein extracted from ginkgo nuts were used as model and real protein sample to verify the digestion efficiency of cMAED. Several conditions including continuous microwave power, the ratio of immobilized trypsin/BSA were optimized according to the analysis of peptide fragments by Tricine SDS–PAGE and LC–MS^n. Subsequently, the ginkgo protein was digested with the protocols of cMAED, MAED and conventional heating enzymatic digestion (HED) respectively and the LC–MS^n profiles of the hydrolysate was compared. Results showed that cMAED combined with immobilized enzyme was a fast and efficient digestion method for protein digestion and microwave power tentatively affected the peptide producing. The cMAED method will be expanded for large-scale preparation of bioactive peptides and peptide analysis in biological and clinical research.

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1. Introduction

During the last decades, number of peptides with interesting pharmaceutical activities have been found and attracted a great deal of attention because of their potential effects in promoting health and reducing disease risk [1]. These bioactive peptides could be produced by in vitro enzymatic digestion of natural resources [2]. However, a suitable method for the production of bioactive peptides with specific functional properties and desired molecular size characteristics is still a challenge [3]. Applying the conventional heating enzymatic digestion (HED) methods [4] for protein digestion often result in enzyme autodigestion, sample loss and time-consuming. Furthermore the enzyme in products would affect the results of the peptides identification and bring difficulty in purify the peptides product [5]. Recently, due to the advantages of high enzyme concentration in limited space, short digestion time and low risk for enzyme autolysis, immobilized enzyme has been widely diverted on and utilized [6], especially in proteomic research [7]. However, the purpose of proteomics is to comprehensively elucidate biological processes by systematically analyzing the proteins expressed in a cell or tissue [8]. Therefore, digested with immobilized enzyme reactor [9] or chip [10] could efficiently produce peptides, and a very small amount of peptides products was enough to be identified because of the speed, accuracy, selectivity, and sensitivity of mass spectrometry (MS) for analyzing the digestion products of proteins. But when it comes to bioactive peptides preparation, fast and efficient methods still need to be further researched and improved. In decades, microwave was applied in many fields [11] and microwave-assisted enzymatic digestion (MAED) would be a potential method to accelerate the process of digestion [12].

Recently, researches [13–15] have conformed that MAED could make the digestion process complete in a few minutes contrasting several hours under conditions of HED. The accelerating digestion ability of microwaves probably attributes to rotation of the bipolar molecules and oscillatory migration of the ionic components of the proteins after absorbing microwave energy [16]. Because microwave has ability to heat the sample in a short time, controlling temperature has become an inevitable problem. In Lin et al.'s work [17], they measured the final temperature of digestion solution with a thermocouple immediately. To avoid this shortcoming, a non-contact infrared continuous feedback temperature system [13,18] was used and short digestion time [7,14,19] (less than 1 min) or low microwave power (1–20 W) was proposed to avoid the denaturation of protein at elevated temperature [20].

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To better understand the assistance capabilities of microwave to the enzyme and protein in the process of microwave-assisted digestion, and to probe into a way to selective protein digestion for bioactive peptides production, we have designed a continuous microwave-assisted enzymatic digestion (cMAED) device and used it for standard protein and real protein samples enzymatic studies. To maintain the continuous power of microwave in cMAED, an external condensing device was proposed to cold the reaction solution so that the digestion temperature keeps stable. BSA were used as model protein to be digested by immobilized trypsin and protein from ginkgo nuts was digested by bromelain with different protocols such as HED, MAED and cMAED.

2. Experimental

2.1. Materials and chemicals

Trypsin (pig pancreas, 1:250), bovine serum albumin (BSA), dithiothreitol (DTT), iodoacetamide (IAA) and N,N′-methylene bisacrylamide were brought from Aladdin Reagent Co., Ltd. (Shanghai, China). Bromelain was obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

The main reagents for Tricine SDS PAGE such as glycerol, sodium dodecylsulphate (SDS), b-mercaptoethanol, bromophenol blue, acrylamide and N,N-methylene bisacrylamide were acquired from Sinopharm Chemical Reagent Co., Ltd.. Formic acid (FA) and acetonitrile were purchased from Dikma Co., Ltd. (CA, America).

The other reagents were of analytical grade, such as tetraethylortosilicate (TEOS, $Si(OC_2H_5)_4$), amino-propyltrimetoxysilane (APTMS, $Si(OCH_3)_3(C_3H_6NH_2)$), ammonia (25% w/w), formaldehyde, glutaraldehyde ($C_5H_8O_2$, 25% w/v aqueous solution), cetyltrimethyl ammonium bromide (CTAB, $C_{16}H_{33}(CH_3)_3NBr$). Water was purified by an arium® 611 system (Sartorius, Germany) with resistance $\geqslant$18.2 MΩ/cm.

2.2. Microwave instrumentation and cMAED setup

MAS-II Smart Microwave Digestion System (Sineo Microwave Chemistry Technology Co., Ltd., Shanghai, China) was employed for the digestion process. The output power can be changed from 100 W to 900 W at the interval of 100 W. The operating frequency is 2450 MHz. This system has a single-mode cavity design, with temperature feedback control. An infrared sensor, which monitors the temperature of the sample, is located above the reaction vessel. An external condensing device (Low-temperature cooling liquid circulating pump, OLSB5/10, Yuhua Instrument Co., Ltd., Henan, China) was applied to cold the reaction solution for the maintenance of continuous microwave power.

Immobilized trypsin was used for cMAED of BSA. Glutaraldehyde activating Fe_3O_4@mSiO_2@nSiO_2-NH_2 was prepared as our present work [15] described. Briefly, 0.0235 g glutaraldehyde activating Fe_3O_4@mSiO_2@nSiO_2-NH_2 was added into 20 mL 0.3 mg/mL trypsin PBS solution (pH = 7.5) for 2 h in a shaker for trypsin immobilization. At last, the final product was washed with deionized water three times and poured in a solution of 20 mM NH_4HCO_3 and 0.02% sodium azide (w/v) for storing at 4 °C before use.

10 mg BSA was dissolved in 1.0 mL Tris–HCl (pH 8.1, 50 mM) solution containing with 8 M urea, and then reduced in 0.1 mL DTT (100 mM) for 20 min at 50 °C. When cooled to room temperature (about 20 °C), BSA was alkylated in the dark in 0.1 mL IAA (100 mM) for 20 min at room temperature, followed by dilution with 8.8 mL Tris–HCl (pH 8.1, 50 mM) to decrease the concentration of BSA to 1 mg/mL. Finally, 2 mL of BSA (1 mg/mL) was digested in the condition of continuous microwave.

The preparation of ginkgo, the process of immobilizing bromelain and the MAED of ginkgo were as our previous work [15]. Briefly, crude ginkgo powder was extracted from ginkgo nuts with the method of alkali-solution and acid-isolation and 0.0073 g glutaraldehyde activating Fe_3O_4@mSiO_2@nSiO_2-NH_2 was used to immobilize bromelain. 0.5000 g of ginkgo protein was first dispersed in 50 mL of Tris–HCl (pH 8.1, 50 mM) solution and then digested by 2250 U immobilized bromelain under the conditions of cMAED at 55 °C with magnetic stirring. After digestion, the solution was separated by centrifugation (3K30, Sigma, Germany) at 14,000 rpm at 4 °C for 5 min after the immobilized bromelain was removed with external magnetic field. Finally, the digestion solution was stored at −20 °C.

2.3. Analysis of protein digestion solution

After digestion, the separated digestion solution was analyzed by Tricine SDS PAGE and LC–MS^n. A solution of solubilizing buffer (SSB), composed of 0.5 mol/L Tris (pH 6.8), 20% glycerol (v/v), 10% SDS (w/v), 10% b-mercaptoethanol (v/v), and 0.1% bromophenol blue (w/v), was prepared freshly. 2 mL of BSA digestion solution was first concentrated to 0.2 mL with the freeze dryer (FD-1CE, Detianyou Technology Development Co., Ltd., Beijing, China) and then all the concentrated BAS solution was mixed with 0.1 mL of SSB, and the mixtures were incubated at 95 °C for 5 min. The samples were run in discontinuous Tricine SDS–PAGE cast with 4% stacking gel, 10% spacer, and 16.5% separating gel (contenting 8 M urea in our test), as described by Schägger [21,22]. The whole process of electrophoresis was as Schägger et al. [21] described.

For LC–MS^n analysis, Aeris PEPTIDE XB-C18 column (Phenomenex, 4.6 mmi.d × 250 mm, 3.6 μm i.d particle size) was used for peptide separation, with the flow rate of 300 μL/min. Water with 0.1% (v/v) FA (Mobile phase A) and CH_3CN (Mobile phase B) were used to generate a 60 min gradient, set as follows: 5% B for 1 min, to 40% B in 33 min, to 95% B in 6 min, kept at 95% B for 6 min, to 5% B in 10 min and kept at 5% B for 4 min. 10 μL of sample was injected for each RPLC-ESI MS^3 analysis. The LTQ-Orbitrap (Thermo-Fisher, San Jose, CA, USA) was operated at positive ion mode. The spray voltage was 3.0 kV, and the heated capillary temperature was 300 °C. The MS was operated in the data-dependent mode, in which a survey full scan MS spectrum (from m/z 100 to 2000) was acquired in the Orbitrap with a resolution of 30,000 at m/z 400. This was then followed by MS^2 scans of the most abundant ions and the MS^3 scans of first, second and third most abundant ions from MS^2. The resulting fragment ions were recorded in the linear ion trap.

2.4. Comparison of cMAED, MAED and HED

0.5000 g of ginkgo protein was first dispersed in 50 mL of Tris–HCl (pH 8.1, 50 mM) solution and then digested by 2250 U immobilized bromelain under the conditions of cMAED or MAED at 55 °C with magnetic stirring for 30 min. The microwave power was 300 W. For HED, 2250 U free bromelain was used to digest 0.5000 g of ginkgo (50 mL, pH 8.1, 50 mM Tris–HCl) at 55 °C in an incubator for 12 h. When the cMAED, MAED and HED of ginkgo finished, each solution was separated by centrifugation at 14,000 rpm at 4 °C for 5 min and stored at −20 °C. But for cMAED and MAED, the immobilized bromelain was removed first by the external magnetic.

2.5. Database searching and peptides identification for LC–MS^n

Protein identifications based on acquired MS^3 spectra were carried out using Xcalibur software (version 2.1) and output as raw files. Then the raw files were converted to mzXML files by X2XML (version 1.3.0.0, free downloaded from http://omics.pnl.gov). Finally,

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