Journal of Molecular Catalysis B: Enzymatic 94 (2013) 23–28

Contents lists available at SciVerse ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

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

The study on microwave assisted enzymatic digestion of ginkgo protein

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ARTICLE INFO

Article history:
Received 22 November 2012
Received in revised form 18 April 2013
Accepted 18 April 2013
Available online 1 May 2013

Keywords:
Microwave-assisted enzymatic digestion (MAED)
Bromelain
Ginkgo protein

ABSTRACT

Microwave-assisted enzymatic digestion (MAED) technique was applied for ginkgo protein digestion with both free and immobilized enzyme. Under the optimized conditions of MAED (0.01 g/mL substrate concentration of bromelain, 4500 U/g enzyme/ginkgo protein, 30 min, 300 W microwave power), a higher digestion rate (7.50%) and a significant increase in antioxidant activity (72.7 mg/g) were obtained in contrast with the conventional methods. With the optimized digestion conditions (0.625% glutaraldehyde (v/v), 0.4 mg/mL initial concentration of bromelain and 4 h of immobilization), the activity and effectiveness factor of immobilized bromelain were respectively 86 U and 81.6%. The results of ginkgo digestion by applying MAED indicated that the digestion rate of immobilized bromelain obtained by MAED method (6.41%) was comparable to that of free bromelain in the conventional digestion (8.13%). In both case with immobilized and free bromelain while applying MAED, a homogeneously abundant distribution of peptide fragments (from 7.863 Da to 5856 Da) and a few different peptide profiles were found. This report brings in conclusion that applying MAED with immobilized enzyme has the potential to obtain the highest number of antioxidant activity peptides.

1. Introduction

Production of bioactive peptides can be achieved by in vitro enzymatic digestion [1]. Applying the conventional methods, e.g., chemical digestion and enzymatic digestion [2] for protein digestion often generate problematic factors such as enzyme autodigestion, sample loss and/or contamination. These common occurrences are undesirable as they can directly affect the analysis of the final products and may render the controlling process rather tricky [3,4]. For mending the flaws brought by the conventional methods, focus has been diverted on many other techniques such as enzyme immobilization [5,6].

Immobilized enzymes offer multiple advantages in contrast to free enzymes for a simpler process, an improved control over product formation, an easier enzyme removal procedure from the reaction mixture and could generate a better environment for enzymatic activity [5–7]. In addition to all this, they have adaptability to various engineering designs [5,6]. Regarding the carriers employed for enzyme immobilization, uses of magnetic particles (MPs) and nanoparticles (MNPs) have been often studied [7–10]. Compared to other types of conventional carriers, MPs and/or MNPs carriers have an additional merit when it comes to selectivity and faster enzyme recovery from the digestion products due to their magnetic property [11]. Having acquired a suitable surface functionalization, MPs and/or MNPs will gain finer recognition ability and better aqueous dispersion in addition to a new property: biocompatibility [12].

Even so, the procedure of attaching desired functional groups with the required amount of quantity and the preferred orientation on nanoparticles surfaces remains a major challenge [13]. As conjugating biomolecules on the surface of MPs and/or MNPs depends heavily on surface functionalization [12], applying core–shell structured MNPs will yield an enhanced success rate that will directly impact surface functionalization due to the following characteristics they possess: a superparamagnetism nature; a high surface area; a large pore volume; and uniformly accessible mesochannels [14].

Recently, core–shell structured microspheres have been synthesized for conducting extensive research in biochemistry analysis [14]. Lin et al. [15] applied a core–shell structured microsphere with a silica-coated magnetite core and ordered mesoporous silica shell for trypsin immobilization under MAED. They have found that peptide fragments produced from BSA and myoglobin in 15 s could be confidently identified. In addition, the immobilized trypsin was easily isolated with the help of an external magnet and could be used repeatedly.

From their experiment, it can be concluded that MAED, recognized in the mid-1980s as an efficient heating source for producing chemical reactions [16–18], is an efficient approach to improve protein digestion speed. Compared to the conventional methods which spanned from several hours lasting till overnight, MAED shortens that record to thousands of times. Sun et al. [19] applied MAED to their experiment and obtained better in-gel digestion

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http://dx.doi.org/10.1016/j.molcatb.2013.04.010

efficiency compared to regular overnight digestion. Izquierdo et al. [20] demonstrated that small peptides produced with higher biological activity could be obtained using pronase, a-chymotrypsin and pepsin to digest bovine b-lactoglobulin by microwave assistance. The high efficiency of MAED has been demonstrated by Pramanik et al. [21,22] and Chen et al. [23] as well. Above all, MAED is successfully applied in identification of microscale amount of protein, but there are few articles focused on applying MAED to digest the largescale amount of real sample of protein and investigate the bioactive of digestion product.

Herein, in order to obtain the highest quantity of antioxidant activity peptides, we have investigated the application of MAED in the largescale amount of real sample of protein and combined MAED with enzyme immobilization for ginkgo protein digestion. First, we have optimized the conditions for employing MAED and picked up bromelain as the experimental enzyme. Then, the immobilized bromelain was applied to MAED for ginkgo protein. Under the observation stage, we have concluded that using MAED was far more efficient than undergoing with the conventional ways.

2. Materials and experimental methods

2.1. Reagents and materials

Crude ginkgo protein powder was extracted from ginkgo nuts in our laboratory as follows: First, the ginkgo nuts were grinded into powder and were sifted with a sieve of 80 meshes. Second, as a ratio of powder to the solvent was 1–10, the powder was incubated at 40 °C in the solution of pH of 9.0 for 90 min. Finally, the supernatant fluid was transfer into acid solution of pH of 4.0 to precipitate the crude ginkgo protein. Right after the extraction, the Kjeldahl method was then applied and ended up detecting 80% of ginkgo protein from the extracted crude ginkgo protein powder.

Tyrosine (CP), bromelain, trypsin and papain were brought from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Flavor protease and alkaline protease were purchased from Novozymes Biotechnology Co. Ltd. (Tianjin, China).

All the reagents for Tricine SDS PAGE were acquired from Sinopharm Chemical Reagent Co., Ltd., and all the reagents for HPLC analysis, such as trifluoroacetic acid (TFA) and acetonitrile were purchased from Dikma Co., Ltd. (CA, USA).

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/w), casein, sodium hydroxide, ammonium sulfate ($(NH_4)_2SO_4$), sulfuric acid, ascorbic acid, sodium phosphate, ammonium molybdate ($(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$), trichloroacetic acid (CCl_3COOH) and cetyltrimethyl ammonium bromide (CTAB, $C_{16}H_{33}(CH_3)_3NBr$).

2.2. Preparation of immobilized enzyme based on magnetic particles.

The synthesis process of the MPs was based on articles [24–27] with some modifications. Under nitrogen protection for 30 min, MPs were formed under the conditions of 63 °C, 300 W microwave heating power of microwave power after a chemical reaction between 100 mL NaOH (4 mol/L) and a solution composed of 40 mL of $FeCl_2$ (0.5 mol/L) and 20 mL of $FeCl_3$ (0.5 mol/L). The product was then washed out and dried by vacuum freeze-drying for a period of 24 h.

First, 0.5 g of MPs was treated with 5 mL of HCl (2 mol/L) under ultrasonic vibration for 5 min. They were then added to a mixture of 70 mL of ethanol, 20 mL of water and 1 mL of ammonia (28 wt%) and were ultrasonicated for 5 min. 3 mL of TEOS was added to the mixing, the mixing then got stirred at room temperature for 12 h to form a SiO_2 coating ($Fe_3O_4@mSiO_2$). Subsequently, the $Fe_3O_4@mSiO_2$ spheres were added to 40 mL of CTMB (5 mg/L) and stirred at 40 °C for 3 h. 6 mL of ammonia and 2 mL of TEOS were added in and stirred for 3 h. The above spheres were redispersed in 100 mL of NH_4NO_3 ethanol solution (10 mg/L) and refluxed at 80 °C for 3 h to remove CTAB to form $Fe_3O_4@mSiO_2@nSiO_2$. Finally, $Fe_3O_4@mSiO_2@nSiO_2$ was added inside a mixture of 1 mL of water, 100 mL of ethanol and 1.5 mL APTMS and stirred at the room temperature for 7 h. After the amino functionalization, $Fe_3O_4@mSiO_2@nSiO_2{-}NH_2$ was formed.

Afterwards, 0.0073 g $Fe_3O_4@mSiO_2@nSiO_2{-}NH_2$ were wash a phosphate buffered saline solution (PBS, 0.1 mol/L, pH 6.8), then added with 20 mL of glutaraldehyde PBS solution and stirred at room temperature for 2 h. Bromelain was added in and the solution was ultrasonicated for 1 min and then agitated for 2 h in a shaker for its immobilization. At last, the final product was washed with deionized water three times before being poured in a solution of 20 mM NH_4HCO_3 and 0.02% sodium azide (w/v) for storing at 4 °C before use. The activity of free and immobilized bromelain was measured according to the method of QB/T 1803-1993 [24].

2.3. MAED for ginkgo protein

MAS-II Smart Microwave Digestion System (Sineo Microwave Chemistry Technology Co. Ltd., Shanghai, China) was used for accelerating the digestion process. At foremost, 0.5000 g of ginkgo protein was dissolved in 50 mL water. Before adding enzymes to different samples of gingko protein solutions, the pH value of each solution was modified to 7.0 with 2% NaOH (w/v). Subsequently, the mixture solution was placed in the microwave oven under the conditions of 300 W at 55 °C with magnetic stirring. After digestion which using free enzymes, the digested solution was boiled at 100 °C for 5 min to eliminate the residue enzyme activity. But for the immobilized bromelain digestion, it was separated first under external magnetic field. Finally each solution was separated by centrifugation (3K30, Sigma, Germany) at 14,000 rpm for 5 min. By using the values of the digestion rate and the antioxidant capacity, it was possible to estimate the efficiency of MAED when applied to ginkgo protein.

2.4. Analytical methods

2.4.1. Determination of enzyme activity

The activity of immobilized bromelain and all free enzymes (such as bromelain, flavor protease, alkaline protease and trypsin), was detected according to QB/T 1803-1993 [28] with some modification which presented in details as following. First, 1% of casein solution (w/v) was prepared in PBS (0.1 mol/L). Second, 2 mL of all free enzymatic solutions were dissolved inside each PBS suitable to their corresponding pH while the immobilized bromelain were dissolved in 2 mL of PBS (pH 6.8). Every solution was incubated at 40 °C for 2 min, and ultimately, 2 mL of casein solution was added inside each solution and digested at 40 °C for 10 min. The digestion was halted by adding 4 mL of CCl_3COOH (4 mol/L). However, before adding 4 mL of CCl_3COOH, the immobilized bromelain was separated in the external magnetic field first. After being percolating through the filter paper, the absorbance value of every digestion solutions was detected with TU-1901 Ultraviolet-visible Spectrophotometer (Beijing Purkinje General Instrument Co. Ltd.) at $\lambda = 275$ nm. Then, it was calculated as the concentration of tyrosine from its working curve. The activity (U) of immobilized bromelain and free enzymes was defined as the amount of tyrosine (μg) produced from digesting casein per min. The specific activity of each enzyme is listed in Table 1.