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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 microspheres. 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.



## 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,

database searching was carried out but inputting mzXML files in MassMatrix (version 2.4.2, free downloaded from http://www.massmatrix.net/mm-cgi/downloads.py). The searching parameters were carefully performed (Supplementary materials, Table 1S). The mechanism of searching could be explained in details in Xu et al's articles [23–26].

Because the molecular mass and species of ginkgo protein are not very clear, the identification of peptides from ginkgo digestion is performed as following rule: the relative intensity of peptides' precursor in MS1 more than 30% was considered but peptides with the associative multiple-charged ion were prior to be considered, e.g. A peptide simultaneously possessed the precursors with state of +2 and +3 charge (Table 2–5S).

## 3. Results and discussion

### 3.1. The design of the device of cMAED

Generally in the laboratory microwave-assisted system, the desired reaction temperature could be maintained by using a sensor-controlled feed back system. But the drawback of this temperature control system was that when the desired reaction temperature reached, the microwave power was weaken seriously. Here we applied an external condensing device (low-temperature cooling liquid circulating pump) to cold the reaction solution so that microwave power keeps continuous. The temperature of condensing liquid (n-octane) in circulating pump (Fig. 1A) was cooled down to an appropriate value before running the microwave instrument. The controlled temperature which measured by the infrared temperature sensor (Fig. 1B) was set at 5 °C higher than the digestion temperature, e.g., if the ideal digestion temperature was 55 °C, the controlled temperature was set at 60 °C. This special design was benefited for digestion because the microwave power could be constantly outputted due to the heat energy was partly transferred by the condensing liquid. The immobilized trypsin and 2 mL BSA solution (1 mg/mL) in 0.05 M of Tris–HCl (pH = 8.0) were added and magnetically stirred (500 r/min) in digestion chamber (Fig. 1F). When the temperature of samples was closed to the preset temperature of 50 °C, the cooled liquid ran in circle in graham condenser (Fig. 1D) to cool down the temperature of the n-octane in cooling chamber (Fig. 1E). Experiments showed that the temperature of digestion system and current speed of the condensing liquid base on our device could be well controlled (Supplementary materials, Table 6S).

### 3.2. The cMAED for BSA protein digestion

The orthogonal array experimental design (OAD) based on three factors and four levels was designed to study the regular pattern for cMAED. Three factors, including the time, the microwave power and the ratio of immobilized trypsin to BSA, and four levels for each factor were studied. The digestion effects were evaluated by the sequence coverage. The significance value (*F*-value) was calculated base on the results of OAD (Supplementary materials, Table 7S) to assess the significance of each factor. Table 1 indicated that the microwave power was statistically significant to the cMAED at $P < 0.01$. The optimal digestion condition for cMAED of BSA was 10 min, 100 W and 3460 U/g immobilized trypsin/BSA. In this condition, more peaks and stronger intensity were presented on LC–MS map (Supplementary materials, Fig. 1S).

There is an interesting phenomenon when we compared the intensity of the identified peptides from the OAD. Fig. 2A illustrated that in the conditions of low microwave power (100 W, 300 W) peptides fragments significantly emerged although the digestion time and the ratio of immobilized trypsin/BSA also affected the digestion effect. Especially in the conditions of 10 min digestion time, 100 W microwave power, and 3460 U/g immobilized trypsin/BSA (band 5), the sequence distribution and the abundance of peptides were the widest (46% sequence coverage) and highest. But in the condition of high microwave power (500 W, 700 W), the intensity of peptides was weak. Therefore, the result suggests that the protocol of cMAED have the potential selectivity of producing peptides in the condition of different microwave power.

To confirm the selective digestion of cMAED, Tricine SDS–PAGE analysis for peptides from all the OAD conditions was carried out as Fig. 2B. The bands of all the lanes of the digestion solutions from 100 W microwave power of cMAED (lane 1, 5, 9, 13, Fig. 2B) were unclear. According their responding LC–$MS^3$ data (Fig. 2A), in the conditions of low microwave power of cMAED, BSA was digested into low molecular weight peptides. For example, when cMAED was applied to digest BSA in the condition of 10 min digestion time, 100 W microwave power, and 3460 U/g immobilized trypsin/BSA, 46% coverage was obtained but there was not any obvious bands appeared in Tricine SDS–PAGE (lane No. 5, Fig. 2B). It reflected that the mass weight of the peptides from the BSA digestion with was beyond the scale from 20,100 Da to 3313 Da. On the other hand, the bands of lane 6, 11, 16 (300 W, 500 W and 700 W respectively, Fig. 2B) were obviously clear. It can deduce from that the protein fragments with mass weight spanning from 3313 to 14,400 existed in the digestion solution. The selectivity of digestion effect of cMAED may due to a reasonable rate for recognition of ligation sites (e.g., next to an arginine or lysine component for trypsin) on the substrate protein by enzyme resulting in selective peptide bond scission [13].

Although in above work only the selective and efficient digestion for BSA has been exemplified applying cMAED protocol, cMAED protocol is easy to extend to real protein digestion. Since our previous work [15] showed that high antioxidant activity peptides could be obtained when controlling digestion conditions

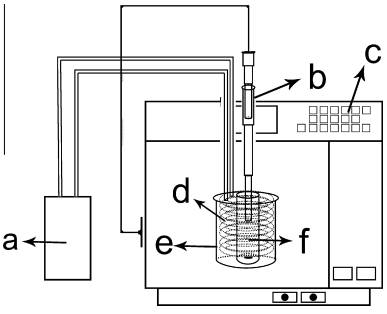


**Fig. 1.** The consist of device of cMAED. (A) Low-temperature cooling liquid circulating pump; (B) Infrared temperature sensor; (C) Microwave system control panel; (D) Graham condenser; (E) Cooling chamber; (F) Digestion chamber.

**Table 1**
Factors and levels of orthogonal test for cMAED of BSA.

| Levels | Factors | | |
|---|---|---|---|
| | A: Time (min) | B: Power (W) | C: Ratio of immobilized trypsin/BSA ($U\ g^{-1}$) |
| 1 | 5 | 100 | 1730 |
| 2 | 10 | 300 | 3460 |
| 3 | 15 | 500 | 5190 |
| 4 | 20 | 700 | 6920 |
| *F* value | 13.14 | 24.23 | 1.54 |

Critical value is 9.78 ($P < 0.01$), 4.76 ($P < 0.05$) and 3.05 ($P < 0.1$). *P* means confidence levels.

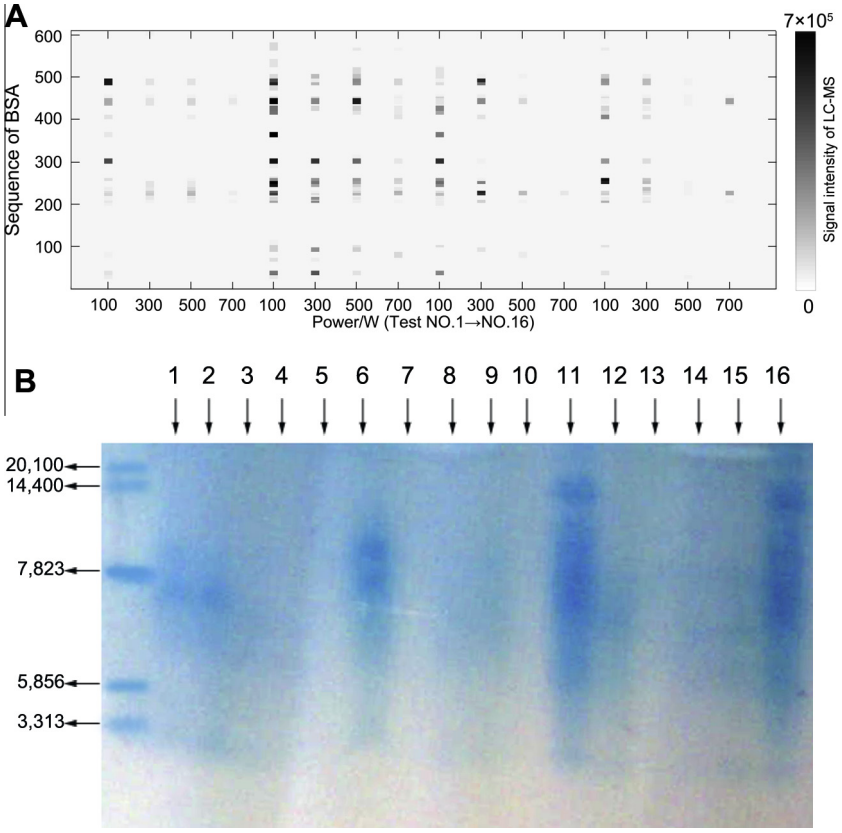


**Fig. 2.** The analysis of products from cMAED of BSA in conditions of OAD. (A) Tricine SDS–PAGE gel analysis. (B) The distribution of sequence and abundance of peptides.

during the process of ginkgo digestion with MAED, ginkgo digestion with cMAED was investigated.

### 3.3. The cMAED for ginkgo protein digestion

Obtaining peptides with specific sequence or molecular mass is desired during the process of bioactive peptides production. The efficiency and selectivity of cMAED was investigated for ginkgo protein digestion. Because the ginkgo protein digestion was performed in large scale volume (50 mL), the continuous microwave power from 300 W to 600 W was applied.

As Fig. 3 showed, the distribution of mass of peptides obtained from different microwave power of cMAED for ginkgo was significantly different with the microwave power increasing. The ginkgo was efficiently digested with the protocol of cMAED. The mass of peptides was mainly distributed below 1000 Da. However, in various microwave power, the enzymatic effect of ginkgo digestion was different. In the condition of 300 W microwave power, the signal intensity of peptides with low mass (below 1000 Da) was strongest. In the conditions of 500 W, the peptides distribution was widest with mass ranging from 1000 Da to 4000 Da but the amount and the intensity of peptides with mass below 1000 Da was lower and weaker than those in 300 W microwave, illustrating the efficient and selective digestion could be obtained by controlling microwave power in the process of cMAED. This implied a possibility for producing bioactive peptides with the protocol of cMAED.

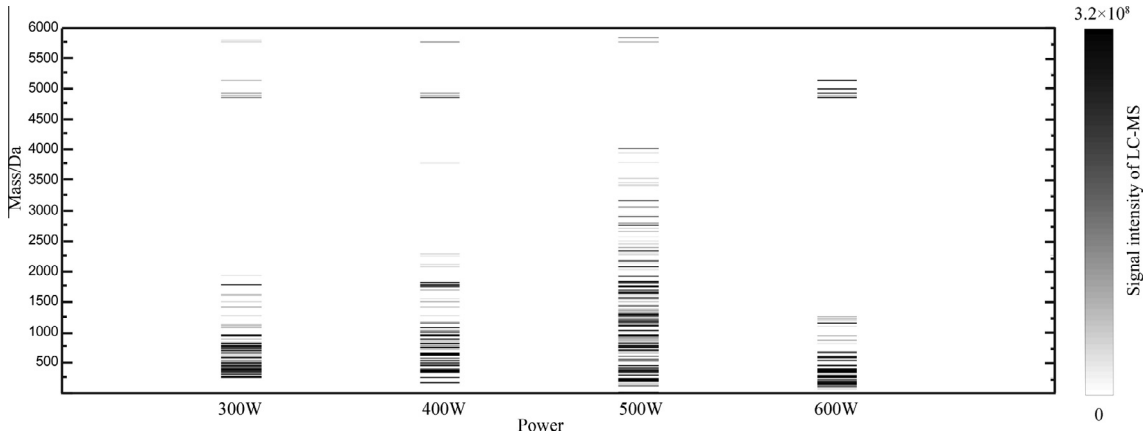


**Fig. 3.** The distribution of mass of peptides obtained from different microwave power of cMAED for ginkgo. Digestion condition: 4500 U/g immobilized bromelain/ginkgo protein, 30 min, 55 °C.

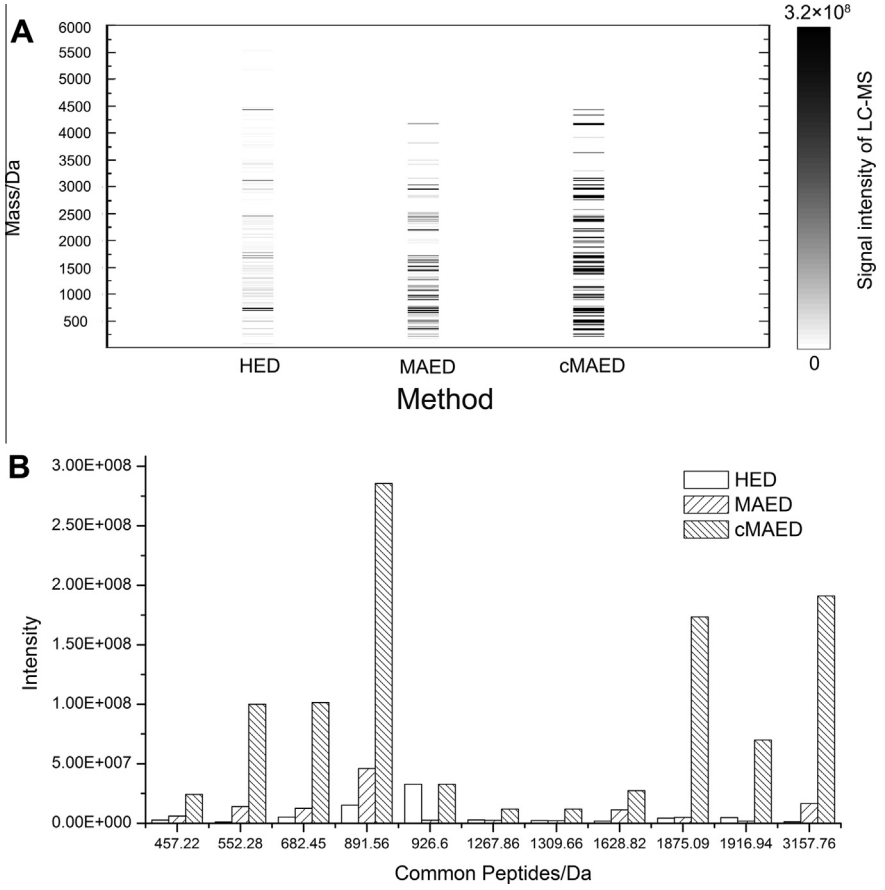


**Fig. 4.** The analysis of peptides from ginkgo digestion with different digestion protocols. (A) The mass distribution of peptides. (B) The common peptides. HED digestion conditions: 4500 U/g free bromelain/ginkgo protein, 12 h, 55 °C; MAED and cMAED digestion conditions: 4500 U/g immobilized bromelain/ginkgo protein, 30 min, 55 °C.

### *3.4. The comparison of cMAED, MAED and HED for ginkgo digestion*

To comfirm the high efficiency of cMAED, the methods of cMAED, MAED and HED for ginkgo digestion was compared. As Fig. 4A shows, the distribution of molecular weight of peptides obtained from cMAED is similar to HED and MAED, but the intensity of peptides is much stronger than the other two methods, which means that cMAED could significantly accelerate the ginkgo protein digestion and produce abundant peptides. Fig. 4B illustrated the intensity of each common peptides obtained from cMAED, MAED and HED. The intensity of peptides from cMAED was the highest of all, especially peptides with the molecular weight of 891.56 Da, 1875.09 Da and 3157.76 Da, implying the method of cMAED was more efficient than that of MAED and HED.

## 4. Conclusions

In this study, we proposed a novel method (cMEAD) for protein digestion. BSA and ginkgo were as substrate to investigate cMAED. Results showed that there existed selectivity digestion of cMAED. The microwave power was statistically significant in the process of BSA digestion. In the condition of different microwave power, the number of peptides obtained from BSA digestion was significantly different. Low microwave power would be the optimized condition for BSA digestion. In the conditions of 10 min digestion time, 100 W microwave power, and 3460 U/g immobilized trypsin/BSA, 46% sequence coverage for cMAED of BSA could be observed, respectively. Tricine SDS–PAGE analysis revealed that in the condition of low power of microwave of cMAED, peptides with low molecular weight (less than 3313 Da) were easily produced. However, during the ginkgo digestion, data showed that high microwave power was suitable for digestion. The intensity of peptides obtained from cMAED was significantly stronger than that from HED and MAED, suggesting that the cMAED could be a fast and efficient protocol for protein digestion.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.02.025.

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