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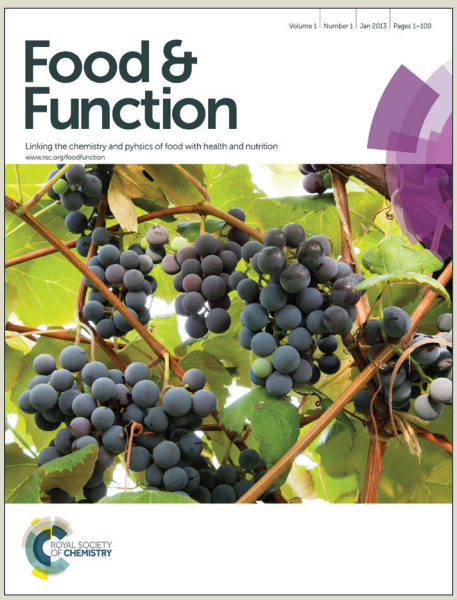

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Title:

Isolation and identification of a novel peptide from zein with antioxidant and antihypertensive activity

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Highlights

- A novel corn peptide was obtained by immobilized enzymes hydrolyzation method.
- The corn peptide was prepared by bioassay-guided isolation procedures.
- The corn peptide showed stability against thermal treatment and simulation digestion.
- The corn peptide showed high antioxidant activity *in vitro* and antihypertensive effects in SHRs.

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Abstract

The aim of this study is to isolate and identify a novel corn peptide (CPs) from zein with antioxidant and antihypertensive activity based on bioactive-guided isolation procedures. Zein was hydrolyzed by double enzymes immobilized with calcium alginate-chitosan beads and then fractionated and purified. The antioxidant and antihypertensive activities of the CPs fractions were screened by the *in vitro* and *in vivo* assays. The *in vivo* animal studies using spontaneously hypertensive rats (SHRs) confirmed the antihypertensive effects of the CPs peptide, and its angiotensin I-converting enzyme (ACE) inhibitory activity was retained after thermal treatment and simulated gastrointestinal digestion. The primary structure of CPs-2-1 was identified by RP-HPLC-MS/MS and the amino acid sequence was determined as M-I/L-P-P with the molecular weight of 452.3 Da. CPs-2-1 showed effective antioxidant and ACE inhibitory activities (IC_{50} values of 220 μg/mL and 70.32 μg/mL, respectively) and it might be a potent candidate for antioxidant functional food or pharmaceuticals for hypertension.

Keywords: *Corn peptides, identification, antioxidant activity, antihypertension activity, stability*

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Introduction

Bioactive peptides are considered specific protein fragments that are inactive within the sequence of the parent protein. After they are released by enzymatic hydrolysis, they may exert various physiological functions.[1] Biologically active peptides can be released after gastrointestinal digestion, food processing and microbial proteolysis of various animals and plant proteins. It is proved that the bioactivity of peptides were usually affected by the structure and amino acid sequences. The peptides may play various roles, such as antioxidative, antithrombotic, opiate-like, mineral binding, immunomodulatory, antimicrobial, hypocholesterolemic and antihypertensive activities.[1] Peptide therapies can range from anticancer and antimicrobial applications to the treatment of the symptoms of Alzheimer's disease.[2]

Bioactive peptides are the main products of hydrolysis of diverse food proteins. They exert various biological roles based on the structural properties and the amino acid composition and sequences, among of which the antioxidant activity and antihypertensive activity of peptides have more information available. There were some reviews on these two functions of peptides in recent years. High blood pressure was considered as a significant health problem worldwide and important advances had been achieved in the identification of dietary compounds that might contribute to cardiovascular health. Among these compounds, peptides with antihypertensive properties received special attention.[3] Plenty of studies had proved reverse relationship between antioxidant intake and diseases. Natural antioxidant peptides had drawn the attention of researchers due to their strong activity and no side effects compared to synthetic antioxidants. Antioxidant activity of bioactive peptides could be attributed to their radical scavenging, inhibition of lipid peroxidation and metal ion chelation properties of peptides.[1] So, more and more studies are focusing on searching for new dietary peptides with antioxidant and antihypertension activities.

Corn gluten meal (CGM), which is a byproduct of the corn wet-milling process, contains about 60 -71% (w/w) of protein. The major protein fraction in CGM was zein, which composed approximately 68% of the total protein weight. Zein is a kind of alcohol soluble protein, which results in the limited applications in food industry. Furthermore, zein is short of lysine and tryptophan so it is poor in nutritional quality.[4] However, the zein hydrolysates, corn peptides (CPs) were reported to have many activities such as the inhibition of angiotensin I-converting enzyme

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(ACE), the alleviation of fatigue, resistance to lipid peroxidation and the facilitation of alcohol metabolism.[5] In recent years, zein had been used to prepare corn peptides (CPs) by the enzymatic hydrolysis or microbial fermentation methods.[4] Some studies had been performed for the prepared hydrolysates from CGM protein on its physiological functional properties. CPs was not only taken up easily by the human body where it could perform special physiological functions, it also had high water-solubility and high utilization. [4] The preparation methods, physicochemical properties, antioxidant activity, amino acid composition and fractionation by ultrafiltration of antioxidant hydrolysates from corn protein had been studied. In the report of Kim et al, hydrolysis of corn gluten by using commercial proteases could improve its water solubility, and the hydralysates showed powerful angiotensin converting enzyme inhibitory (ACEI) activity.[6] It was reported that the processing functionality and antioxidative activities of CGM hydrolysates could be proved by using a commercial protease Alcalase, Protamex, or fermentation with Bacillus natto.[7-9] In the study of Zhou et al, corn protein hydrolysates, prepared by three types of microbial proteases, were separated by sequential ultra-filtration into several fractions, and the fraction of 1-3 kDa showed the highest activity in scavenging peroxyl radicals. [4] Protamex catalyzed corn gluten meal (CGM) hydrolysis peptides (CHP) were prepared and it was found that the antioxidant activities of CHP were highly correlated to small peptide molecules and high content of antioxidative amino acids.[10] Compared with the commonly used commercial protease, enzyme immobilization was a promising method for high enzyme recovery and extended use of enzyme in different solvents at extreme pH values and temperatures as well as at exceptionally high substrate concentrations.[11] In our previous study, a novel enzyme immobilization method, double enzymes (alcalase and trypsin) immobilized in a calcium alginate-chitosan carrier (ATCC) had been used to obtain corn hydrolysates from zein in an ethanol solution.[12] The hydrolysates was found to have good antioxidant activities. However, the structure and the antihypertension activities of CPs are still unknown.

In recent years, much work have been done to evaluate the *in vitro* activity of food derived peptides on the angiotensin-I-converting enzyme (ACE), which plays an important role in the regulation of blood pressure. The ACE (dipeptidyl carboxpeptidase, EC3.4.15.1) can cause hypertension by destroying the balance of the two systems the Rennin Angiotensin System (RAS) and Kallikrein Kinin System (KKS). [13-14] Antihypertensive peptides were reported to inhibit ACE

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activities by combining the active radical of Zn^{2+} in the obligatory bind site of ACE, so they could exert prominent antihypertensive action. The antihypertensive peptides were usually detected on the *in vitro* antihypertensive studies on ACE inhibitory activities. However, the complete correspondence between *in vitro* and *in vivo* effects and the stability on thermal treatment and simulated gastrointestinal environment treatment had not been demonstrated in many of the published studies.

The aim of this study was to identify the primary structure of the CPs obtained from zein by our developed enzyme immobilization method. The antioxidant activities, antihypertensive effects in spontaneously hypertensive rats (SHRs) and ACE inhibitory activity of CPs were evaluated, and the stability on the ACE inhibitory activity was also investigated after the thermal treatment and simulated gastrointestinal environment treatment.

Materials and methods

Materials

Zein was obtained from Gaoyou Rixing Medicinal Materials Co (Jiangsu, China).The alcalase (from bacillus subtilis fermentation), trypsin (from pig pancreas), pepsin (from gastric chief cell) were from Tianjin Nuoao Enzyme Preparation Technology Co. (Tianjin, China). The angiotensin I converting enzyme (ACE, from rabbit lung), hippuryl-histidyl-leucine (HHL) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The ultra-filtration system (Model No. 8050) and cellulose membranes for zein hydrolysates preparation were purchased from Millipore Co. (Billerica, MA). Sephadex G-10 was purchased from GE Healthcare Bio-Sciences Corp (Uppsala, Sweden). All other chemicals and reagents were purchased locally and were analytical grade. Experimental procedures conformed to the China legislation on Protection of Animals Used for Experimental and other Scientific Purposes.

CPs preparation and purification

The hydrolysis and preparation of CPs was carried out according to our previous studies.[12] Briefly, 2.5% (m/v) of zein solution (in 85% ethanol) was hydrolyzed by ATCC beads (containing both alcalase and trypsin simultaneously immobilized within calcium alginate-chitosan composite carrier) at the conditions of pH of 8.5 and the substrate and enzyme mass ratio was 1:1, [S/E]. The hydrolysis

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reaction was performed at 55°C for 120 min and terminated by filtering out the ATCC beads, then the pH was adjusted to 7.0. The ethanol was removed and the zein hydrolysates were obtained. The hydrolysates were collected and sequentially ultra-filtered through a Millipore 8050 ultrafiltration unit using cellulose membranes with 4 kDa and 6 kDa molecular weight (Mw) limits. Three peptide fractions, fraction 1 (F1), composed of hydrolysate peptides with Mw >6 kDa , fraction 2 (F2), composed of hydrolysate peptides with Mw between 4 and 6 kDa and fraction 3 (F3) composed of hydrolysate peptides with Mw < 4 kDa were obtained, each fraction was collected and lyophilized. Based on the antioxidant activity screening studies, the most active fraction was selected for further purification using a Sephadex G-10 chromotography (50 × 3.0 cm, I.D.), eluted with water at a flow rate of 10 mL/h, and elution peaks were monitored at 280 nm. The main fractions were collected by BS-100A automatic fraction collector (Huxi Analysis instrument Co. LTD, China) and named as CPs-1 and CPs-2, respectively. The fraction with the highest ACE inhibitory activity was dissolved in distilled water, and separated by reversed-phase high performance liquid chromatography (RP-HPLC) on a YMC-Pack ODS-A column (C18, 250 mm×10 mm I.D.YMC Co., Ltd. Japan). For RP-HPLC analysis, mobile phases used in the gradient elution consisted of eluent A consisting of 0.1% trifluoroacetic acid (TFA) in distilled water (v/v); and eluent B of 0.1% trifluoroacetic acid (TFA) in acetonitrile. The separation was performed with a linear gradient from 5 to 50% eluent B at a flow rate of 1.0 ml/min in 40 min. The UV absorbance of the eluent was monitored at 280 nm. Finally, the fraction with the antioxidant and ACE inhibitory activity was collected. This was followed by identification of the amino acid sequence of the peptide.

Scavenging activity against DPPH free radicals

The DPPH scavenging activity of CPs was detected in accordance with our previous study.[12] One hundred microliters of CPs fractions was mixed with 2900 μL DPPH solution (120 μM) in ethanol and incubated in the dark at 37°C for 30 min. The absorbance was recorded at 517 nm. The percentage (%) inhibition of CPs on free radical production by DPPH was calculated and ascorbic acid was used as positive control.

ACE inhibitory activity *in vitro*

The ACE inhibitory activity of CPs was analyzed by HPLC method according to the assay developed by Wu et al.[15] Briefly, the substrate HHL was dissolved (5 mM) in 0.1 M sodium-borate

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buffer (pH 8.3) containing 0.3 M NaCl. The assay was performed by mixing 80 μL of substrate solution with 30 μL of CPs fractions (or borate buffer for control). After 10 min of incubation at 37°C, 10 μL of ACE solution (0.1 U/mL) were added and the solution was further incubated at 37°C for 30 min with continuous agitation at 450 rpm. The reaction was stopped by addition of 150 μL of 1 M HCl and the solution was filtered through a 0.45 μm nylon syringe filter before being analyzed by RP-HPLC.

The HPLC analysis was performed on a C18 column (150 × 3.0 mm I.D.), particle size 5 μm with a varian chromatographic system and analytes were detected at the wavelength of λ=228 nm. The column was eluted at a flow rate of 0.4 mL/min with a two solvents system: (A) 0.05% TFA in water and (B) 0.05%TFA in MeOH. The gradient consisted 5-60% B in 10 min, maintained for 2 min at 60% B, then returned to 5% B for 1 min. This was followed by isocratic elution for 4 min at 5% B. The evaluation of ACE inhibition was based on the comparison between the concentration of CPs in presence or not and the degree of ACE inhibition (%) was calculated.[16] All the experiments were performed in triplicate.

Thermal stability assays of the CPs on ACE inhibitory activity

CPs fractions (1.0 mg/mL) with different Mw distribution (F1, F2, F3, CPs-1, CPs-2) were thermal treated at different temperatures（30, 50, 70, 90, 110 ºC）for 6 min. All solutions were cooled to room temperature before the ACE inhibitory activity analysis *in vitro*. The activity of the CPs prior to treatment was considered as 100%. All the experiments were performed in triplicate.

Stabilities against simulated gastrointestinal digestion on ACE inhibitory activity

Stabilities of CPs fractions against gastric proteases *in vitro* were assessed using pepsin and pancreatin according to the reported methods of Elizabeth et al. [17] and Escudero et al. [18]with some modifications. Pepsin (0.2 mg) was added to aqueous solutions of CPs (1:100; enzyme to substrate ratio), the mixture was adjusted at pH 2.0 using 1 M HCl and incubated at 37ºC. After 120 min, the pH was adjusted to 7.5 by adding 1 M $NaHCO_3$. Then, pancreatin (0.4 mg) was added at a 1:50 enzyme to substrate ratio, and the solution was further incubated at 37 ºC for 120 min. The reaction was stopped by heating at 95ºC for 10 min in a water bath, followed by cooling at room temperature. After the simulated gastrointestinal digestion, the peptides were freeze-dried and then reconstituted for ACE inhibitory activity determination. Experiments for each sample were done in triplicate. The

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inhibitory activity of the CPs prior to digestion was considered as 100%.

***In vivo* assay of CPs on antihypertensive activity**

Antihypertensive effects of the purified peptides were carried out in male SHRs (Age10-15 weeks, Weight 220-260 g, No.11003800004211, Beijing HFK Bioscience Co., China). All rats were cared and fed in accordance with the standards for laboratory animals established by the People's Republic of China (GB14925-2001) and Animal handling followed the Declaration of Helsinki and the Guiding Principles in the Care and Use of Animals. The rats were allowed to acclimate a week prior to the experiments. Tail systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured by the noninvasive tail-cuff method[19] with a computer-assisted BP-2010 Series tail measurement equipment (Softron Beijing Biotechnology Co., Ltd.). SHRs with tail SBP higher than 175 mmHg and DBP higher than 120 mmHg were used in this study. The SHRs were divided into four groups with four rats each and administered the following treatments: (a) Zein hydrolysate before ultrafiltration dissolved in physiological saline at the dose of 50 mg/kg body weight, (b) CPs-2 dissolved in physiological saline at the dose of 10 mg/kg body weight, (c) the positive control captopril dissolved in physiological saline at the dose of 10 mg/kg body weight, and (d) physiological saline only. Each group received a 1 mL dose of each treatment via oral gavage. The SBP and DBP were measured both before administration (zero time) and 2, 4, 6, 8, 12 and 24 h post-administration. Physiological saline alone and captopril served as negative and positive controls, respectively. To measure BP, SHRs were pre-warmed at 37°C for 10 min in a warming box.

HPLC-MS/MS analysis

Amino acid sequence of the purified peptide from zein hydrolysate was determined by RP-HPLC-MS-MS using Mass Spectrometry (mior OTOF-QII MS, Bruker Dalton Company, USA) with an electro-spray ionization (ESI) source. The elution was performed using a mobile phase consisting of water + 0.1% trifluoroacetic acid (eluent A) and acetonitrile + 0.1% trifluoroacetic acid (eluent B). Gradient elution was carried out according to the following process: 0-40 min, A 95-50%, B 5-50%. Mass spectrometry conditions: ESI^+ ion source; spray voltage was 4500 V; Set dry heater was 180°C; the set nebulizer gas (N_2) was 0.8 Bar; the flow rate of drying gas (N_2) was 60.0 L/min; spectra were recorded over the mass/charge (m/z) range of 50-1500. The peptide sequencing was

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262 performed by processing the MS-MS spectra using the mass spectrometry database (Swiss Prot) as
263 well as manual calculation.

264 **Statistical analyses**

265 The values were expressed as the means ± standard deviation (SD) of three replicates. Statistical
266 analysis of the data was done using the SAS software (SAS Institute Inc., Cary, NC, USA). The
267 differences in mean were calculated using the Duncan's multiple-range tests for means with 95%
268 confidence limit ($P < 0.05$).

269

270 **Results and discussion**

271 **Purification of peptide CPs-2-1**

272 Based on our previous studies, the CPs fraction with Mw < 4 kDa, F3, showed the stronger
273 antioxidant activities than the other two fractions F1, > 6 kDa and F2, 4 kDa - 6 kDa.[12] Herein, the
274 most active fraction F3 was selected for the further purification using a Sephadex G-10
275 chromotography in the study, and two fractions CPs-1, CPs-2 (Fig.1A) were collected and
276 lyophilized for the determination of their ACE inhibitory activity. The active fraction CPs-2 was
277 then selected for the further isolation and preparation on RP-HPLC chromatography and the main
278 fraction CPs-2-1 was obtained (Fig. 1B).

279 **ACE inhibitory activity of CPs *in vitro***

280 ACE activity leads to an increase in the blood pressure by producing the vasoconstrictor peptide
281 angiotensin II and by degrading the vasodilator peptide bradykinin. Inhibitors of ACE are thus used
282 as therapeutic agents against hypertension. Many kinds of peptides derived from food proteins are
283 known to exhibit ACE inhibitory activity and are considered to be milder and safer as compared to
284 synthetic drugs.[20] Furthermore, these peptides are multifunctional and easily absorbed in the
285 gastrointestinal tract.[21] The ACE inhibitory activity of different CPs fractions was presented in Fig.2.
286 The results showed that the ACE inhibition capacity was increased with decrease of CPs Mw
287 distribution when all the fractions were detected at the same concentration of 1.0 mg/mL. The
288 highest activity was exhibited by fraction CPs-2 with the ACE inhibitory activity of 79.3%, which
289 had the smallest molecular weight distribution. While the faction F1, with the highest molecular
290 weight distribution, showed the lowest inhibitory effects (48.6%). The results were in accordance

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to the report of Huang et al., in which the corn peptide fraction with Mw < 3 kDa showed the highest ACE-inhibitory activity.[22] In other studies on plant protein and peptides such as grass carp protein and peanut protein hydrolysate, it was also found that ACE inhibition capacity was increased with the increase of hydrolysis and the Mw< 3 kDa fraction showed the higher ACE inhibitory activity than the fractions with Mw of 3-10 kDa or above 10 kDa.[20,23] It could be suggested that the molecular size of the peptide played an important role in its ACE-inhibitory activity and most of them were less than 3 kDa.

Effect of thermal treatment on ACE inhibitory activity of CPs

The generated bioactive peptides can be directly ingested either raw or heat treated, or incorporated into other processed products. In this case, it is necessary to consider the stability of the ACE inhibitory activity among the typical temperature conditions. To investigate the thermal stability of CPs, the peptides fractions were subjected to incubation at temperature 30, 50, 70, 90 and 110 ºC for 6 min and the residual activity was measured. The ACE inhibitory activity of the CPs prior to thermal treatment was used as control and considered as 100%. All the assayed CPs fractions remained ACE inhibitory activity after the thermal treatment at different levels (Fig.3A). CPs-2 showed the highest relative ACE inhibitory retention rate (RACEI) after 50 ºC treatment (179.6%). ACE inhibitory rates of CPs-2 increased than that of the original one when it was thermal treated at 30, 50, 70 and 90 ºC (138.9%, 179.6%, 142.4%, 152.0%), and 99% relative activity was still remained after the thermal treatment at 110 ºC. ACE inhibitory activity of F1 was also increased with the treatment from 50 to 110 ºC (131.0%, 114.6%, 104.3%, 113.1%, respectively) except treatment at 30 ºC (40.1%). ACE inhibitory activities of F2 were increased after 50 ºC (141.3%) and 110 ºC (122.3%) treatment. The inhibitory rate was increased when F3 was heated at 50 ºC to 90 ºC (130.3%, 119.1%, 137.8%), and the rate was increased when CPs-1 was treated at these temperatures except for that at 50 ºC. For the CPs fractions F1, F2 and F3, thermal treatment at 30 ºC resulted in the decrease of ACE inhibitory activity with the retention rates of 40.1%, 80.1% and 75.6%, respectively. Different fractions showed different activity retention tendency, among which CPs-2 showed the highest stability against thermal treatment. The reason might be due to the different composition and molecular weight distribution.

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Effect of gastrointestinal **digestion *in vitro* on ACE inhibitory activity of CPs**

Proteolytic enzymes can generate bioactive peptides during digestion that were inactive within the parent protein.[24]ACE inhibitory peptides could exert *in vivo* antihypertensive effect if they reach the blood stream in an active form.[25]So, after oral ingestion, peptides need to resist complete hydrolysis by gastrointestinal enzymes and brush border peptidases, and be able to pass through the intestinal wall with preserving their biological activity.[18] To evaluate the stability under digestion by gastrointestinal enzymes, the CPs fractions was incubated with various gastrointestinal proteases including pepsin and pancreatin, and then the ACE inhibitory activity was detected. Compared with the ACE inhibitory activity of the undigested control (considered as 100%), the relative ACE inhibitory retention rates (RACEI) of digested CPs fractions by gastric proteases were 82.8%, 126.6%, 76.8%, 63.2% and 72.1% for F1, F2, F3, CPs-1 and CPs-2, respectively. Though there were some decrease on the ACE inhibitory activity of F1, F3, CPs-1 and CPs-2, they were all above 63%. Especially, the activity of fraction F2 was increased to 126.6% after the *in vitro* digestion treatment (Fig.3B), suggesting that the peptides might be resistant to digestion in the gastrointestinal tract or they might be partially degraded into smaller peptides with the antihypertensive biological activity. These results were agreed with the previous studies on other peptides, in which it was showed that small peptides still presented ACE inhibitory activity after digestion.[18] These results indicated that CPs was relatively stable against gastrointestinal proteases of pepsin and pancreatin. The related mechanism will need further study.

Antihypertensive effects of CPs in SHRs

The antihypertensive effect of CPs was evaluated by measuring the changes in the SBP and DBP of SHR during a 24 h observation period following a single gastric intubation (Fig.4). In the negative control group (normal saline), no significant changes were found in the SBP and DBP of SHR during the 24 h after administration. After administration of zein hydrolysate, there were decrease on SBP (-24.5 mmHg) and DBP (-19.8 mmHg) at 8 h after administration, while, there was no significant changes were found on SBP during the 24 h after administration (3.8 mmHg). Furthermore, administration of CPs-2 (10 mg/kg) and captopril (10 mg/kg) caused significant decrease of the blood pressure (BP) at 2, 4, 6, 8 and 12 h after administration ($p<0.05$). CPs-2 administration resulted in the decrease of 42.5 mmHg of SBP change and 17.1 mmHg of DBP change at 8 h and

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44.6 mmHg of SBP change and 10.2 mmHg of DBP change at 12 h, which was even better than the positive control captopril (SBP with 22.7 mmHg change and DBP with 13.9 mmHg change at 8 h, SBP with 17.7 mmHg change and 7.9 mmHg change of DBP at 12 h). The *in vivo* effects of CPs were in accordance with the results of ACE inhibitory activity of CPs. In the review of Martinez-Maqueda et al., it was stated that the complete correspondence between *in vitro* and *in vivo* effects had not been demonstrated in many of the published studies because of the discrepancy between ACE inhibitory (ACEI) and antihypertensive activity of peptides.[3] The reason might be due to their further degradation during gastrointestinal digestion, the impossibility to reach the target organ in the organism in a sufficient amount or because other mechanisms different than ACE inhibition might be involved. In this study, the peptide CPs showed relatively good gastrointestinal digestion stability and so there was correspondence between *in vitro* and *in vivo* effects. Results suggested that the ACE inhibitory peptide obtained through double enzymes immobilized with calcium alginate-chitosan beads could provide an obvious antihypertensive effect in SHRs at a dosage of 10 mg/kg per rat.

Identification of CPs-2-1 by RP-HPLC-MS/MS.

The fraction CPs-2, with the strongest ACE inhibitory activity was then separated by reversed-phase RP-HPLC, and the main components CPs-2-1 was obtained and subsequently subjected to RP-HPLC-MS/MS for peptide sequence identification. The accurate relative molecular mass of the peptide, deduced from the m/z value of $[M+H]^+$ by subtraction of one mass unit for the attached proton, was 452.3 Da (Fig. 5A). The $[M+H]^+$ ion at m/z 453.3 suggested a tetrapeptide composed of the amino acids Met, Ile/Leu, Pro and Pro. As shown in Fig. 5B, the m/z 453.3-322.3=131.0, which indicated the first amino acid from the N-terminal was a Met (M) residue and m/z 322.3 was the y_3 ion. Similarly, m/z 322.3 (y_3) - 209.2 (y_2) =113.1, thus we could infer that the second amino acid from the N-terminal was a Leu/Ile (L/I) residue, in which L and I were isomers. Next, y_2-y_1= m/z 209.2 -114.1=95.1, which suggested that the third amino acid of the N-terminal was Pro (P) residue. Finally, the y_1=114.1, suggested that the C-terminal was Pro (P) residue. From the manual calculation process above, the amino acid sequence of CPs-2-1 was determined as M-I/L-P-P. The results of peptide CPs-2-1 sequencing were subjected to a manual evaluation as described by Chen et al.[26] the mass signal and corresponding fragmentation spectra matched to a single peptide

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fragment by manual calculation. In this study, the amino acids analysis of the peptide CPs-2-1 was agreed with the study on amino acids composition of zein, in which zein was rich in Leu (20%), Pro (10%) and Ala (10%) and lacked of alkaline and acidic amino acids.[27] The molecular weight and the amino acids composition were accord with the reports that the bioactive peptides were in the size of 2-20 amino acids and molecular masses of less than 6000 Da.[1]

DPPH radical scavenging effects and ACE inhibitory activities of CPs-2-1

The DPPH radical scavenging effects and ACE inhibitory activities of CPs-2-1 were shown in Table 1. Peptide CPs-2-1 showed antioxidant and ACE-inhibitory activities in dose dependent manner with the IC_{50} values of 220 μg/mL and 70.32 μg/mL, respectively. The antioxidant activity of CPs-2-1 was stronger than the previous report of Wang et al., in which corn gluten meal (CGM) was hydrolyzed using two proteases (Alcalase and Protamex) to produce the antioxidant peptide with the EC_{50} value of 950 μg/mL on scavenging activity against DPPH free radicals.[26] In the study of Huang et al., [22] IC_{50} values of ACE-inhibitory activities of the corn peptides with different molecular weight were 440, 290, 1270 μg /mL for Mw< 1 kDa permeate, Mw< 3 kDa permeate, Mw< 5 kDa permeate, respectively, which were larger than the IC_{50} value of CPs-2-1, suggesting that CPs-2-1 showed more effective ACE-inhibitory activities than the reported one. The difference might be due to the different composition, structure, and hydrophobicity between the CPs-2-1 and the reported peptides. CPs-2-1 was a potent candidate for antioxidant and ACE-inhibitory peptide product. The purified peptide might be used in functional food products as a bioactive component with good antioxidant and ACE-inhibitory activities.

It was reported that Tyr, Trp, Met, Lys, Cys, and His were examples of amino acids that could cause antioxidant activity.[29]Amino acids with aromatic residues could donate protons to electron deficient radicals. This property could improve the radical-scavenging properties of the amino acid residues. [30-31] In the amino acid composition of CPs-2-1, aromatic amino acids, Phe, was also presented, which had been shown to act positively as direct radical scavengers. The amino acid sequence of CPs-2-1 was agreed with the report that at penultimate C-terminal position, aliphatic (V, I, and A), basic (R) and aromatic (Y and F) residues were frequently found in ACE inhibitory peptides, while aromatic (W, Y, and F), proline (P) and aliphatic (I, A, L and M) residues were

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preferred at the ultimate C-terminal end of peptides.[32] Jun et al. reported that oral administration of Ile-Gln-Pro at a dosage of 10 mg/kg showed significant decreases of SBP and DBP in SHR.[33] The amino acids composition and sequence M-I/L-P-P might play great roles to the antioxidant and antihypertensive activities. This was the first time that the peptide M-I/L-P-P was isolated from food protein with high ACE inhibitory activity.

Conclusion

Under the bioassay-guided isolation procedure, M-I/L-P-P, a peptide with effective antioxidant and antihypertensive activity, was identified by RP-HPLC-MS/MS. This peptide was from zein hydrolysate fraction by double enzymes immobilized with calcium alginate-chitosan beads in the ethanol phase with the molecular weight of 452.3 Da. The antihypertensive activities were relatively well retained after thermal treatment and simulated gastrointestinal environment treatment. There were good correspondence for the *in vitro* and *in vivo* studies of CPs on antihypertensive effects. The new peptide might be a potent candidate for functional food or pharmaceuticals for hypertension.

Acknowledgements

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552 **Related table**

553 **Table 1** The IC_{50} values of CPs-2-1 on DPPH radical scavenging effects and ACE inhibitory
554 activities (μg /mL)

Sample	DPPH radical scavenging activities	ACE inhibitory activities
CPs-2-1	220 ±2.63	70.32 ±0.61

555 Values are presented as means ±SD (n=3).

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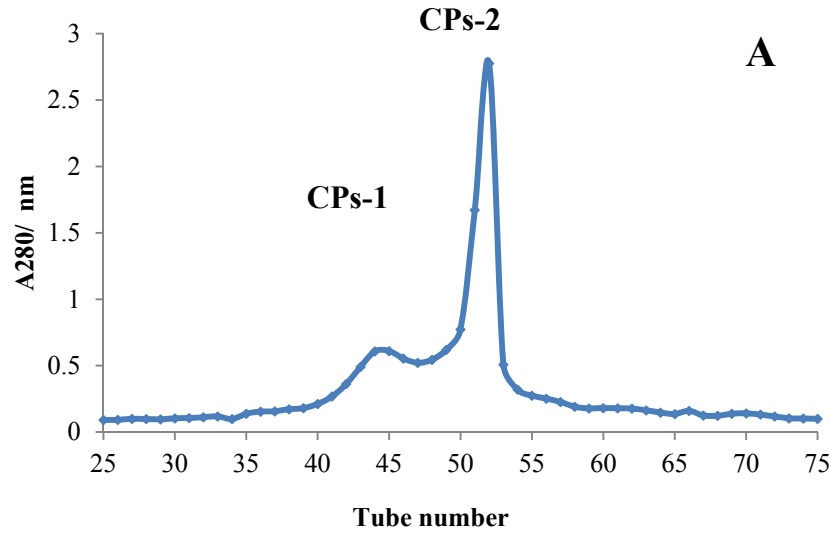
578 **Related Figures**



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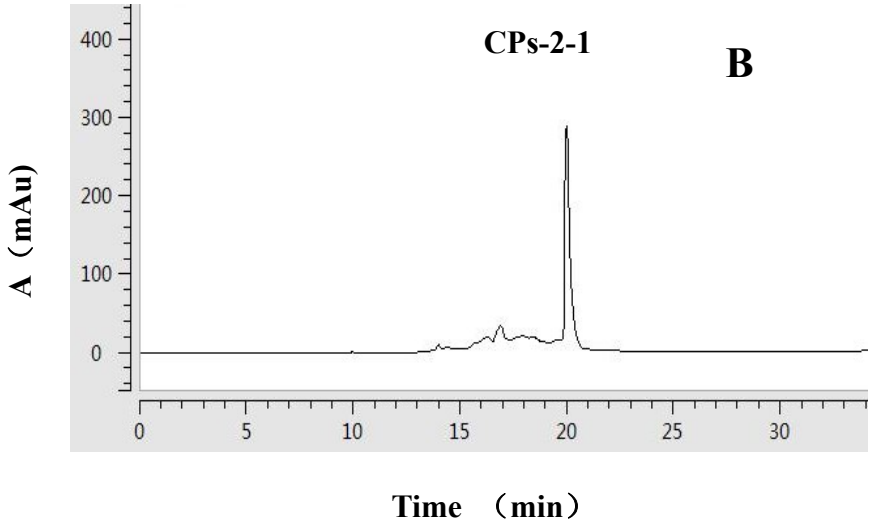

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590 **Fig.1.**Purification of peptide CPs-2-1. (A: Sephadex G-10 chromatogram; B: RP-HPLC

591 chromatogram).

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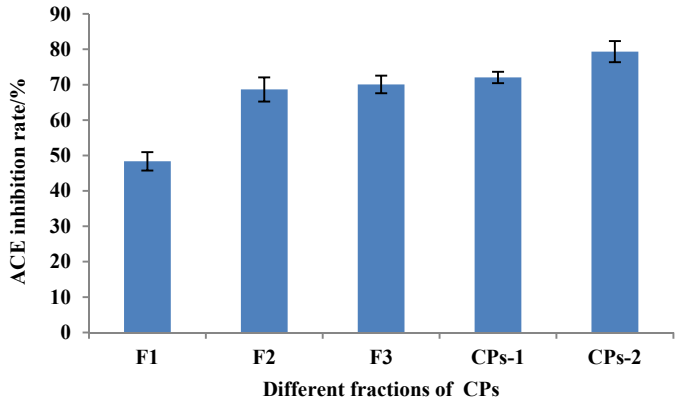

Fig.2. The angiotensin-I-converting enzyme(ACE) inhibitory activities of CPs fractions.

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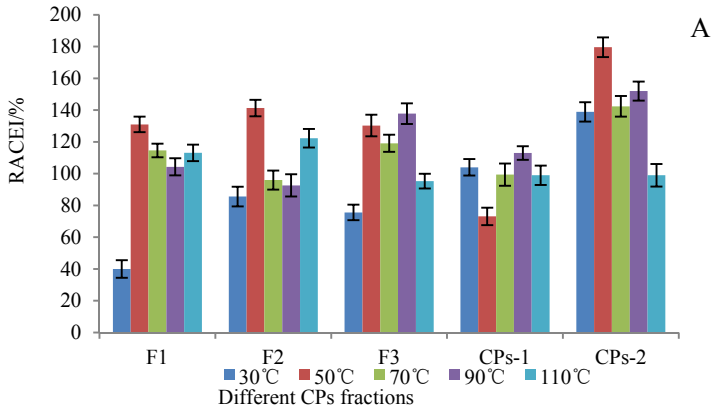

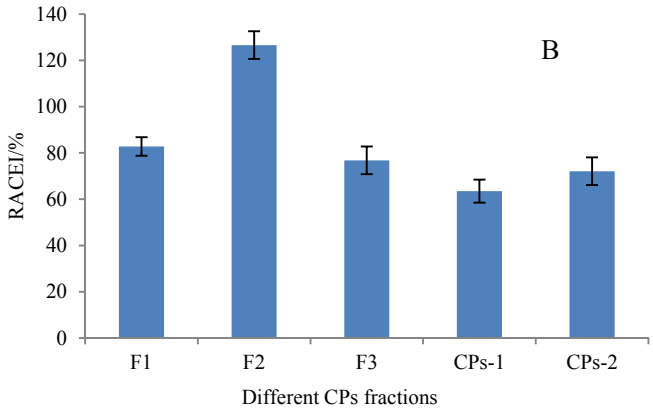

Fig.3. Stabilities of angiotensin-I-converting enzyme (ACE) inhibitory activities of CPs fractions. (A) CPs with incubation at different temperatures; (B) CPs digestion *in vitro* by gastrointestinal proteases. RACEI: Relative ACE inhibitory retention rate; The activity of the CPs prior to treatment was considered as 100%.

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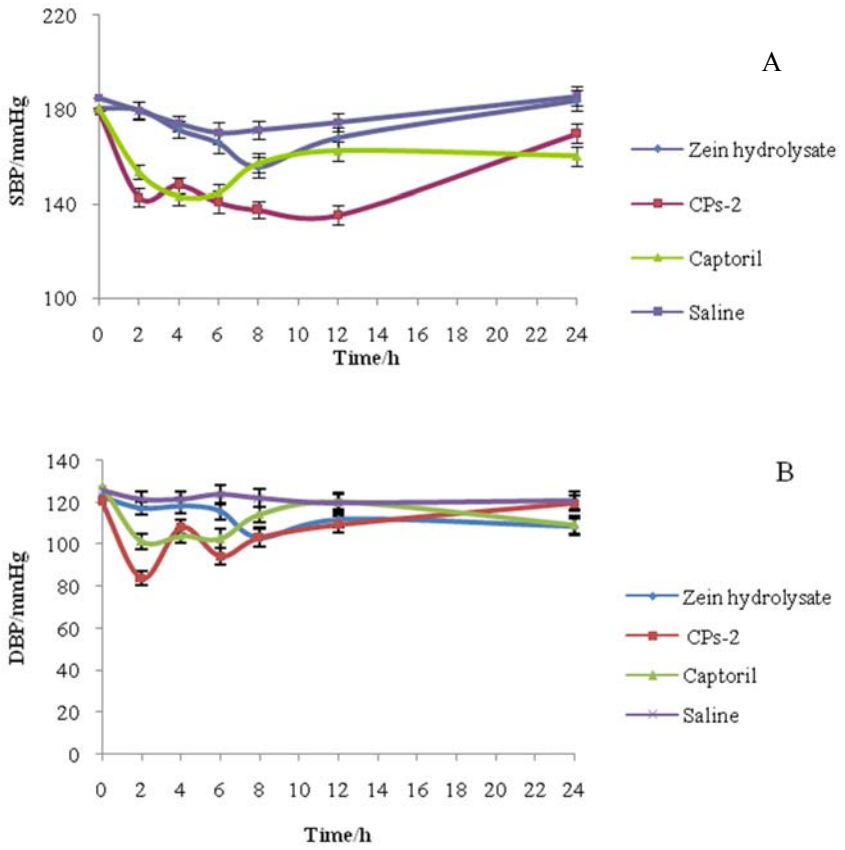

Fig.4. Antihypertensive effects of CPs on spontaneous hypertensive rats (SHRs). The actual systolic blood pressure (SBP)(A) and diastolic blood pressure (DBP) (B) over the same time period following administration of the zein hydrolysate (50 mg/kg), CPs-2 (10 mg/kg), a positive control (captopril,10 mg/kg), and a saline solution (negative control). Date shown were the means ±SD (n = 4).

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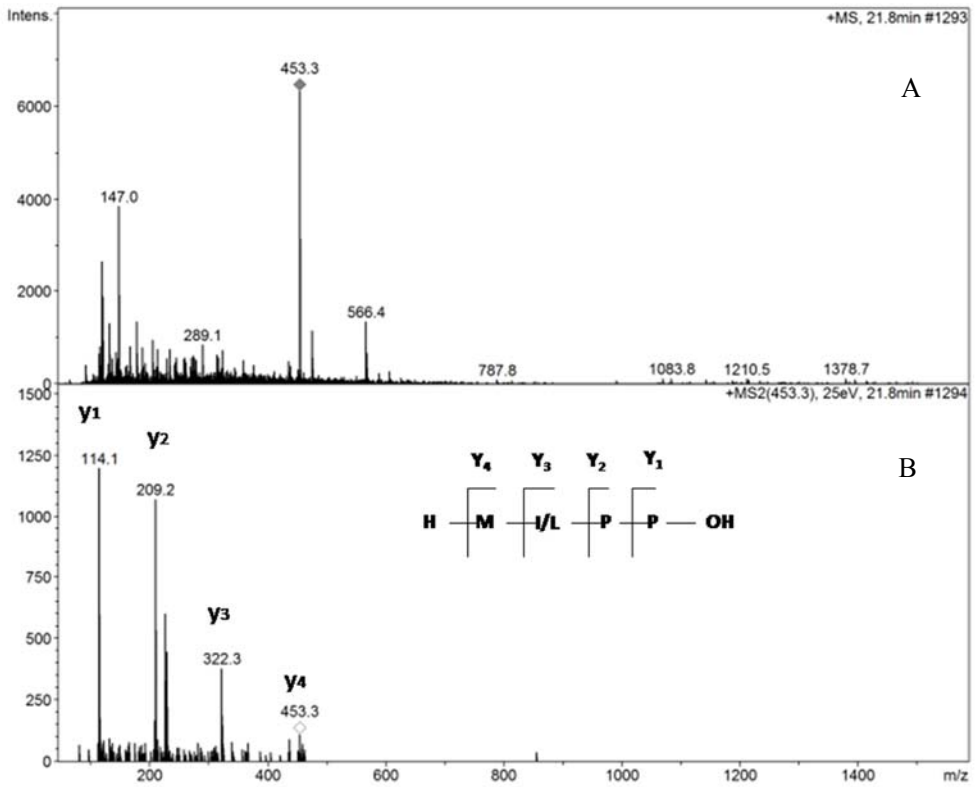

Fig. 5. Identification of molecular mass and amino acid sequence of CPs-2-1. (A) IT-MS spectrum of CPs-2-1. (B) IT-MS/MS spectrum of m/z 452.3.

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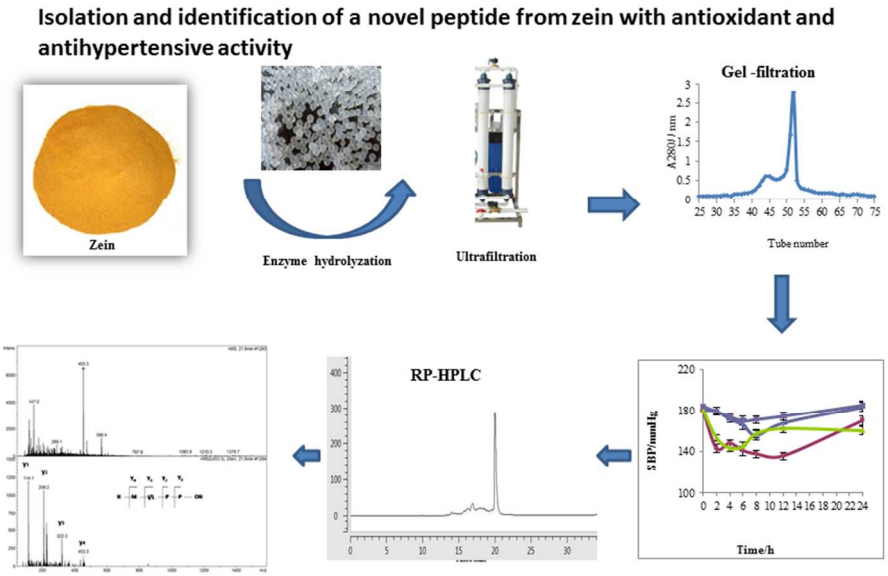

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