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Isolation and Characterization of Angiotensin I Converting Enzyme (ACE) Inhibitory Peptides from Rice Bran Proteins and Evaluation of Activity and Stability

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YL designed the study and purchased materials. BD performed experimental work, analyzed the data and wrote the article. YL and LF helped in figures. YM and YS provided testing equipment.

Key words
ACE inhibitory peptides, Purification, RB protein, Simulated digestion

ABSTRACT

The aim of this study was to investigate anti angiotensin I converting enzyme (ACE) activity of the rice bran (RB) and comparison of its activity with selected commercial antihypertensive pharmaceutical. A new peptide with ACE inhibition properties was obtained by using alkaline protease to enzymatic hydrolysis of RB crude protein purified by ultrafiltration, size exclusion chromatography and RP-HPLC. Using consecutive chromatographic techniques successively, the acidic hydrolysates of RB proteins were fractionated and the new ACE inhibitory triplet was isolated and identified. The amino acid sequence of the ACEI was identified as Ile-Thr-Leu or Leu-Thr-Ile. *In vitro,* ACE inhibition assays showed that the IC_{50} value of the peptide was 0.0118 mg·ml^{-1}. The inhibitory activity of the peptide slightly increased after incubation with gastrointestinal proteases. It was revealed that the ACE inhibiting activity of isolated peptide was lower than that of e nalapril maleate, however, higher than that of all the other antihypertensive pharmaceutical tested. Moreover, the peptide with stable and strong ACEI activity has the potential application in functional and novel foods, dietary supplements or even pharmaceuticals as an antihypertensive agent.

INTRODUCTION

Angiotensin I-converting enzyme (ACE) plays an important physiological role in regulating blood pressure, which belongs to the class of zinc proteases that need zinc and chloride for their activity. Specific inhibitors of ACE have proved to be useful antihypertensive drugs. Many synthetic ACE inhibitors including captopril, enalapril, lisinopril and others are available for clinical use (Brown *et al*., 1998; Raia *et al*., 1990). ACE inhibitors are well tolerated by most patients, however some undesirable side effects may occur such as cough, loss of taste, renal impairment and angioneurotic edema (Alemán *et al*., 2011). The peptides derived from food proteins are considered to be milder and safer compared with synthetic drugs. Furthermore, these peptides usually have multifunctional properties and are easily absorbed by humans (Raia *et al*., 1990; Brown *et al*., 1998).

ACE inhibitory peptides can be classified into three groups (Iroyukifujita *et al*., 2000). The first group comprises of true inhibitors, whose activity is not changed by pre-incubation with ACE. The second group comprises of substrates for ACE, which converts them to inactive peptides. The third group comprises of the so-called pro-drug peptides, which are converted to true inhibitors by ACE or gastrointestinal proteases, resulting in increased activity. Studies have shown that only true inhibitors and pro-drugs have the ability to lower blood pressure (Fujita *et al*., 1999; Iroyukifujita *et al*., 2000; Gu *et al*., 2011).

Many studies have been directed toward enzymatic hydrolysates of different food proteins. Peptides that inhibit ACE in vitro have been identified in hydrolysates of various food sources such as casein (Gobbetti *et al*., 2000; Silva *et al*., 2005; Lee *et al*., 2012), whey protein (Pihlanto *et al*., 2000; Vermeirssen *et al*., 2004; Pan *et al*., 2012), soybean (Wu *et al*., 2002; Kuba *et al*., 2005; Vercruysse *et al*., 2008), rapeseed (Marczak *et al*., 2003), mushroom (Lee *et al*., 2004), seafood (Je *et al*., 2005; Tsai *et al*., 2008; Zhao *et al*., 2009; Zhang *et al*., 2009; Hwang *et al*., 2010; Rojas-Ronquillo *et al*., 2012) and porcine myosin B (Muguruma *et al*., 2009). However, still little is known about the ACE inhibitor of the rice bran (RB).

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Purification of ACE inhibitory peptides (ACEIPs) is generally accomplished by using one or a combination of different chromatographic techniques. For example, ultrafiltration can be used to get a roughly separate into a high or a low molecular weight fraction (Hyun *et al.*, 2000). Alternatively, gel filtration also separates peptides according to their molecular weight. Normally, gel filtration is followed by reversed phase high performance liquid chromatography (RP-HPLC) using C18 columns (Zhang *et al.*, 2009; Guo *et al.*, 2009; Liu *et al.*, 2010). One or more HPLC runs may be necessary to obtain the satisfactory degree of purification.

RB is rich in proteins and the first byproduct of rice is often wasted as animal feed. RB proteins that have low sensitivity and are easily absorbable and can therefore be used as a cheap source of protein for humans.

Therefore, converting RB into economically valuable products is desirable. This study describes the extraction and characterization of a novel ACE inhibitor peptide from RB protein, which can be used as an antihypertensive drug. The ACE inhibitory activity of the hydrolysis was also determined *in vitro*. In addition, by using size exclusion chromatography followed by RP-HPLC, a new ACE inhibitory tripe tide from the hydrolysis was isolated.

MATERIALS AND METHODS

Materials

The RB was obtained from Xi Jie of Bei Da Huang, China. ACE (from rabbit lung), the triplet peptide (Hip-His-Leu), Sephadex G-15 was purchased from Sigma Chemical Co., USA.. Alcalase (EC 3.4.21.62, from *Bacillus licheniformis*) was purchased from Novo Co., Denmark. Ultrafiltration membrane was purchased from Tianjin Mo-tian-mo Co., China. All other reagents used in this study were analytical grade chemicals, purchased from Tianjin Tian-xin Fine Chemical Development Center.

Preparation of RB protein hydrolysates

Preparation of protein extract

RB protein was extracted by using hexane. The ratio of RB to hexane was 1:8 (w·v^{-1}), then which was mixed using a magnetic force blender (Model SHJM-1, Xiandai, China) for 30min, and then centrifuged at 3000 rmin^{-1}, 10min, the pellet was taken in order to obtain defatted RB. The ratio of defatted RB to distilled water was 1:10 (w·v^{-1}). The mixture was adjusted to pH 9.45 by using 1 mol ml^{-1} sodium hydroxide, and was then incubated at 50 °C for 3 h. The pH of the mixture was monitored every 10 min and adjusted to 9.45 if required. The mixture was centrifuged at 3500 rmin^{-1} for 15 min, the supernatant was collected, and then pH was adjusted to 4.1 ~ 4.5 by 0.5 mol·l^{-1} hydrochloric acid. After 0.5 mol·l^{-1} HCL for pH adjustment, the mixture was centrifuged at 3000 rmin^{-1} for 10 min. The resulted pellet was washed three times with distilled water.

Preparation of protein hydrolysates

Protein solution in distilled water (3.3%) was digested with alcalase (3209 U·g^{-1}) at pH 9.1 and 40 °C for 4 h. The reaction was terminated by boiling the mixture and the resulted hydrolysis was collected for further analysis.

Measurement of ACE inhibitory activity in vitro

The ACE inhibitory activity was measured using the method of Cushman (Cushman *et al.*, 1971) with slight modifications. A sample solution (25 µl) with 50 µl substrate (8.3 mmol·ml^{-1} Hip-His-Leu in 0.1 mol·ml^{-1} sodium borate buffer containing 0.3 mol·ml^{-1} NaCl at pH 8.3), at 37 °C for 5 min. Then ACE solution (25 mU·ml^{-1}) was added and incubated at 37 °C for 60 min. The reaction was terminated by the addition of 1.0 mol·ml^{-1} HCl (200 µl). The resulting hip uric acid was extracted with 1.0 ml of ethyl acetate. After centrifugation (4000 rmin^{-1}, 15 min), 1 ml supernatant was transferred into a test tube, and left to evaporate at 120 °C for 30 min in a drying oven. The hip uric acid was dissolved in 4.0 ml of distilled water, and the absorbance was determined at 228 nm using a spectrophotometer (Model UV-2550, Shimadzu, Japan). The IC_{50} value was defined as the concentration of the inhibitor that could inhibit 50% of the ACE activity.

Determination of peptide concentration

The peptide concentration of the samples was measured by mixing a 50 ml sample with 2 ml formaldehyde reagent (Church *et al.*, 1983). After incubation for 2 min at 25 °C, absorbance was measured at 340 nm. A standard curve (0~1000 mg·ml^{-1}) of casein peptone was used to calculate the peptide concentration of the samples.

Purification of ACE inhibitory peptide

Ultrafiltration

The protein hydrolysis was isolated by using ultrafiltration (UF) system with 10 kDa and 6 kDa molecular weight (MW) cut-off UF membranes (Motianmo, Tianjin, China), successively. Three fractions with molecular weights of > 10 kDa (I), 6 ~ 10 kDa (II), < 6 kDa (III) were subjected to SDS-PAGE in order to determine the MW of the proteins in each fraction.

Size exclusion chromatography

The column used for gel filtration was a Sephadex

G-15 gel column (φ16 mm×300 mm) and eluted with 0.01 mol·ml^{-1} hydrochloric acid at a flow rate of 0.5 ml·min^{-1} at room temperature. Fractions of 3 ml were collected with a fraction collector. The absorbance of the eluate was measured at 280 nm. The highest active fractions were pooled and lyophilized, immediately.

Reversed phase high-performance liquid chromatography

The samples that showed the highest activity were further separated by RP-HPLC on a C18 column (Shim-Pack PREP-ODS, 12 µm, 250 mm×4.6 mm, Varian, Sint-Katelijne-Waver, Belgium). Mobile phase A was 5% acetonitrile containing 0.1% trifluoroacetic acid (TFA), and mobile phase B was 80% acetonitrile containing 0.1% TFA. Separation was done with a linear gradient of Mobile phase A and B (0~20 min, 0~80% B; 20~24 min, 80%~0 B; 25~60 min, 100% A; in 60 min) at a flow rate of 0.5 ml·min^{-1} and UV absorbance was monitored at 220nm using a diode array detector (Thermo Electron). The ame dose of sample (100.0 µl) was injected every time, fractions were collected, pooled and then peptide concentration and ACE inhibitory activity were measured *in vitro*. One active fraction was saved for peptide identification.

Then the active fraction was separated with a linear gradient (0~60 min, 0~80% B) at a flow rate as 1.0 ml·min^{-1}. The UV absorbance of the fractions were monitored at 220 nm.

Identification and analysis of the purified peptide

Amino acid composition

The 40.0 µl of sample was oven dried and hydrolyzed in vacuum-sealed glass tubes at 110 °C for 24 h in the presence of boiling 6 mol·ml^{-1} HCL containing 0.1 % phenol with norleucine as internal standard. The volume of hydrolytic acid was adjusted to 50 ml. After filtration, 1 ml of the filtrate was subjected to vacuum drying and then was added to 1 ml of 0.02 mol·ml^{-1} HCl and finally, was air dried for 30 min. An automatic amino acid analyzer (L-8800, Japan) was used for the determination of amino acid composition of the purified peptide.

MALDI-TOF MS analysis

Sample solution (1 µl) was added to 1 µl of saturated solution of α-cyano-4-hydroxycinnamic acid, and 1 µl of the mixture was spotted on a 192 hole stainless steel MALDI target plate and air dried. Trypsin hydrolysis of myoglobin was used as internal standard. MALDI manufacturer and model (e.g. Ultraflexx II, Bruker).

Stability of peptide for gastrointestinal proteases

The stability of the ACE inhibitor peptide (ACFIP) against gastrointestinal proteases was assessed *in vitro*. Twenty milligram of freeze dried purified peptide was dissolved in 4 ml of 0.05 mg·ml^{-1} pepsin. The pepsase was prepared by using 0.1 mol·ml^{-1} KCI-HCl, pH 2.0 and incubation in a 37 °C water bath for 4 h. The reaction was stopped by boiling the mixture; the pH of the mixture was then adjusted to pH 8 by adding NaOH. Two milliliter of the solution was centrifuged (12000 rmin^{-1}, 5 min). The supernatant was used for ACE inhibitor activity assay. The remaining solution was subjected to further digestion with 2 ml of 0.05 mg·ml^{-1} pancreatic proteases at 37 °C for 4 h. The enzyme was inactivated by boiling for 5 min. The mixture was then centrifuged (12000 rmin^{-1}, 5 min). The supernatant used for ACE inhibitory activity determination.

Comparison of other antihypertensive compounds with RB extract in vitro

Purified peptide E-9 compared with seven different types of synthetic antihypertensive drugs, including enalapril maleate, valsartan, nifedipine, losartan, hydrochlorothiazide, prazosin hydrochloride, atenolol, and reserpine triamterene. Then 50 µl of 3.0 mg·ml^{-1} purified RB peptide fraction E-9 was used to measure ACE inhibition activity.

Assessment of the stability of ACE inhibitor peptides

Based on the above, 20 µl of 2.0 mg·ml^{-1} ACE inhibitory peptide was incubated with 20 µl (4 mU) of ACE at 37 °C for 3 h, and the activity was measured every 30 min. The relative ACE inhibition activity was compared before and after incubation.

RESULTS AND DISCUSSION

ACE inhibitory activity of ultrafiltration fractions

Firstly, RB protein was hydrolyzed using alcalse, which was fractionated by ultra filtration into three components (Table I). Sub-fraction I showed the molecular weight (MW) of greater than 10 kDa, with an IC_{50} value of 14.47 mg·ml^{-1}. The Sub-fraction II and III showed the components with 6 ~ 10 kDa and < 6 kDa, the IC_{50} values were 3.516 and 0.8848 mg·ml^{-1}, respectively. However, the fraction III showed the most significant ACE inhibitory activity (Byun *et al*., 2001; Je *et al*., 2005; Alemán *et al*., 2011; Pan *et al*., 2012). The ACE inhibitory activity of sub-fraction II was higher than I (Table I). After ultrafiltration, the peptide recovery rate was 84.62% and the activity recovery was 82.14%.

Prior to the use of dextran gel, high-performance liquid chromatography and other methods were used to determine the MW range of the components III. The MW

of fraction III was estimated, the value was 5.8 kDa. These results were in line with the theoretical properties of the peptides on interest. The MW range of ACE inhibiting peptide was wide. Other investigations on ACE inhibitory peptides indicated that they are consisted of 2 to 15 peptides with MW from 200 to 1500 Da (Byun *et al*., 2001; Je *et al*., 2005; Alemán *et al*., 2011; Pan *et al*., 2012).

Table I.- Analysis of RB proteinous fractions after ultrafiltration.

Fraction number	Molecular weight (MW)	Peptide content (μg·ml^{-1})	ACE inhibition rate (%)	IC_{50} (mg·ml^{-1})
I	> 10kDa	3.05	10.53	14.47
II	6~10kDa	1.69	23.96	3.51
III	< 6kDa	1.31	73.80	0.88

Size exclusion chromatography

ACE inhibiting peptides are hydrophobic, therefore with Sephadex gel might exist between strong hydrophobic interactions. In order to avoid the abnormal peak shape of on Sephadex G-15 column chromatogram, 0.01 mol·ml^{-1} HCL was used to reduce the ionic strength of the salt solution. The Sephadex G-15 gel column was also used for further fractionation. The separated fractions of sub-fraction III were designated as A to E (Fig. 1). Collection of the corresponding peak components, peptide concentration and *in vitro* ACE inhibitory activity were measured for fractions A to E (Table II).

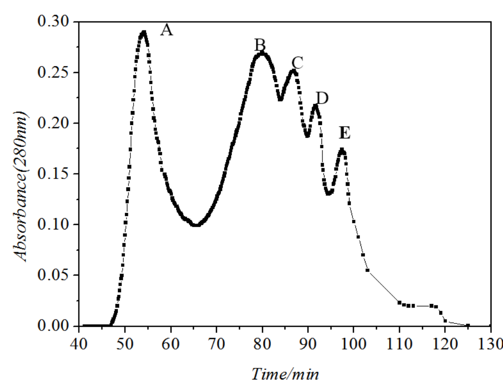

Fig. 1. Absorbance maximum of peptides separated from the RB sub-fraction III by using Sephadex G-15 chromatography.

According to the molecular weight of the substance, Sephadex G-15 chromatography separated the compounds. The column size was limited up to 1.5 kDa. Because of the potential blockage, some molecules could be trapped in the column, the filler particle clearance was followed. There was no ACE inhibitory activity for fraction A (the first fraction eluted from sephadex column) (Table II). This might also indicate instability of the ACE substrate, hippuryl-L-histidyl-L-leucine (HHL) triplet or presence of a degrading compound in fraction which breaked down HHL and releases HL.

Although ACE inhibitory activity was widely observed in all fractions, fraction A and E had the highest peptide concentration (Table II). Fraction E was last to elute that exhibited the highest ACE inhibitory activity with an IC_{50} value of 0.0546 mg·ml^{-1}. After Sephadex G-15 column chromatography, the fraction E peptide recovery rate reached 84.15 %, and the activity recovery was 90.02 %.

Table II.- Properties of five components of RB sub-fraction III by Sephadex G-15.

Peak	Peptide concentration (μg·ml^{-1})	ACE inhibition rate (%)	IC_{50}(mg·ml^{-1})
A	102.5	-	0.0938
B	84.7	52.76	0.0803
C	60.3	38.51	0.0783
D	51.2	34.34	0.0746
E	86.0	78.69	0.0546

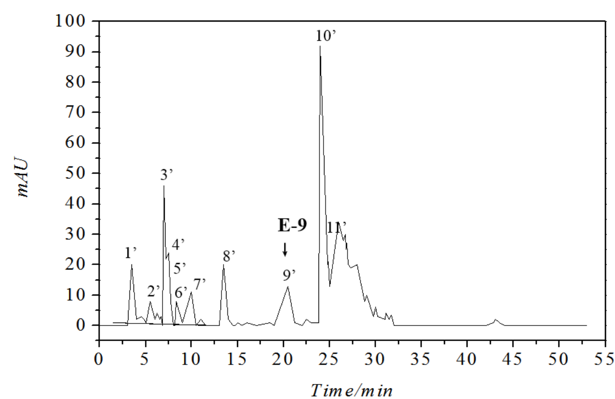

Fig. 2. RP-HPLC chromatogram of peptides separated from fraction E.

Reversed phase high-performance liquid chromatography

The fraction E was further purified by using preparing RP-HPLC on a SP reversed phase semi-preparative column with gradient elution several times. Chromatographic profile of fraction E at 220 nm indicated that this fraction consisted of a mixture of peptides. Twenty-three

components were sub-fractionated from fraction E (Fig. 2). The peaks at 12'~23' had relatively short retention time, so the number of these peaks was not noted. The relevant 11 isolated peptides were designated fractions E-1~E-11 and their concentrations and ACE inhibitory activities were determined (Table III). The highest ACE inhibition was observed for fraction E-9 with an IC_{50} value of 0.0118 mg·ml^{-1}. This fraction was lyophilized to determine molecular mass of the peptide.

Table III.- RP-HPLC analysis results of eleven components of the RB fraction E (E-1~E-11).

Peak	Retention time (min)	Peptide concentration (μg·ml^{-1})	ACE inhibition rate (%)	IC_{50} (mg·ml^{-1})
E-1	3.604	47.2	23.56	0.1002
E-2	5.392	—	—	—
E-3	6.067	31.7	27.23	0.0582
E-4	6.322	—	—	—
E-5	7.788	41.5	36.92	0.0562
E-6	8.453	70.4	57.11	0.0616
E-7	11.307	68.8	66.17	0.0520
E-8	11.590	61.3	49.33	0.0621
E-9	17.615	21.0	89.03	0.0118
E-10	23.616	32.1	54.44	0.0295
E-11	24.299 ~ 28.501	72.7	32.17	0.1129

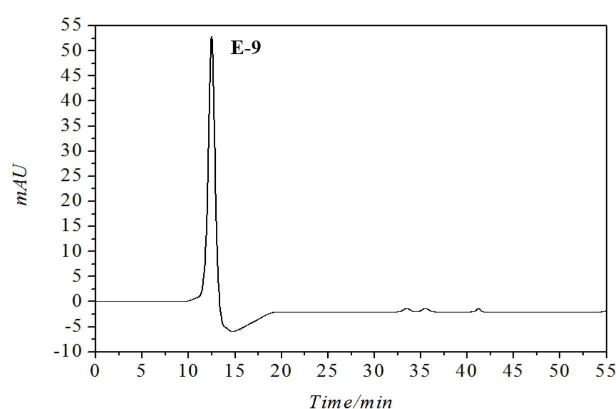

Fig. 3. RP-HPLC analysis result of E-9.

Fraction E-9 was eluted by using RP-HPLC on the Shim-Pack PREP-ODS reverse column (Agilent Co., USA.) with gradient elution several times. Figure 2 shows the chromatographic profile of the fraction E at 220nm, indicating that this fraction is still a mixture. Further RP-HPLC analysis of the E-9 fraction revealed that it was a single compound. After RP-HPLC chromatography, peptide recovery rate of sample reached 97.06%, the activity recovery was 95.32% (Fig. 3).

Using a three-step purification procedure from RB protein hydrolysis with ACE inhibitor activity in sequence, including ultrafiltration, gel filtration and reverse-phase high-performance liquid chromatography respectively, the fraction E-9 (IC_{50} value was 0.0118mg·ml^{-1}) was purified. Peptide recovery rate was 69.11% and the activity recovery was 70.48 %. While, during the purification steps, typical results obtained are summarized in Table IV.

Table IV.- The three steps of separation and purification of RB anti ACE peptide.

Fraction	Purification method	IC_{50} (mg·ml^{-1})	Purification fold
III (< 6 kDa)	Ultrafiltration	0.8848	1.00
E	Sephadex G-15	0.0546	16.21
E-9	RP-HPLC	0.0118	74.98

Identification of the purified peptide

Amino acid composition

Fraction E-9 was subjected to analysis by an automatic amino acid sequence after complete hydroxylation. The analysis results (Fig. 4) showed that the fraction E-9 was a pure peptide which consisted of three amino acids: threonine (Thr, T), isoleucine (Ile, I) and leucine (Leu, L).

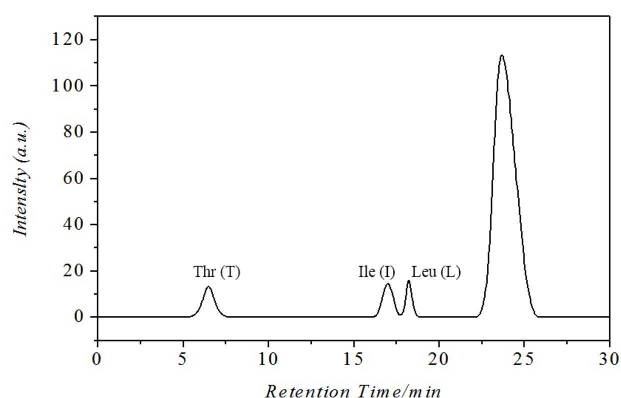

Fig. 4. Peak intensities shown by amino acid HPLC analysis from ACEI (fraction E-9).

MALDI-TOF MS analysis

The mass charge ratio ($m·z^{-1}$) of the purified peptide E-9 was determined by MALDI-TOF mass spectrometry. The panels A and B in Fig.5 showed the mass spectrum of

the ions generated from fraction E-9 that was fragmented into ions of 345.238 and 329.445 $m{\cdot}z^{-1}$. This also indicated that the sample was a purified peptide which was separated by RP-HPLC. The parent ion at 345.238 $m{\cdot}z^{-1}$ was fragmented into amino acid ions, as shown panel B in Figure 5. The MALDI-TOF analysis indicated that the amino acid sequence of E-9 fraction was Ile-Thr-Leu (ITL) or Leu-Thr-Ile (LTI). By looking up the database, it is a novel ACE inhibitory peptide potentially, with an IC_{50} value of 0.0118 mg·ml^{-1}.

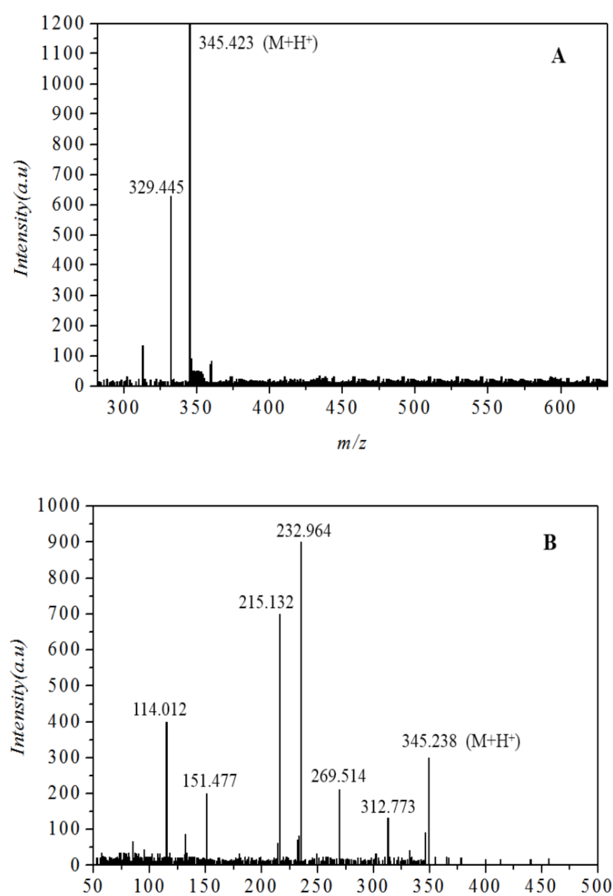

Fig. 5. MALDI-TOF MS (A) and MS/MS spectra of fraction E-9 (B).

It is reported that most of the bioactive peptides contain 2 to 20 amino acids (Iroyukifujita *et al.*, 2000; Gobbetti *et al.*, 2000; Pihlanto *et al.*, 2000; Vermeirssen *et al.*, 2004; Liu *et al.*, 2010; Alemán *et al.*, 2011). Furthermore, there are indications that only *di*- and *tri*-peptides can be absorbed intact into the human circulatory system via the intestinal peptide transporter PEPT1 (Vercruysse *et al.*, 2008; Zhang *et al.*, 2009). It is necessary for ACEIPs to exert their physiological function (Muguruma *et al.*, 2009). What's interesting to us is that the RB protein obtained by alkaline protease hydrolysis is purified by a series of methods, ACE inhibiting peptide chain length of *tri*-ptides also is in the range that most bioactive peptides contain 2 to 20 amino acids.

Stability of peptide against gastrointestinal proteases

In order to inspect the stability of ACE inhibitor, RB antihypertensive peptides (RBAP) were enzymatically digested by intestinal enzymes, pepsin and trypsin, respectively (Fig. 6). Fraction E-9 was subjected to digestion by pepsin. The digested material showed higher ACE inhibitory activities than the original fraction. There might be two explanations for the observed increase in ACE inhibition activities. On one hand, certain components with the original ACE could show higher activities to inhibit ACE (Zhao *et al.*, 2009). On the other hand, some fractions did not inhibit the activity of ACE, but some new active components might be produced by the effect of pepsin which could exhibit better inhibitory activity. As a result, the IC_{50} value changed slightly smaller, from 0.0118 to 0.0104 mg·ml^{-1}. This was also conserved that after digestion with pepsin the ACE suppression activity of the RB was increased.

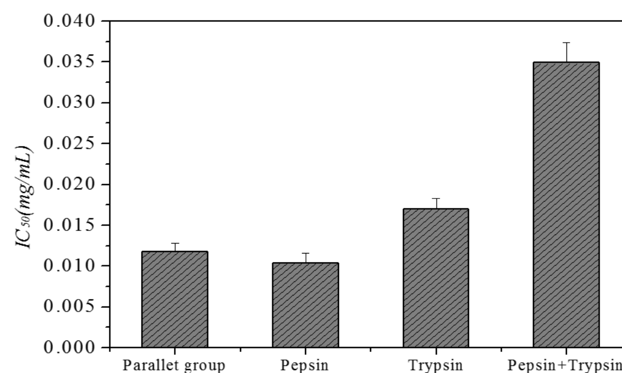

Fig. 6. The IC_{50} values of fraction E-9 after gastrointestinal digestion.

After digestion of the fraction E-9 by trypsin alone, the IC_{50} value of the product increased up to 0.0170 mg·ml^{-1}. Moreover, the peptide digested by pepsin and trypsin in sequence, the product showed less ACE inhibition activity, and IC_{50} value increased to 0.0349 mg·ml^{-1}. The main active antihypertensive peptides of RB peptides could resist enzymatic hydrolysis by pepsin, trypsin, and other digestive enzymes significantly, so they still retained their ACE inhibitor activity. Therefore, these suggested that RBAP might have lowering blood pressure significantly after oral administration.

Table V.- The comparison of E-9 with seven synthetic antihypertensive drugs *in vitro.*

Test No.	Antihypertensive drugs	Property	Effective concentration (mg·ml^{-1})	ACE inhibition (%)	IC_{50} value (mg·ml^{-1})
1	E-9	—	3.0	42.21	0.0118
2	Enalapril maleate	ACEI	3.0	77.47	0.0034
3	Valsartan	Angiotensin II receptor blockers (ARB)	3.0	NA	NA
4	Nifedipine	Calcium antagonists	3.0	NA	NA
5	Losartan hydrochlorothiazide	diuretic	3.0	NA	NA
6	Prazosin hydrochloride	α - receptor inhibitors	3.0	NA	NA
7	Atenolol	α - receptor inhibitors	3.0	NA	NA
8	Reserpine triamterene	compound antihypertensive drug	3.0	22.80	0.0658

NA=Not available

Comparison of activity of the fraction E-9 with antihypertensive drug in vitro

ACE inhibiting properties of the fraction E-9 was compared with seven different types of antihypertensive drugs as shown in Table V. Because there were a few components in reserpine triamterene (antihypertensive drug), which might be an angiotensin converting enzyme inhibitor. Under warm environment, it will have a certain low level of inhibition on ACE with an IC_{50} value was 0.0658 mg·ml^{-1} *in vitro*. The main ingredient of Enalapril maleate tablets is angiotensin converting enzyme inhibitors (ACEI), at the same dose, compared to other types of antihypertensive drugs, and had the strongest inhibition against ACE *in vitro*, with an IC_{50} value of 0.0034 mg·ml^{-1}. Since the IC_{50} of antihypertensive peptide E-9 was 0.0118 mg·ml^{-1}, this may well be due to the fact that the peptide E-9 and drug ACEI react with different active sites of ACE. On the other hand, some compositions of drugs ACEI may also contain other compounds, which still have high ACE inhibition *in vitro*. The ACE inhibition of valsartan, nifedipine, losartan and hydrochlorothiazide, prazosin hydrochloride, atenolol and other antihypertensive drugs were not measured *in vitro,* due to these compounds were different with ACEI which did not contain any ACE inhibitor or blood pressure lowering properties. These compounds suggestted that hypertensive effects that cannot be expressed *in vitro*, so did not detect any ACE inhibitor activity *in vitro*.

Stability of E-9 fraction against ACE

RB ACE purified peptides (fraction E-9) interacted with ACE at 37°C, the relative ACE inhibition rate was remained more than 90% during 3 h. Its activity had only slight changes and the comparative ACE inhibitor activity trend was shown in Fig. 7. The results illustrated clearly that ACE inhibition of E-9 fraction of RB in this study retained high stability relatively after each time point.

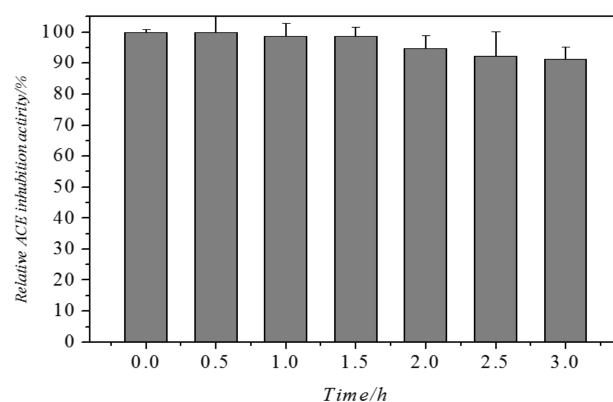

Fig. 7. The relative ACE inhibition rate of fraction E-9.

CONCLUSIONS

This study demonstrates that RB protein can be used as a source of ACEIPs after hydrolysis with alkaline protease. The novel type of ACE inhibitor tripeptide was purified by ultrafiltration, size exclusion chromatography and RP-HPLC successively. The amino acid sequence of the ACEI was found to be Ile-Thr-Leu (ITL) or Leu-Thr-Ile (LTI). The purified peptide was evaluated for ACE tolerance. RB ACE purified peptides of fraction E-9 interacted with ACE at 37 °C during 3 h, its activity had only slight changes and the relative ACE inhibition rate remained more than 90 %. Moreover, seven different blood pressure lowering drugs were compared with the E-9 at the same dose. Enalapril maleate showed the strongest inhibition to ACE *in vitro*, with an IC_{50} value of 0.0034 mg·ml^{-1}. However, the IC_{50} value of antihypertensive peptide E-9 was 0.0118 mg·ml^{-1}. While the antihypertensive drug had a certain low level of inhibition on ACE with an IC_{50} value was 0.0658 mg·ml^{-1}. This peptide has the potential application in functional

foods, dietary supplements or even pharmaceuticals as an antihypertensive agent.

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Statement of conflict of interest

Authors have declared no conflict of interest.

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