



Purification and characterization of angiotensin I-converting enzyme inhibitory peptides from enzymatic hydrolysate of hen egg white lysozyme

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ARTICLE INFO

Article history:

Received 21 August 2011

Accepted 10 December 2011

Available online 22 December 2011

Keywords:

Hen egg white lysozyme

Angiotensin I-converting enzyme

ACE inhibitory peptide

Gastrointestinal enzymes

ABSTRACT

The aims of this study were to explore new functional bioactivity of hen egg white lysozyme (HEWL) protein hydrolysate and to identify potential angiotensin I-converting enzyme (ACE) inhibitory peptides from HEWL. The protein was hydrolyzed by gastrointestinal enzymes, including pepsin, α -chymotrypsin and trypsin, under simulated physiological conditions, and the hydrolysate exhibited potent ACE inhibitory activity with an IC_{50} value of $15.6 \pm 1.4 \mu\text{g/mL}$. Three novel ACE inhibitory peptides, specifically, Met-Lys-Arg, Arg-Gly-Tyr and Val-Ala-Trp with IC_{50} values of 25.7 ± 0.2 , 61.9 ± 0.1 and $2.86 \pm 0.08 \mu\text{M}$, respectively, were isolated from the hydrolysate of HEWL in two stages of reverse-phase high-performance liquid chromatography (RP-HPLC), and their sequences were analyzed by UPLC-MS/MS. Kinetics studies suggested that the purified peptides were all competitive inhibitors; preincubation experiments implied that the peptide Arg-Gly-Tyr was a true substrate and that the other two peptides were true ACE inhibitors. These results indicate that HEWL may become an effective source of ACE inhibitory peptides. This study reveals the antihypertensive bioactivity of HEWL protein hydrolysate and provides further evidence that eggs are an excellent source of health-promoting food.

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1. Introduction

Hypertension is related to the incidence of coronary heart disease and treatment of it effectively reduces the risk of cardiovascular disease and related ailments (Collins et al., 1990). The rennin-angiotensin system is one of the important mechanisms regulating blood pressure. A key enzyme in this system is angiotensin I-converting enzyme (ACE; EC.3.4.15.1). ACE increases blood pressure by converting inactive angiotensin I to the potent vasoconstrictor angiotensin II as well as by inactivating the vasodilator bradykinin (Rohrbach, Williams, & Rolstad, 1981). Therefore, the inhibition of ACE activity is effective to reduce blood pressure. From this perspective, many synthetic ACE inhibitors, including captopril, enalapril, lisinopril, nifedipine, losartan and others, have been developed to treat and prevent hypertension. Although presenting obvious antihypertensive effects, these drugs have some undesirable side effects, such as dry cough, taste disturbances, skin rashes and organ dysfunctions (Brown & Vaughan, 1998; Glasser, 2001). For the last four decades, the natural food-derived ACE inhibitory peptides have attracted much attention, due to their predominance over synthetic drugs in terms of mildness, safety, reliability, absorbability and lack of effect on normal blood pressure (De Leo, Panarese, Gallerani, & Ceci, 2009). To date, a large number of ACE inhibitory peptides with

various amino acid sequences have been found in hydrolysates from food proteins, including egg proteins (Miguel & Aleixandre, 2006), that were digested with different proteases under different hydrolysis conditions. The ovalbumin (Fujita, Yokoyama, & Yoshikawa, 2000) and ovotransferrin (Lee, Cheng, Enomoto, & Nakano, 2006; Liu et al., 2010) that are derived from egg-white proteins have been frequently reported to contain ACE inhibitory peptides. Nevertheless, the peptides with ACE inhibitory activity derived from hen egg white lysozyme (HEWL) are not reported. It is likely that the active peptides from HEWL are overlooked when screening for ACE inhibitory peptides from hen egg protein by enzymatic hydrolysis, due to the low level of HEWL in eggs (about 3.5% of total hen egg protein) (You, Udenigwe, Aluko, & Wu, 2010).

HEWL is an antimicrobial protein that has been commercialized for use as a natural preservative to control bacterial growth in foods (You et al., 2010). Except for the antibacterial action of HEWL itself, enzymatic hydrolysates of HEWL showed antibacterial activity (Abdou, Higashiguchi, Aboueleinin, Kim, & Ibrahim, 2007), antioxidant activity (You et al., 2010), and inhibitory activity against calmodulin-dependent phosphodiesterase (You et al., 2010). However, no knowledge is available regarding the ACE inhibitory activities or antihypertensive effects of these hydrolysates. The amino acid composition analysis, conducted using on-line software (ProtScale), shows that the HEWL protein contains high proportion of hydrophobic (43.4%) and basic amino acid residues (13.7%) in its sequence. The presence of the hydrophobic or basic amino acid residues enhances the inhibitory potency (Wu, Aluko, & Nakai, 2006a, 2006b). This

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finding suggests that research on the potentially bioactive peptides with ACE inhibitory activity from HEWL should be conducted.

To exert antihypertensive potency *in vivo*, the peptides must reach target sites in an active form. Gastrointestinal digestion and transport are the main barriers to the bioavailability of ACE inhibitory peptides (Vermeirssen, Van Camp, & Verstraete, 2004). Hydrolysis with gastrointestinal proteases can be used to produce ACE inhibitory peptides with the advantage that the formed peptides will resist the physiological digestion after oral administration (Matsui et al., 2002). Many studies have demonstrated that a simulated gastrointestinal digestion is notably effective in the formation of ACE inhibitory peptides, and a number of these peptides have been verified by animal experiments to lower blood pressure significantly *in vivo* (Matsui et al., 2002; Yang, Marczak, Yokoo, Usui, & Yoshikawa, 2003). In the present study, an HEWL hydrolysate was prepared by gastrointestinal enzymes, fractionated by reversed phase chromatographic method, and characterized by LC–MS. Three ACE inhibitory peptides derived from HEWL are identified, and the inhibition mechanisms and stability of the peptides on ACE were investigated. Furthermore, the structure–activity relationship of the isolated peptides is also discussed.

2. Materials and methods

2.1. Materials

HEWL was purchased from Dalian Greensnow Egg Products Development Co., Ltd. (Dalian, China). Angiotensin I-converting enzyme (EC 3.4.15.1) from rabbit lung, pepsin from hog stomach, α -chymotrypsin from bovine pancreas, and trypsin from New Zealand-sourced pancreas were purchased from Sigma. Hippuryl-histidyl-leucine (HHL) was obtained from the Peptide Institute (Osaka, Japan). All other reagents, unless otherwise specified, were of analytical or guaranteed reagent grade.

2.2. *In vitro* digestion of HEWL

The two-stage process of producing HEWL enzymatic hydrolysate was carried out according to the method of Matsui et al. (2002) with some modifications. HEWL was dispersed in a 0.9% (w/w) NaCl solution to give a concentration of 0.2% (w/v), and the pH of the protein solution was adjusted to 2.0. After heating in a water bath at 80 °C for 15 min, the protein slurry was cooled at 37 °C. Afterwards, the sample was incubated with pepsin (1:50, w/w) for 5 h at pH 2.0 and 37 °C followed by hydrolysis with α -chymotrypsin and trypsin (1:100, w/w) for 5 h at pH 7.5 and 37 °C. Hydrolysis was carried out in a shaking bath (thermally controlled incubator) under constant stirring (100 g), and the reactions were stopped by heating at 95 °C for 10 min followed by cooling to room temperature. At different time intervals during the hydrolysis process, samples were withdrawn and placed in a boiling water bath for 10 min to inactivate the enzyme. The samples were then centrifuged (10,000 g, 20 min, 4 °C), and the supernatants were collected, lyophilized and stored at –20 °C until further analysis.

2.3. Purification of ACE inhibitory peptides

The gastrointestinal digest of HEWL was fractionated using preparative Waters 1525 HPLC (Waters Corporation, Milford, MA) on a Sunfire Pre C18 column (19 × 150 mm, Waters) with a linear gradient of acetonitrile (5–65% in 60 min) at a flow rate of 7 mL/min. The injection volume was 1 mL, and the injected sample had a peptide concentration of 6 mg/mL. The same injection was conducted for five times. The elution fractions were detected at a wavelength of 214 nm, collected, pooled and vacuum-dried using a centrifugal concentrator (Labconco, USA), and were re-dissolved in 1 mL of deionized

distilled water to assay the peptide concentration and the ACE inhibitory activity. The resultant fractions that exhibited the strongest ACE inhibitory activities were subjected to analytical RP-HPLC for further peptide separation in an XBridge™ C18 column (4.6 × 150 mm, Waters, USA). The flow rate of the mobile solvent was set at 0.8 mL/min, and the linear gradient of acetonitrile concentration for each sample was based on the polarity of the fractions from preparative RP-HPLC. The elution peaks were detected at a wavelength of 214 nm, and the major peaks were collected, pooled and vacuum-dried using a centrifugal concentrator (Labconco, USA) for the determination of ACE inhibition.

2.4. Analysis by online UPLC–MS/MS

The collected peak with the highest ACE inhibition was subjected to ultraperformance liquid chromatography (UPLC) for peptide separation in an Acquity UPLC BEH C18 column (2.1 × 120 mm) (Waters Corporation, Milford, MA). Ten microliters of each peptide sample (0.5 mg/mL) was loaded for each elution. The elution was made in linear gradient mode from 0.1% formic acid to 40% acetonitrile containing 0.1% formic acid (20 min) at a flow rate of 0.3 mL/min. Identification of the sequence of peptides present in the fraction exerting remarkable ACE inhibitory activity was performed by matrix-assisted laser desorption/ionization time-of-flight/time-of-flight mass spectrometry (MALDI-TOF-TOF MS). Both MS and MS/MS data were acquired with a Waters Synapt Mass Quadrupole Time-of-Flight Mass Spectrometer (Waters Corporation, Milford, MA). Spectra were recorded over the mass/charge (*m/z*) ranges of 100–1500 in both MS and MS/MS modes. Peptide fragmentation in the MS and MS/MS mode was respectively achieved by collision-induced dissociation (CID) using atmospheric air as the collision gas, and the collision energies were set at 6 eV and 20 eV for MS and MS/MS, respectively. The signal threshold to perform auto-MS/MS in the data-dependent acquisition was 20 counts/s in the total ion current, and the precursor ions were isolated within a range of *m/z* 3.0. Instrumental control and data analysis were performed using MassLynx software version 4.1 (Micromass U.K. Ltd., Wythenshawe, Manchester, U.K.). Both the peptide sequencing module of the software and manual calculations were used to process the MS/MS data and to perform peptide sequencing. The peptide sequences were matched to the published sequence of the HEWL protein derived from protein data in UniProtKB.

2.5. Assay of ACE inhibitory activity

The ACE inhibitory activity of the peptides were measured *in vitro* following the spectrophotometric assay described by Cushman and Cheung (1971), with some modifications as explained below. A 10–100 μ L solution of each sample was mixed with 80 μ L of a 5 mM hippuryl-L-histidyl-L-leucine (HHL) borate buffer containing 100 mM borate and 300 mM NaCl (pH 8.3), and the volume was subsequently increased to 190 μ L with 100 mM borate. The borate buffer (100 mM, pH 8.3) was used as a blank. The above mixture was preincubated at 37 °C for 5 min before adding 10 μ L of ACE solution (3 mU). The reaction mixture was incubated for 30 min at the same temperature and was terminated by the addition of 1 N HCl (200 μ L). The released hippuric acid was extracted with 1.2 mL of ethyl acetate. After centrifugation (4500 g, 5 min), 0.8 mL of the upper layer was transferred to a test tube and vacuum-dried at 80 °C for 30 min by using a centrifugal concentrator (Labconco, USA). The hippuric acid was redissolved in 0.8 mL of deionized distilled water, and absorbance was measured at 228 nm using a spectrophotometer (Shimadzu, Japan). Tests were performed in triplicate for each sample. The concentration of each peptide that was required to inhibit 50% of the ACE activity was defined as the IC₅₀ value.

2.6. Peptide synthesis

The peptides Met-Lys-Arg, Arg-Gly-Tyr, and Val-Ala-Trp were chemically synthesized by GenScript Corporation (Nan Jing, China). The purity of the synthesized peptide was verified by analytical HPLC coupled with MALDI-TOF MS to be higher than 99%.

2.7. Inhibitory kinetics study

To clarify the ACE-inhibition pattern, two different concentrations of peptides Met-Lys-Arg (25 and 50 μM), Arg-Gly-Tyr (50 and 100 μM), and Val-Ala-Trp (2.5 and 5 μM) were added to each reaction mixture according to the method of Katayama et al. (2008) with slight modifications. The enzyme activities were measured at different substrate concentrations (0.5, 1, 2, and 3.5 mM). The inhibition kinetics of ACE in the presence of each peptide was determined with a Lineweaver–Burk plot.

2.8. Stability of the synthetic peptide inhibiting ACE

The stability of the synthesized peptides inhibiting ACE was assessed in vitro according to a previously described method (Yang et al., 2003) with some modifications. Briefly, 100 μL of the 1.5 mM peptide solution were treated with 40 μL of ACE (12 mU) and 160 μL of 0.1 M borate (pH 8.3) for 3 h at 37 $^{\circ}\text{C}$, and the reaction was stopped by heating the solution in a water bath at 95 $^{\circ}\text{C}$ for 10 min; later, the reaction mixtures were cooled to room temperature. Subsequently, the reaction mixtures were centrifuged at 10,000 g for 20 min, and the supernatants were evaluated for ACE inhibitory activity.

2.9. Measurement of peptide content

A spectrophotometric measurement using *o*-phthalaldehyde (OPA) was used to determine the contents of the hydrolysates according to the method of Church, Swaisgood, Porter, and Catignani (1983) with several modification. Briefly, 100 mL of reagent was prepared by mixing 50 mL of 100 mM borax, 5 mL of 20% (w/w) sodium dodecyl sulfate, 80 mg of OPA solution (dissolved in 1 mL of methanol) and 200 μL of β -mercaptoethanol; the volume was subsequently adjusted to 100 mL with deionized water. A 2 mL aliquot of the OPA reagent was mixed with 100 μL of a sample. After incubation for 2 min at ambient temperature, the absorbance at 340 nm was measured using a spectrophotometer (Shimadzu, Japan). The peptide concentration was quantified using casein tryptone (Oxoid) as a standard, and the value was expressed in mg equivalent of casein tryptone/mL.

2.10. Determination of degree of hydrolysis

The OPA method used to measure the degree of hydrolysis (DH) was a modification of that of Church et al. (1983). Both the preparation of the OPA reagent and assay of absorbance are the same as in the measuring method of the peptide content described as above. A standard curve was prepared using L-isoleucine (0–2 mg/mL). The DH, defined as the percent ratio of the number of peptide bonds (n) to the total number of peptide bonds in the substrate studied (N), was calculated as outlined below.

$$\text{DH}(\%) = \frac{n}{N} \times 100 \quad (N = 128 \text{ for HEWL protein}) \quad (1)$$

$$n = \frac{\Delta \text{AbsMd}}{\epsilon C} \quad (2)$$

where ΔAbs is the difference between the absorbance value at 340 nm of the test sample and the non-hydrolyzed sample; M is the relative molecular weight ($M = 14,304$ for HEWL); d is the dilution factor corresponding to the assay procedure; ϵ is the molar extinction

coefficient at 340 nm ($6000 \text{ mol}^{-1} \text{ cm}^{-1}$); and C is the concentration of HEWL.

Therefore, according to formulas (1) and (2), the DH is expressed as follows:

$$\text{DH}(\%) = \frac{1.86d\Delta \text{Abs}}{C} \quad (3)$$

This formula was used for all subsequent calculations of the DH of the samples.

2.11. Statistical analysis

All of the tests were conducted in triplicate for determination of the ACE inhibitory activities of samples. Data were expressed as means $\pm$ standard errors. Statistical analyses were performed using SPSS version 13.0 software (SPSS Inc., Chicago, IL, USA), and the significant differences were determined with a 95% confidence interval ($p < 0.05$) using Tukey's test.

3. Results

3.1. ACE inhibitory activities of digests

To obtain potential ACE inhibitory peptides from HEWL, 5-h hydrolysis with pepsin followed by another 5-h hydrolysis with α -chymotrypsin and trypsin was conducted under simulated physiological conditions. Many studies have indicated the importance of DH of a protein in relation to ACE inhibitory activity of its hydrolysate (Balti, Nedjar-Arroume, Adje, Guillochon, & Nasri, 2010). Therefore, the effect of DH of HEWL on ACE inhibitory activity of its hydrolysate was investigated first in this study. As shown in Fig. 1A for peptic

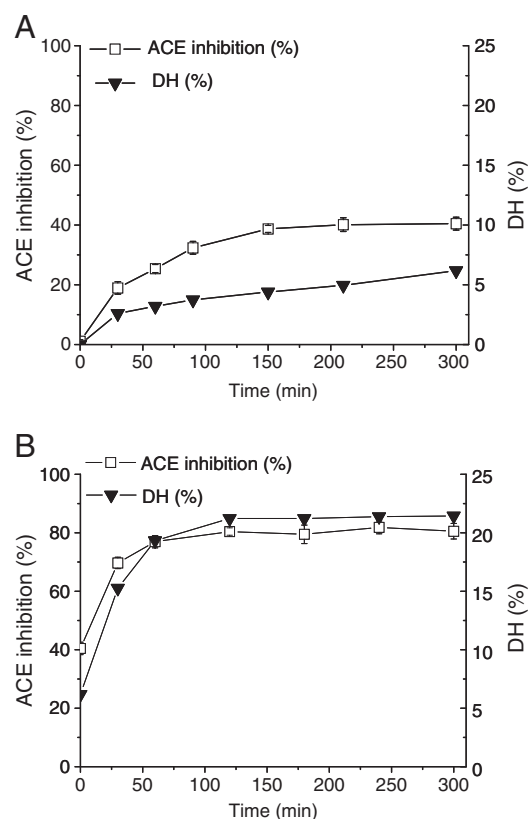


Fig. 1. The effect of time on ACE inhibitory activity and DH of gastrointestinal hydrolysate of HEWL. (A) Pepsic hydrolysate; (B) α -Chymotrypsin-tryptic hydrolysate after pepsin.

hydrolysis, undigested HEWL exhibited nearly no ACE inhibitory activity. The DH of HEWL and ACE inhibitory activity of its hydrolysate increased with increasing hydrolysis time in the first 2.5 h. The highest inhibitory activity against ACE (40%) was obtained with a DH of 4.5%. Further digestion did not result in an increase in the ACE inhibitory activity. After the peptic hydrolysis of 5 h, ACE inhibitory action was observed with the IC_{50} value of 144.2 ± 1.3 $\mu\text{g/mL}$, but the corresponding DH was only 6.2%. It was reported that HEWL is notably resistant to peptic digestion, due to the existence of an α -helical secondary structure of high degree, resulting the lower DH (You et al., 2010).

After 5-h peptic digestion of HEWL, subsequent hydrolysis with α -chymotrypsin and trypsin was undertaken (Fig. 1B). A dramatic increase in ACE inhibitory activity was observed in the first 2 h of hydrolysis with α -chymotrypsin and trypsin and the activity increased with the increase of DH, but further hydrolysis did not result in an increase in ACE inhibitory activity and DH. The resulting hydrolysate (DH=21.5%) showed the highest ACE inhibition of 80.5% with the IC_{50} being 15.6 ± 1.4 $\mu\text{g/mL}$. The DH value and the IC_{50} value respectively increased nearly 3.5 fold and 10 fold compared to the first peptic hydrolysate, suggesting that the digestive enzymes in the intestinal tract are necessary for the release of the shorter peptides with higher activities. Similar effects by two-stage hydrolysis of gastrointestinal enzymes have also been reported for pea and whey proteins (Vermeirssen, van der Bent, Van Camp, van Amerongen, & Verstraete, 2004). Compared with the previously reported values for food proteins hydrolyzed by gastrointestinal enzymes ranging from 48 to 1733 $\mu\text{g/mL}$ (Matsui et al., 2002; Pihlanto-Leppala, Koskinen, Piilola, Tupasela, & Korhonen, 2000; Vermeirssen et al., 2004; Yang et al., 2003), the HEWL protein hydrolysate exhibited great ACE inhibitory activity. It was possible that hydrophobic (43.3%) and basic (13.7%) amino acid residues of high proportion and their positions in HEWL protein sequence played an important role in forming ACE inhibitory peptides.

Moreover, the two-step hydrolysis of gastrointestinal enzymes greatly contributed to the release of these active peptides. The result reveals the importance of the dual select of the protein sources and proteases to prepare peptides with high ACE inhibitory activities.

3.2. Isolation and purification of ACE inhibitory peptides from HEWL hydrolysates

As reported in Fig. 2A, twenty-nine fractions were separated in a Sunfire Pre C18 column for gastrointestinal hydrolysates of HEWL, and the fractions were individually collected and designated as F1–F29. Except for the fractions F5, F15 and F16, all fractions displayed ACE inhibition as shown in Fig. 2B. The ACE inhibition of nearly half of the 29 fractions was above 50%, and the ACE inhibition of one fourth of the fractions was above 70%. Among of the higher active peaks, the fractions F4, F13 and F26 were found to possess the lowest IC_{50} values at 5.93 ± 0.2 , 13.77 ± 0.11 and 6.75 ± 0.05 $\mu\text{g/mL}$, respectively. Therefore, the fractions F4, F13 and F26 were further purified in an analytical C18 reverse phase column (Fig. 3). Three major sub-fractions were collected individually from both F4 (Fig. 3A) and F13 (Fig. 3B), and four major subfractions were obtained from F26 (Fig. 3C) for determination of their ACE inhibitions. The most potent fractions, specifically, F4-P2 ($51.2 \pm 2.6\%$ inhibition), F13-P1 ($73.5 \pm 3.3\%$ inhibition) and F26-P1 ($86.5 \pm 3.5\%$ inhibition), were analyzed by MALDI–TOF–TOF to identify the peptide sequences.

3.3. Identification of ACE inhibitory peptides

The most potent ACE inhibitory fractions obtained after purification by two-stage RP–HPLC were subjected to UPLC MALDI–TOF–TOF MS analysis for the determination of both the molecular masses and the sequence of the peptides contained in the fractions. Fig. 4 shows

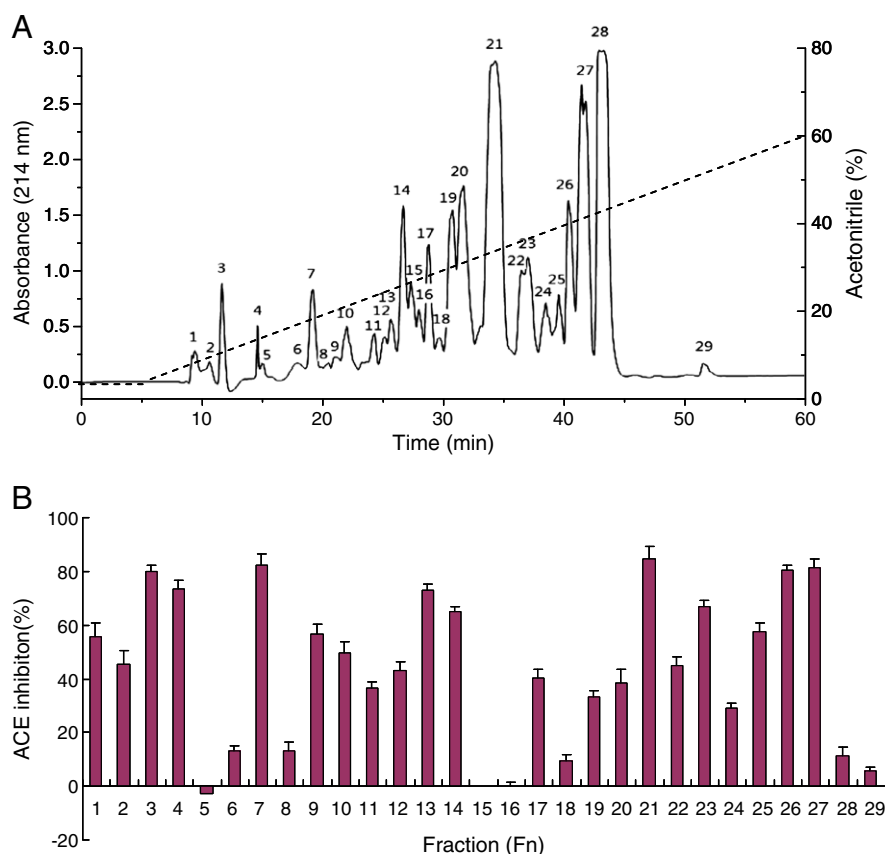


Fig. 2. (A) Preparative RP–HPLC profile of gastrointestinal digest of HEWL and (B) ACE inhibitory activities of the collected fractions.

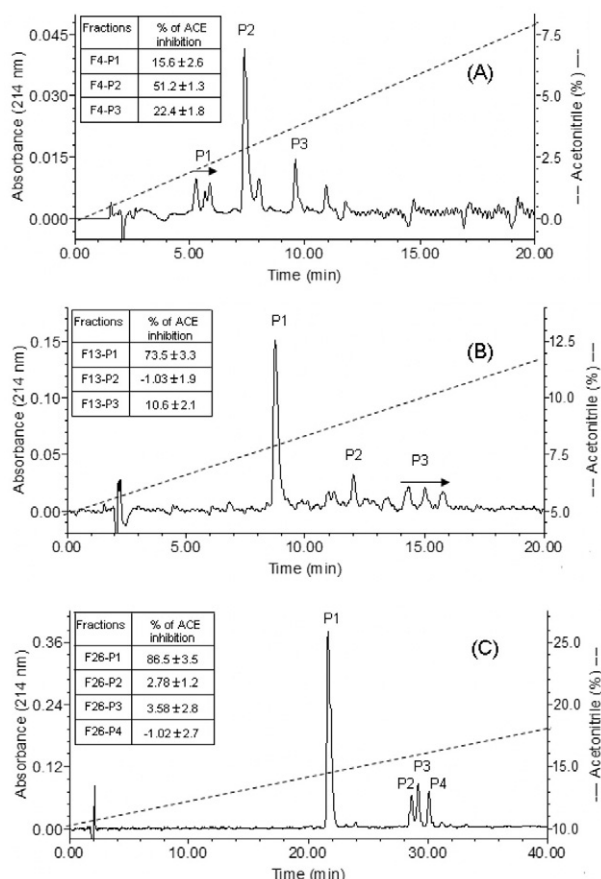


Fig. 3. Chromatography of the peptides in the fractions F4 (A), F13 (B), and F26 (C) from preparative RP-HPLC separated by analytical RP-HPLC, respectively. The inset table is the ACE inhibitory percentage values of the collected subfractions.

the MS and the MS/MS spectra of the most active fraction (F26-P1). The accurate relative molecular mass of the peptide, deduced from the m/z value of $(M+H)^+$ (Fig. 4A) by subtraction of one mass unit for the attached proton, is 374.21 Da. According to this molecular mass and the tandem MS shown in Fig. 4B, the amino acid sequence of peptide identified is Val-Ala-Trp. Likewise, peptide sequences in the other two active fractions (F4-P2 and F13-P1) were identified as Met-Lys-Arg and Arg-Gly-Tyr, respectively. Table 1 also indicates the exact positions of the identified peptides within HEWL including their observed and calculated masses as well as their sequences. The peptides Met-Lys-Arg, Arg-Gly-Tyr and Val-Ala-Trp were chemically synthesized, and the assay of ACE inhibitory activity showed that their IC_{50} values were 25.7 ± 0.2 , 61.9 ± 0.1 and $2.86 \pm 0.08 \mu M$, respectively (Table 1). To the best of our knowledge, all identified peptides are novel peptides with previously unreported ACE inhibitory activity.

3.4. Determination of ACE inhibition pattern

The inhibition mechanisms of the tripeptides Met-Lys-Arg, Arg-Gly-Tyr and Val-Ala-Trp were evaluated by kinetic studies. As shown in Fig. 5, the Lineweaver–Burk plots with an intersection on the vertical axis indicated that all peptides were competitive inhibitors, and the K_i values were determined to be 16.3 ± 0.18 , 52.1 ± 0.13 and $2.4 \pm 0.09 \mu M$, respectively. Most of the ACE inhibitors that were derived from food protein hydrolysates (Katayama et al., 2008; Ruiz, Ramos, & Recio, 2004) belong to the competitive type. These competitive inhibitors are able to enter the active center of ACE, interact with the active sites and prevent substrate binding.

3.5. Classification of the purified peptides by the preincubation method

On the basis of interaction with ACE, ACE inhibitory peptides can be classified into three types: (1) true inhibitors that are resistant to cleavage of ACE; (2) real substrates that are transformed into inactive or reduced activity fragments by ACE; (3) pro-drugs that are converted into highly active fragments after ACE incubation. The latter two types are generally known as the “substrate-type” (Marczak

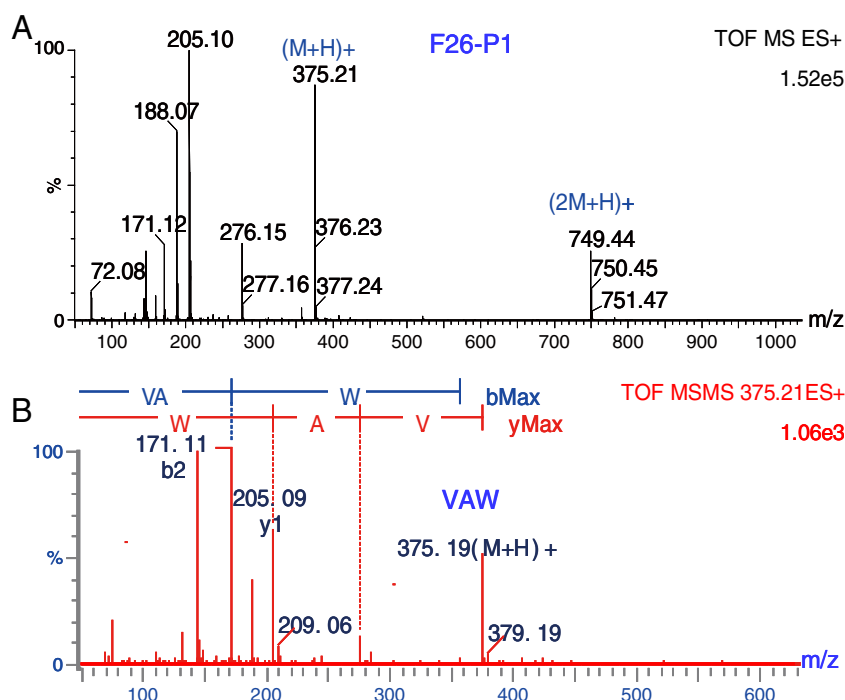


Fig. 4. Identification of peptide sequence. (A) MS spectrum of the subfraction F26-P1 from analytical RP-HPLC; (B) MS/MS spectrum of the precursor ion m/z 375.21 from MS of F26-P1.

Table 1
Structural characterization of ACE inhibitory peptides derived from HEWL.

Fraction	Sequence	m/z	q ^a	Obsd mass ^b	Calcd mass ^c	Position	IC ₅₀ (μM)
F4-P2	Met-Lys-Arg	434.25	1	433.19	433.25	f (12–14)	25.7 ± 0.2
F13-P1	Arg-Gly-Tyr	395.20	1	394.19	394.20	f (21–23)	61.9 ± 0.1
F26-P1	Val-Ala-Trp	375.20	1	374.17	374.20	f (109–111)	2.86 ± 0.08

The sequence of HEWL was as follows (the identified active peptides are underlined):KVFG RCELAAMKRHGLDNYRGYSGLGNWVCAAKFESNFNTQATNRNTDGDSTGYILQINS RWWCNDGRTPGSRNLCNIPCSALLSSDITASVNC AKKIVSDGNGMNAWVAWRNRCKGTDVQAWIRGRL (The sequence comes from protein data UniProtKB, and the record number is P00698).

^a Charge state of the precursor ion.

^b Molecular ion mass observed in the MALDI-TOF-TOF system calculated in Daltons (Da).

^c Calculated monoisotopic mass of the matched peptide.

et al., 2003). In this study, we analyzed the ACE inhibitory activity of the synthetic peptides before and after ACE incubation. As shown in Table 2, the IC₅₀ values of Met-Lys-Arg and Val-Ala-Trp barely changed indicating that these two peptides are true inhibitors for ACE. However, the IC₅₀ value of Arg-Gly-Tyr doubled (from 61.9 ± 0.1 to 126.2 ± 0.3 μM) after preincubation, suggesting that this peptide is a real substrate. ACE is a type of peptidyl-dipeptidase that hydrolyzes

C-terminal dipeptides from various peptides (Rohrbach et al., 1981). In our study, the peptide Arg-Gly-Tyr was possibly hydrolyzed into Arg and Gly-Tyr by ACE based on the dipeptidase properties of ACE, although further experiment is needed to confirm this assumption. Gly-Tyr was previously reported in silk fibroin hydrolysate by alcalase with ACE inhibitory activity and antihypertensive effects (Zhou, Xue, & Wang, 2010).

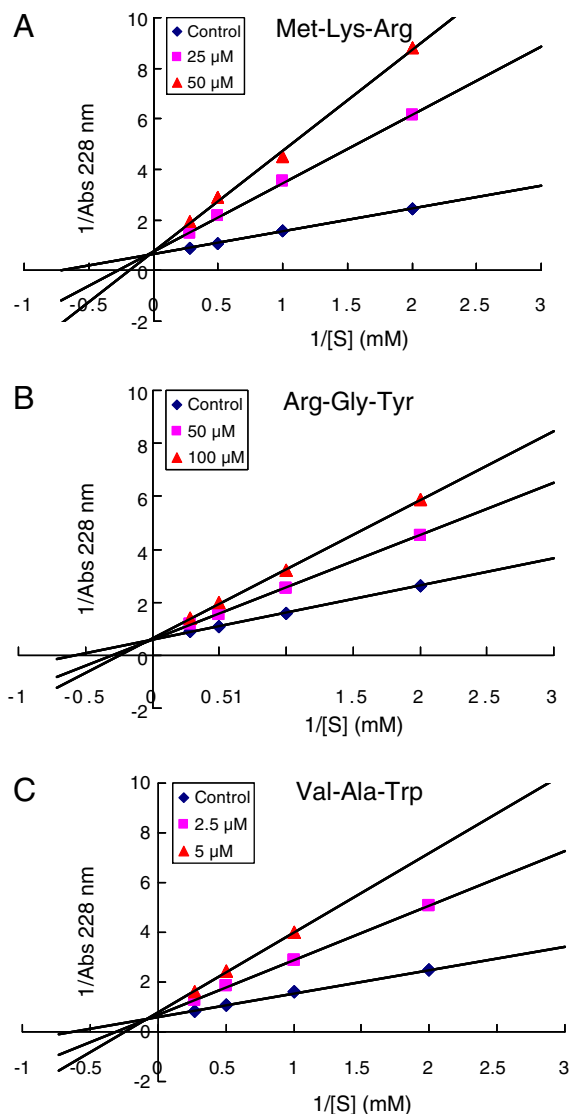


Fig. 5. Lineweaver–Burk plots of ACE inhibition by the synthetic peptides: (A) Met-Lys-Arg, (B) Arg-Gly-Tyr, and (C) Val-Ala-Trp. The ACE inhibitory activities were measured in the presence or absence of the synthetic peptides. 1/[S] and 1/Abs represent the reciprocal substrate concentration and absorbance of reaction mixture at 228 nm after the inhibitory reaction finished, respectively.

4. Discussion

Eggs are broadly recognized as a valuable source of protein in human diets and currently known to contain many substances with biological functions beyond basic nutrition (Kovacs-Nolan, Phillips, & Mine, 2005). Recently, many peptides derived from egg proteins have been found to possess potent ACE inhibitory activities. For instance, Phe-Phe-Gly-Val-Arg-Cys-Val-Ser-Pro (0.4 μM), Glu-Arg-Lys-Ile-Lys-Val-Tyr-Leu (1.2 μM), Leu-Trp (6.8 μM), and Phe-Arg-Ala-His-Pro-Phe-Leu (3.2 μM) were obtained from the peptic digestion of ovalbumin (Fujita et al., 2000), and Lys-Val-Arg-Glu-Glu-Thr (9.1 μM) was derived from the chymotryptic digestion of ovotransferrin (Lee et al., 2006). Most of the reported ACE inhibitory peptides from eggs are produced by single enzymatic hydrolysis and are relatively large peptides. The results of this study show that a two-step hydrolysis of HEWL with gastrointestinal enzymes promoted the formation of higher DH and more active peptides compared to a single peptic hydrolysis. Moreover, three potent ACE inhibitory peptides, specifically, Met-Lys-Arg (2.86 ± 0.08 μM), Arg-Gly-Tyr (61.9 ± 0.1 μM) and Val-Ala-Trp (25.7 ± 0.2 μM), with low molecular weight (MW) were purified and identified from digests of HEWL protein after a two-stage hydrolysis. Majumder and Wu (2009) also reported that digestion of cooked eggs in vitro generated a number of potent, low-MW ACE inhibitory peptides. Short-chain peptides, especially di- and tri-peptides, are more easily absorbed in the intestinal tract than larger ones (Mathews & Adibi, 1976). Furthermore, the ACE inhibitory peptides derived from digestion by gastrointestinal enzymes have the advantage that the formed peptides would resist physiological digestion after oral intake (Matsui et al., 2002; Yang et al., 2003). Accordingly, the active peptides produced by the gastrointestinal digestion of HEWL in this study may have the potency to lower blood

Table 2

IC₅₀ values of identified peptides derived from lysozyme before and after ACE incubation.

Peptide sequence	ACE incubation	
	IC ₅₀ (μM)	
	Before	After
Val-Ala-Trp	2.86 ± 0.08 ^a	2.9 ± 0.05 ^a
Arg-Gly-Tyr	61.9 ± 0.1 ^a	126.2 ± 0.3 ^b
Met-Lys-Arg	25.7 ± 0.2 ^a	26.5 ± 0.14 ^a

^{a–b} Means within the same row without a common letter differ significantly ($P < 0.05$) with Tukey's test.

pressure in vivo, although further animal experiments are needed to be conducted to confirm this assumption.

The field of quantitative structure activity relationship (QSAR) studies the relationship between biological activity and chemical structure (Wu et al., 2006a). To date, hundreds of ACE inhibitory peptides with various sequences derived from food proteins have been reported, and their inhibitory activities are in the range of 0.21–1682 μM (De Leo et al., 2009). The discovery of these inhibitory peptides has greatly advanced understanding of the structure and activity relationships of peptides. The conventional method of producing and identifying ACE inhibitory peptides refers to the selection of a wide range of proteins, the selection of various enzymes, the optimization of enzymatic hydrolysis conditions, and the further purification and characterization of peptides. Although this process is labor-intensive and costly, it promotes discoveries of many new ACE inhibitory peptides with different structures and expands the database of active peptides.

Among the identified peptides, the peptide Met-Lys-Arg possesses a moderate IC_{50} value of $25.7 \pm 0.2 \mu\text{M}$. This peptide contains a C-terminal Arg, which is important for ACE inhibition due to the positive charge of the guanidine group (Meisel, 1998). Moreover, it has been reported that the positively charged amino acids Lys/Arg in the middle position facilitate peptide-enzyme binding and, therefore, reinforce the inhibitory potency of the tripeptides (Majumder & Wu, 2010; Rohrbach et al., 1981; Wu et al., 2006a). For example, Ile-Arg-Tyr, due to the presence of a positively charged amino acid residue in the middle position, showed inhibitory activity five times stronger than Ile-Gln-Tyr (Majumder & Wu, 2010). From this respect, the Lys residue in the peptide Met-Lys-Arg probably plays an important role in the ACE inhibitory power of this peptide. ACE prefers to have substrates or inhibitors that contain hydrophobic amino acid residues at the first three C-terminal positions (Cheung, Wang, Ondetti, Sabo, & Cushman, 1980; Wu et al., 2006a). Compared with the other hydrophilic and basic amino acid residues, the N-terminal Met residue in the peptide Met-Lys-Arg should play a crucial role in entering the hydrophobic active center of ACE. Therefore, the residues in the three positions of Met-Lys-Arg all contribute to the inhibitory potency of the peptide. This peptide shows partial similarity with Met-Arg-Trp (0.6 μM) from spinach rubisco, and the peptide Met-Arg-Trp was reported to possess a remarkable antihypertensive activity (Yang et al., 2003).

Of all of the purified tripeptides, the peptide Val-Ala-Trp shows the strongest ACE inhibitory activity with an IC_{50} value of $2.86 \pm 0.08 \mu\text{M}$. In general, tripeptides with a Pro or aromatic residue (Trp, Tyr, or Phe) at their C-terminus and a branched aliphatic (Val, Ile, or Leu) amino acid residue at the N-terminus have potent ACE inhibitory activities (Cheung et al., 1980; Wu et al., 2006a). The sequence of the peptide Val-Ala-Trp has the structure-activity relationship described as above. Many similar tripeptide sequences in other reports, such as Val-Ala-Pro (2 μM) (De Leo et al., 2009) and Ile-Met-Tyr (1.8 μM) (Matsui et al., 2002), also have low IC_{50} values.

It is worth noting that the peptide Arg-Gly-Tyr contains an aromatic amino acid residue (Tyr) at the C-terminus but not a branched aliphatic residue at the N-terminus. Accordingly, this peptide shows weaker inhibitory activity against ACE than Val-Ala-Trp. In addition, Arg-Gly-Tyr can be served as a substrate of ACE (Table 2). This peptide sequence exhibits a similar structure to that of Arg-Ile-Tyr (Marczak et al., 2003), which also can be cleaved by ACE, thereby releasing the C-terminal dipeptide. A previous study by Rohrbach et al. demonstrates that the antepenultimate position at the C-terminal in a tripeptide is the most important for specificity of peptide substrate against ACE, and the preferable amino acids of the enzyme for this position in terms of substrate specificity are Phe, Ala, Ser, Arg, Pro, and Ile (Rohrbach et al., 1981). As for the sequences Arg-Gly-Tyr and Arg-Ile-Tyr, the basic amino acid residue (Arg) probably plays an important role in the cleavage of ACE against this type of tripeptide.

5. Conclusions

In the present study, we first reported the ACE inhibitory activity of HEWL protein hydrolysate and expanded the functional activities and application of HEWL protein hydrolysate. Our research suggested that the two-stage hydrolysis of HEWL under simulated physiological conditions was more likely than single peptic hydrolysis to produce short potent ACE inhibitory peptides. Three novel peptides (Met-Lys-Arg, Arg-Gly-Tyr and Val-Ala-Trp) were identified in the gastrointestinal digestion of HEWL after consecutive RP-HPLC. The peptide Arg-Gly-Tyr is found to be a true substrate and the peptides Met-Lys-Arg and Val-Ala-Trp are true inhibitor against ACE, and all of these peptides are competitive inhibitors. Compared to other reported ACE inhibitory peptides derived from eggs, the peptide Val-Ala-Trp showed high inhibitory potency, and the peptide Met-Lys-Arg showed moderate inhibitory power. Nevertheless, further research is needed to determine if these peptides can be effectively transported through the intestinal wall into the bloodstream and exert antihypertensive activities *in vivo*.

Acknowledgments

Financial support from National 863 Programs of China (member: 2007AA10Z330) is fully acknowledged. Special thanks to GuanJun Tao (senior engineer) for its valuable contribution in the mass spectrometry analysis.

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