Isolation and Characterization of a Novel Angiotensin-Converting Enzyme-Inhibitory Tripeptide from Enzymatic Hydrolysis of Soft-Shelled Turtle (Pelodiscus sinensis) Egg White: In...

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A novel angiotensin converting enzyme inhibitory peptide derived from proteolytic digest of Chinese soft-shelled turtle egg white proteins

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ABSTRACT

In this study, soft-shelled turtle (*Pelodiscus sinensis*) egg white (SSTEW) proteins were digested by thermolysin and the resulting small peptides were further fractionated by reverse phase chromatography. Peptides with angiotensin I-converting enzyme inhibitory (ACEI) activity from these fractions were screened. A lysozyme-derived peptide, IW-11, from the fraction with the most effective ACEI was identified by liquid chromatography–tandem mass spectrometry (LC–MS/MS) and its purified form showed effective ACEI activity in vitro (IC_{50} = 4.39 ± 0.31 μM). The Lineweaver–Burk plots indicated that the inhibition towards ACE caused by this peptide is a competitive inhibition. The molecular docking study further revealed that the ACEI activity of IW-11 is mainly attributed to the formation of hydrogen bonds between the N-terminal residue of IW-11 and the S1 pocket (Ala354 and Tyr523) and the S2′ region (His513 and His353) of ACE. Moreover, the digestion parameters were further optimized and the target peptide (82% purity) was readily obtained (15% yield) without any cumbersome purification procedure. Notably, lysozyme C is the most abundant protein in SSTEW, which implies that an efficient production of this ACEI peptide from SSTEW is promising.

Biological significance
Inhibition of ACE has proven to be an effective strategy in prevention and treatment of hypertension and related diseases. Unlike typical synthetic ACE inhibitors which exert well described side effects, food-derived peptides with ACE inhibitory activity may be safer alternatives for hypertension treatment. In this study, we comprehensively identified peptides derived from SSTEW digest using a proteomic approach. IW-11, which is derived from lysozyme, the most abundant protein in SSTEW, showed remarkable inhibition

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towards ACE. This peptide has been demonstrated to have a competitive inhibitory property which is able to bind to ACE active site and found to be a true inhibitor against ACE according to Lineweaver–Burk plots. Using an optimized thermolysin condition, IW-11 can be readily obtained without any complex purification step, which will benefit its further application to prevention or treatment of hypertension.

1. Introduction

Angiotensin I converting enzyme (ACE or kinase II; EC 3.4.15.1) is a dipeptidyl-carboxypetidase containing zinc ion in its active site. ACE plays important roles in renin–angiotensin and kallikrein–kinin systems by catalyzing the conversion of the inactive decapeptide, angiotensin I into the potent vasoconstrictor, octapeptide angiotensin II. Furthermore, it inactivates vasodilator nonapetide brandykinin and results in an increase of blood pressure [1]. Inhibition of ACE has proven to be an effective strategy in prevention and treatment of hypertension and related diseases. Some synthetic ACE inhibitory drugs such as captopril, enalapril or benazepril are currently used in the treatment of hypertension and heart failure. However, as those classic ACE inhibitors (ACEI) exert well described side effects such as allergic reactions, skin rashes, cough, or taste disturbances [2], there is a constant interest in new, safe compounds with ACEI potential as alternatives to synthetic drugs.

Recently, many studies have been carried out to discover ACEI in various biological sources, e.g. medicinal plants and food sources such as soybean, cheese, milk, soya milk, whey protein, sweet potato, algae protein, oyster protein, canola meal, porcine collagen, or egg white [3–6]. Among these biological sources, an increasing number of new peptides are continuously reported in the literature isolated from hen egg white protein components notably ovotransferrin [7], ovalbumin [8] and lysozyme [9–11]. These findings highlight the importance of eggs in human health as it contains a particularly high protein content and functional peptides.

Chinese soft-shelled turtle (*Pelodiscus sinensis*) egg has been viewed as a tonic food for a long time in China. Now soft-shelled turtle has been an economically important aquaculture species and large-scale farming of Chinese soft-shelled turtles in East Asia and Southeast Asia has been growing rapidly. According to Lu's report, enzymatic hydrolysate derived from defatted proteins of Chinese soft-shelled turtle (SST) showed antihypertensive effect on spontaneously hypertensive rats (IC_{50} = 2.8 mg/mL) [12]. Recently, Liu et al. further improved the antihypertensive effect to IC_{50} = 190 ± 5 μg/mL by membrane ultra-filtration [13]. These results imply that proteins from the SST are potential biological sources for ACEI active peptides. The medicinal effects of SST eggs have been mentioned in *Materia Medica*, a standard reference book of information on medicinal substances used in Chinese herbal medicine [14]. However, functional peptides sourced from the SST egg white (SSTEW) proteins have not been studied extensively. Due to their potential beneficial health effects, our present study was focused on isolation of bioactive peptides with antihypertensive activity derived from the SSTEW proteins. Enzymatic digest of SSTEW proteins were comprehensively analyzed using a proteomic approach and ACEI active peptides were screened by ACE assay. The inhibition mechanism was further proposed according to the results of enzyme kinetics and molecular docking experiments. To the best of our knowledge, the present work is the first study focused on screening of ACEI-active peptides from SSTEW proteins.

2. Material and methods

2.1. Materials and chemical reagents

Chinese soft-shelled turtle eggs (SSTEW) were obtained from Linluo Township, Pingtung County, Taiwan. ACE (angiotensin I converting enzyme) from rabbit lungs, hippury-L-histidyl-L-leucine (HLL), hippuric acid (HA), TFA (trifluoroacetic acid), acetonitrile (ACN), the enzymes thermolysin (from *Bacillus thermoproteolyticus rokko*) and trypsin (from bovine pancreas) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Formic acid (FA), sodium chloride, sodium hydroxide, boric acid and ammonium bicarbonate (ABC) were purchased from J. T. Baker (Phillipsburg, NJ, USA). A Pierce BCA Protein Assay Kit (#LC142892) was obtained from Thermo Fisher Scientific Inc. (Rockford, IL, USA). The synthetic peptide (IVRDPNGMGAW) was obtained from MDBio, Inc. (Taipei, Taiwan). Molecular weight cut-off (MWCO) ultra-filtration membranes with a 3 kDa cut-off were procured from Millipore (Bedford, MA, USA). All other chemicals used were of analytical grade.

2.2. Purification and SDS-PAGE analysis of lysozyme C

Freeze-dried turtle egg white was dissolved in 20 mM tris base (pH 10.4) buffer at a concentration of 1.5 mg/mL and filtered through a 0.45 μm filter (Millipore). 1 mL filtrate was applied to a HiTrap Q HP anion exchange column (5 × 1 ml, GE Healthcare, Sweden) coupled with an ÄKTA prime FPLC system (GE Healthcare, Sweden). After washing the column with deionized water for 30 min, the column was equilibrated with buffer A (20 mM tris base, pH 10.4) and sample was eluted with buffer B (1 M NaCl in 20 mM tris base, pH 10.4) using a linear gradient of NaCl concentration from 0% to 100% at a flow rate of 1 mL/min within 50 min. The elution was monitored at 280 nm under an UV detector. Fractions were collected and passed through the molecular weight cut-off 3 kDa ultrafiltration membrane to obtain the lysozyme in the retentate. To examine the protein profile of SSTEW and the purity of the lysozyme C in the obtained fractions, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed using 12.5% polyacrylamide resolving gel (pH 8.8) and 4% stacking gel (pH 6.8). The protein bands were stained with Coomassie brilliant blue R-250. The molecular weight calibration kit for SDS electrophoresis (Amersham Biosciences, USA) was used as protein molecular weight markers.

2.3. Preparation of SSTEW protein hydrolysate

SST egg white was separated from yolk by pipette tips, the egg white then was lyophilized prior to hydrolysis. The resulting powder (100 mg) was dispersed in ammonium bicarbonate buffer (ABC, 50 mM, pH 8.5) to obtain 10% protein slurry (w/v) and homogenized by centrifugation at 10,000 *g*, 4 °C for 15 min. Enzymatic digestion was performed in two stages, using a commercial enzyme namely thermolysin. The mass ratio of protein substrate to protease was 885:1. The egg white (6.2 mg) digestion using thermolysin (7 μg) was conducted in 50 mM ABC at 60 °C for 10 h. The enzymatic digestion was stopped by centrifugation (14,000 *g*, 10 min, 4 °C), and the filtrate was transferred to fresh tubes for subsequent studies. For the optimized generation of target peptide, SSTEW was separated from yolk by pipette tips, the egg white then was lyophilized prior to hydrolysis. The resulting powder (10 mg) was dispersed in 1 ml ammonium bicarbonate buffer (50 mM, pH 8.5) to obtain 1% protein slurry (w/v) and sonicated 4 pulses for 15 s each pulse at 40 Hz. The protein slurry was then centrifuged (14,000 *g*, 15 mins, 4 °C) and the supernatant was used for subsequent hydrolysis. The enzymatic hydrolysis using thermolysin was conducted in a 3 kDa MWCO ultra-filtration membrane using enzyme to substrate ratio of 1:200 at constant temperature of 60 °C for 3 h. The enzymatic digestion was stopped by centrifugation (14,000 *g*, 10 min, 4 °C), and the permeate (<3 kDa) was lyophilized for subsequent studies.

2.4. ACE inhibitory activity assay

The ACE inhibitory activity assay was performed based on the method described by Cushman et al. [15] using HLL as the substrate with slight protocol modifications. In each test sample, the assay mixture was composed of the following components: 20 μLof ACE (100 mU/mL), 10 μL of hydrolysate or peptide (1 mg/mL) and 30 μL of HLL (2.5 mM). Control sample contained 20 μL of ACE (100 mU/ml), 30 μL of HLL (2.5 mM) and 10 μL of borate buffer (200 mM, pH 8.3) containing 300 mM NaCl. The same buffer was used for the preparation of Captopril®, substrate, and hippuric acid solutions. All solutions were incubated at 37 °C for 30 min in a thermostatically controlled shaker incubator prior to mixing, and for an additional 30 min at the same temperature after mixing. The reaction was stopped by adding 70 μl of 1 M HCl and then 20 μl of the reaction mixture was separated on Hitachi IL system equipped with an L-7100 pump (Hitachi Inc., Japan). The Hippuryl-L-histidyl-L-leucine (HHL) and ACE-hydrolyzed product hippuric acid (HA) were injected into a BioBasic C18 column (Thermo Scientific, 250 mm length, 4.6 mm i.d., 5 μm particle size) and separated using an isocratic elution of 21% ACN containing 0.5% TFA with a flow rate of 1.0 ml/min. The separated HHL and HA were detected on a UV–VIS L7420 detector (Hitachi Inc., Japan) at 228 nm and the peak areas were integrated using D-700 chromatography data station software (Hitachi Inc., Japan). The inhibitive activity degree was calculated as follows:

$$\text{ACE inhibition}(\%) = [1-(\Delta A_{\text{inhibitor}}/\Delta A_{\text{control}})] \times 100$$

where $\Delta A_{\text{control}}$ was the peak area of reaction blank, the reaction blank mixture contained the same volume of the buffer solution instead of the sample; $\Delta A_{\text{inhibitor}}$ was the peak area of the reaction in the presence of both ACE and enzymatic peptide sample, and the IC_{50} value was defined as the concentration of inhibitor inhibiting 50% of the ACE activity under the assayed conditions.

Definition of ACE activity: 1 unit (U) of ACE activity was defined as the amount of enzyme required to catalyze formation of 1 μmol of HA from HHL per minute at 37 °C.

2.5. Fractionation of ACE inhibitory peptides from crude digests

Freeze-dried crude digest that has been passed through MWCO 3 kDa ultrafiltration membrane was dissolved in 5% ACN and 0.2% TFA in deionized water (v/v). After homogenized, the filtrate was fractioned on a PepClean™ C_{18} Spin Column containing C_{18} resin (Thermo Scientific, USA). After washing the column with 5% ACN and 0.1% TFA, the sample was eluted with 200 μl of linear gradient of 5%, 25%, 50%, 80% and 100% eluent B (5% deionized water and 0.1% TFA in ACN) and centrifuged 10,000 ×*g* for 10 min. Fractions were collected, freeze dried and ACE inhibitory activities of fractions were determined.

2.6. IC_{50} determination of the crude digests and purified peptides

The IC_{50} value is defined as the concentration of crude digest or purified peptide that is able to inhibit 50% of the ACE activity. Five different concentrations of inhibitory peptide were selected and evaluated for their % ACE inhibitory activity. The IC_{50} of the different peptides was determined by plotting the % ACE inhibition against the different concentrations of peptide. Experiments were done in triplicate.

2.7. Identification of purified peptides by LC/MS-MS

Freeze dried purified peptides were dissolved in 5% ACN and 0.1% FA in deionized water for LC–MS/MS analysis. LC–MS/MS analysis was performed using a Thermo LCQ DECA XP MAX system with an electrospray ionization (ESI) source (Thermo Scientific Inc., USA). Samples were loaded onto a BioBasic C_{18} column with diameter 150 × 2.1 mm, particle size 5 μm. Separation of the peptides was performed using a gradient going from 5 to 70% acetonitrile in 0.1% formic acid over 90 min. Mass spectral data was acquired using Thermo-Xcalibur™ data acquisition. The sheath gas flow rate was 50 arb, spray voltage applied for full mass scan was 4 kV and the capillary voltage was 20 V with capillary temperature of 300 °C. MS scan from m/z 100 to m/z 1000 was performed, with a flow rate of 200 μl/min. The MS/MS raw data were obtained using Thermo-XCalibur™ (Thermo-Scientific) then processed into MGF files using Mascot Distiller v2.3.2.0 (Matrix Science, London, UK). The resulting MGF files were searched using the Mascot search engine v2.3 (Matrix Science, UK) with the following search parameters: (1) protein database was set to be a home-made database Testudines which was established from the combined Fasta files of Testudines; (2) the enzyme was set as none enzyme; (3) the precursor and product ion mass tolerance was set at 2 Da/1 Da; (4) the significance threshold was $p < 0.05$. The

peptide sequence was identified through database matching as well as the manual interpretation of its MS/MS spectrum. Peptides with ion scores more than the identity threshold (score > 35) were regarded as identified peptides. In addition to LC–MS/MS identification, the identity of the peptide with the best ACE inhibition was also confirmed by a synthetic peptide with sequence of IVRDPNGMGAW by comparison with their LC retention time, m/z and MS/MS spectra.

2.8. Inhibitory kinetics study

To determine the type of ACE inhibition of IVRDPNGMGAW, the Lineweaver–Burk plot was used to distinguish competitive, non-competitive and uncompetitive inhibition caused by this peptide. The kinetics of ACE inhibition of IVRDPNGMGAW was determined according to a previous study [11]. The basic conditions of the experiment were the same as those for the ACE-inhibitory assay. Activity of ACE in the assay was kept constant at 2 mU. The enzyme activity was measured at various substrate (HHL) concentrations (0.5, 1, 2, and 3.5 mM) in the absence and presence of an inhibitor at two different concentrations (10 and 20 μM). The kinetic parameters of V_{max} and K_m in the absence and in the presence of an inhibitory peptide were determined using constructed Lineweaver–Burk plot of 1/[S] versus Vi. The slope represented Km values while y-intercept represented Vmax values. The dissociation constant (Ki) was calculated by plotting slope vs inhibitor concentration.

2.9. Characterization of inhibitor type by preincubation method

The inhibitor type of ACEI peptide was determined by preincubating the peptide with ACE prior to addition of HHL as the substrate, following the method described by Ruiz et al. [16]. The peptide prepared at 1 mg/mL in 100 mM borate buffer and 300 mM NaCl (pH 8.3) was incubated with ACE (6 mU) for 3 h at 37 °C. The reaction was stopped by heating at 95 °C for 10 min. IC_{50} value was compared before and after preincubation. To verify the result, the peptide was also applied to LC–MS.

2.10. Molecular docking

The model for ACE used in this study was imported from the Protein Data Bank (1O8A.pdb) which represented the crystal structure of the human angiotensin converting enzyme–lisinopril complex at 2 Å resolution [17]. The structures of the peptides were constructed using Accelrys Discovery Studio Visualizer version 3.0 and energy minimized with CHARMm program. The molecular docking study of the peptides at the ACE binding site was run using an automated docking software, Molegro Virtual Docker version 6.0 (Molegro Computational drug discovery, Denmark, http://www.molegro.com/). Before the docking procedure, water molecules and the inhibitor lisinopril were removed whereas the cofactors zinc and chloride atoms were retained in the active site throughout the docking process. The binding site was determined by detecting the cavities within a spacing of x: 37.53, y: 35.243, and z: 45.223 with a radius of 20 Å [18]. The ligands (peptides) were then individually docked and their interactions monitored based on the energy estimation. A value of population size and maximum interactions of 100 and 10,000 respectively were used for each run and the software returned data for the 10 best poses obtained according to its binding affinity. The software Accelrys Discovery Studio Visualizer 3.0 was utilized for identification of hydrogen bonding and hydrophobic interactions established between residues at the ACE active sites and the peptide poses.

3. Results and discussion

3.1. SDS-PAGE pattern of SSTEW proteins

Unfertilized soft-shelled turtle eggs were divided into egg white and yolk, the protein distribution in soft-shelled turtle egg white (SSTEW) was analyzed by SDS-PAGE. Compared to hen egg white (HEW), which is dominated by a few highly abundant proteins such as ovalbumin (45 kD) and ovotransferrin (76 kD), SSTEW is rich in lysozyme C (14 kD), as shown in Figure S1 (A) in the Supplemental Information. The protein identities for the spots shown on the SDS-PAGE were confirmed using in-gel trypsin digestion followed by liquid chromatography–tandem mass spectrometry (LC–MS/MS) and database matching. The protein distribution of SSTEW shown in Figure S1(A) is consistent with the previous report [19] which indicates that lysozyme C is the most abundant protein in SSTEW. Therefore, the major peptides derived from SSTEW protein hydrolysate will be different from those derived from hen egg white and the discovery of novel egg-derived ACEI peptides is possible. The purified SSTEW lysozyme C (SSTEWL) can be readily obtained by fast protein liquid chromatography (FPLC) using anion exchange column, as shown in Figure S1(B) in the Supplemental Information. Recently, some novel ACEI peptides derived from hen egg white lysozyme C (HEWL) have been reported [9–11], however the low abundance of lysozyme C in hen egg white may limit a large-scale production of these ACEI peptides. Generation of ACEI peptides from SSTEWL may be more practical, although the full amino acid sequence of SSTEWL is slightly different from that of HEWL (the full sequences of SSTEWL and HEWL are shown in Figure S2 in the Supplemental Information).

3.2. Screen and identification of ACEI peptide from SSTEW protein hydrolysate

Since lysozyme C is the most abundant protein in SSTEW, the screen of ACEI peptide from SSTEWL can be simply carried out from SSTEW protein hydrolysate without protein-level separation step. SSTEW proteins were digested by thermolysin, trypsin or the combination of these two enzymes. The hydrolysate was filtered with ultrafiltration membrane (3 kD) and the resulting peptides with molecular weight less than 3 kD obtained from the filtrate were subjected to in vitro ACE inhibitory assay. The hydrolysate obtained from thermolysin digestion showed the best IC_{50} value (49 μg/mL) among three kinds of enzymatic digests (data was not shown here). Through LC–MS/MS analysis and database-assisted identification, peptides derived from SSTEW are summarized in Table 1. The

Table 1 – Peptides identified from thermolysin digest of SSTEW using LC–MS/MS analysis and Mascot database search.

Identified protein (GI number)	Identified peptide	Position Start–end	Observed m/z	Mr (Calc)	Mascot score
Soft-shelled turtle lysozyme [*Pelodiscus sinensis*] (GI:149241704) (Sequence coverage = 87%)	GKIYEQCELARE	1–12	720.1	1437.7	63
	IYEQCELARE	3–12	627.6	1252.6	64
	FKRHGMDGYHG	13–23	653.3	1303.6	36
	FKRHGMDGYHGYS	13–25	777.9	1153.7	38
	HGMDGYHGYS	16–25	562.3	1122.4	46
	LGDWVCTAKHESN	26–38	730.5	1458.6	62
	LGDWVCTAKHESNFNT	26–41	911.6	1820.8	50
	LGDWVCTAKHESNFNTAATNYNRGDQSTDYG	26–56	1146.2	3434.5	43
	NTAATNYNRGDQSTDYG	40–56	924.6	1846.8	61
	TAATNYNRGDQSTDYG	41–56	867.2	1732.7	76
	AATNYNRGDQSTDYG	42–56	816.7	1631.7	80
	ATNYNRGDQSTDYG	43–56	781.4	1560.6	39
	ATNYNRGDQSTDYGILQ	43–59	958.6	1914.9	65
	TNYNRGDQSTDYGILQ	44–59	923.1	1843.8	49
	YNRGDQSTDYGILQ	46–59	815.4	1628.7	56
	NRGDQSTDYGILQ	47–59	733.9	1465.7	69
	ILQINSRW	57–64	515.3	1028.6	49
	INSRWW	60–65	431.3	860.4	35
	ACGIECSELLKAD	77–89	677.2	1350.6	37
	LKADITA	86–92	366.3	730.4	36
	LKADITAA	86–93	401.7	801.5	44
	ITAAVNCAKR	90–99	524.2	1045.6	43
	IVRDPNGMG	100–108	480.2	957.5	46
	IVRDPNGMGAW	100–110	608.2	1214.6	89
	IVRDPNGMGAWVAW	100–113	787.0	1570.8	49
	VRDPNGMGAW	101–110	552.2	1101.5	53
	VAWTKY	111–116	384.3	766.4	40
	VAWTKYCKGKDVSQW	111–125	900.6	1797.9	45
Beta-actin [*Pelodiscus sinensis*] (GI:189473615) (Sequence coverage = 11%)	SYELPDGQVITIGNER	101–116	896.6	1789.9	47
Immunoglobulin D heavy chain constant region [*Pelodiscus sinensis*] (GI:255969727) (Sequence coverage = 1%)	DYLPSAEKIQW	121–131	675.5	1348.7	40
Liver growth hormone receptor [*Pelodiscus sinensis japonicus*] (GI:12003321) (Sequence coverage = 3%)	FPWLLIIVFG	239–248	402.8	1203.7	36
	SEDNQSKLPFAD	427–438	676.1	1349.6	37
L-Lactate dehydrogenase A [*Pelodiscus sinensis japonicus*] (GI:13650168) (Sequence coverage = 4%)	AHSKLVIITAGARQ	87–100	488.4	1463.9	38
Cytochrome oxidase subunit I [*Pelodiscus sinensis*] (GI:336038756) (Sequence coverage = 5%)	VWSVVITAVLLLL	169–181	713.5	1424.9	37

home-made database of *P. sinensis* was established from the combined Festa files of *P. sinensis*. Most identified peptides (sequence coverage = 87%) were derived from lysozyme C (*P. sinensis*) implied that this protein is abundant in SSTEW which is consistent with the data shown in SDS-PAGE, as shown in Figure S1 (A). Due to the broad specificity of thermolysin digestion, peptides with redundant sequences were simultaneously identified. The sequence redundancy may increase the difficulty level for the downstream peptide separation. The filtrate from thermolysin digest was roughly fractionated into five fractions using reverse phase liquid chromatography (RP-LC), as shown in Fig. 1(A). ACEI activity for each fraction was examined by in vitro ACE inhibitory activity assay and the result indicated that fraction 4 showed the best ACE inhibition activity (63% inhibition). This fraction was further analyzed using LC-MS/MS, the molecular weight of major peak (*m/z* 608.7, +2) was 1215.4 Da, as shown in Fig. 1(B). The sequence of this peptide was characterized as IVRDPNGMGAW (IW-11) according to database-assisted identification as well as the manual interpretation of its MS/MS spectrum shown in Fig. 1(C). IW-11 was derived from lysozyme C (residue 100–110), which demonstrated that the ACEI activity from the hydrolysates of SSTEW was mainly attributed to this SSTEWL-derived peptide. In addition to LC–MS/MS identification, the sequence of the peptide was also confirmed by a synthetic peptide with the same sequence by comparison with their LC retention time, m/z and MS/MS spectra. To determine the IC_{50} value of this peptide, the synthetic peptide (IVRDPNGMGAW) was used for in vitro ACE inhibitory assay. The IC_{50} of IW-11 towards ACE was determined to be 4.39 ± 0.31 μM (Figure S3 in the Supplemental Information) which showed that ACE inhibitory activity was superior to most active peptides discovered from hen egg white hydrolysate [8,20] or even those derived from hen egg lysozyme [9–11]. The explanation of the better IC_{50} value of IW-11

compared to other long peptides is that it contains hydrophobic amino acid residues at the N-terminus, positively charged amino acids at the middle, and aromatic amino acids at the C-terminus. It has been previously reported that peptides containing proline 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 [21]. Positive charge of Lys and Arg (basic

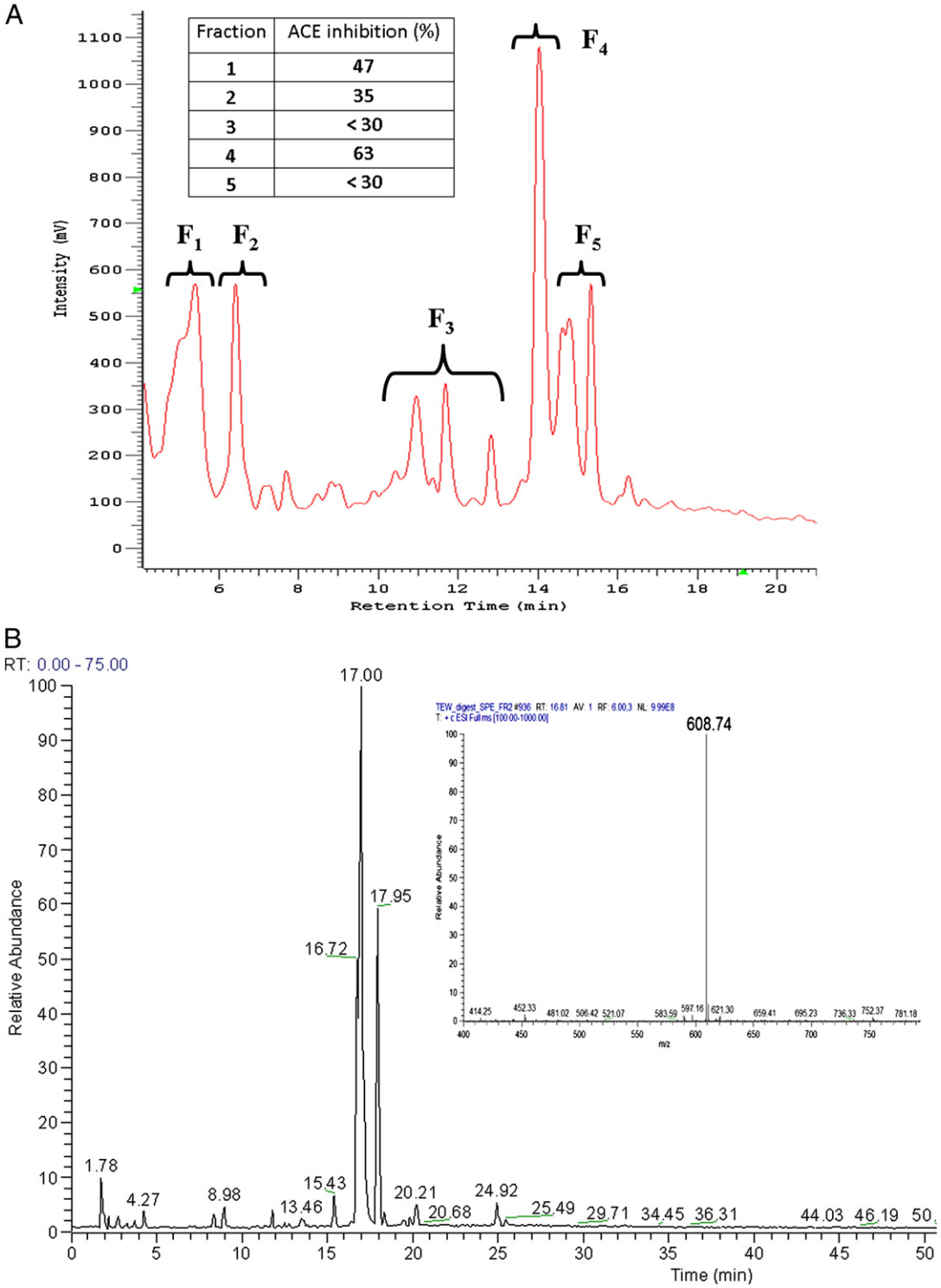

Fig. 1 – (A). RP-HPLC chromatogram of SSTEW hydrolyzed by thermolysin applied to a C_{18} column. The inset table shows the ACE inhibitory percentage value of each fraction; (B). LC–MS chromatogram of fraction 4 obtained from analytical RP-HPLC. The inset figure shows the precursor ion of the most intense peak at m/z 608.74; (C). ESI-MS/MS spectrum of precursor ion m/z 608.74.

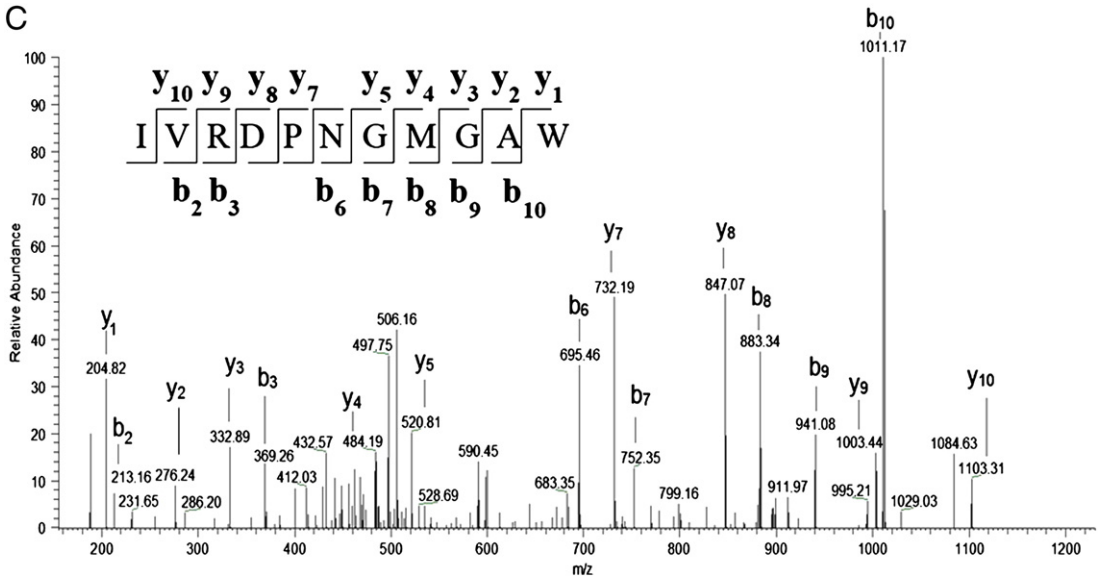

Fig. 1 (*continued*).

amino acid) may also contribute to the inhibitory potency [22]. This result demonstrated that it is a potent food derived antihypertensive peptide.

3.3. *Optimized production of IW-11 from SSTEW*

In order to isolate the ACEI peptides from crude hydrolysate, tedious purification procedures such as gel filtration, ion exchange chromatography or two step reverse phase chromatography are required [9,11,14], which are less practical for a mass production of ACEI peptide. To circumvent this limitation, the digestion parameters in terms of enzyme-to-protein ratio, digestion time and temperature were optimized for the target-specific peptide formation from SSTEW. The effect of enzyme to substrate ratio (1/50, 1/100, 1/200, 1/400, 1/800, 1/900 w/w) on the formation of the target peptide is shown in Figure S4 in the Supplemental Information. The data acquired from RP-HPLC were then used to count the yield and purity of target peptide. The amount of IW-11 was calculated based on its peak area fitting on the calibration curve obtained from the standard peptide (peak area vs. concentration). The yield of the hydrolysate was calculated based on the mole of starting protein and the mole of peptide formed during this hydrolysis. The purity of the target peptide was determined by the comparison of peak area of target peptide and the sum of all peak areas. The amount of thermolysin used played a dominant role in releasing IW-11 from SSTEW. Raising the level of enzyme resulted in increasing the released peptides yet decreased the purity and yield in the hydrolysate. While at a ratio 1/200, the peak of target peptide IW-11 was present as the most abundant peak (purity = 82%) in the hydrolysate with the yield of 15%, as shown in Table 2. According to a previous report, the enzyme to substrate ratio in thermolysin digestion was an important factor due to the broad specificity of this enzyme [23]. As a consequence, a 1/200 E/S ratio was chosen for optimization of other digestion parameters. As seen in Table 2, longer incubation time did not increase the yield but decreased the purity of target peptide from 82% to 24%. This might be due to further digestion of target peptide by thermolysin (Figure S5). Thermolysin specifically hydrolyzes peptide bonds on the amino side of bulky hydrophobic residues such as Ile, Leu, Val, and Phe [24]. This result indicated that hydrolysis time of 3 h might be the most suitable time to release the target peptide. The phenomena of further digestion of target peptide by thermolysin due to longer incubation time also happened in all enzyme-to-substrate ratios. Moreover, the target peptide even ceased to be visible when the hydrolysis time was prolonged. Therefore, we continued optimizing the hydrolysis parameter using E/S ratio of 1/200 and hydrolyzed for 3 h. Similarly, the effects of hydrolysis temperature (40, 50, 60, and 70 °C) on the yield and purity of target peptide in SSTEW hydrolysate were also examined (Figure S6 in the Supplemental Information). As seen in Table 2, hydrolysis of SSTEW in 40 °C was not able to release the target peptide while hydrolysis at 50 °C could release a small

Table 2 – Effect of enzyme to substrate ratio (w/w), hydrolysis time (h), and hydrolysis temperature (°C) on purity and yield.

E/S ratio (w/w)	Hydrolysis time (h)	Hydrolysis temperature (°C)	Peptide amount (μg)	Yield (%)	Purity (%)
1/50			5.91	7.17	3.7
1/100			6.34	7.69	22.68
1/200	3	60	12.35	14.97	82.30
1/400			3.88	4.70	87.96
1/800			2.32	2.81	89.72
1/900			1.10	1.33	88.56
	1		1.74	2.11	18.41
1/200	3	60	12.35	14.97	82.30
	5		4.68	5.67	32.23
	10		6.69	8.11	24.48
		40	0	0	0
1/200	3	50	1.61	1.95	74.69
		60	12.35	14.97	82.30
		70	9.07	11.00	61.52

amount of the target peptide. Hydrolysis at 70 °C decreased the purity from 82% to 61%, suggesting that many other peptides were also released. Previously reported ACEI peptides derived from thermolysin digest of other protein sources were applied at varied temperatures. Lei et al. [7] applied thermolysin digestion at 55 °C to release active peptide from hen egg ovotransferin, while Ono et al. [25] used 37 °C to release active peptide from salmon muscle using thermolysin. In our case, the optimum temperature to obtain our peptide was found at 60 °C. Using this optimized digestion condition (hydrolysis under 1/200 E/S ratio at 60 °C for 3 h), the target peptide with more than 80% purity can be readily obtained (15% yield) without any cumbersome purification procedure, as shown in Fig. 2. The purity and identity of IW-11 in this SSTEW hydrolysate was also confirmed by LC–MS and LC–MS/MS.

3.4. *Inhibitory kinetic study and inhibition type classification of IW-11*

The ACE inhibition pattern of the active peptide was analyzed by the Lineweaver–Burk plot. As shown in Fig. 3, the Lineweaver–Burk of peptide converged at the y-intercept with that for the reaction containing no inhibitor and the slope altered in the presence of IW-11 while the y-intercept on 1/[V] was unchanged. This suggests a competitive inhibition by the peptide which means peptide can interact with the active site of ACE and prevent substrate binding. Previously reported ACE-inhibitory peptides from hen egg white lysozyme also acts as competitive inhibitor [11]. The competitive inhibitors can bind to the active site to block it or to the inhibitor-binding site that is remote from the active site so as to alter the enzyme conformation such that the substrate no longer binds to the active site [26]. The difference in the structure of the inhibitory peptides can lead to have various ACE inhibition patterns. The ACE inhibitor also might act as a noncompetitive or uncompetitive inhibitor. A noncompetitive inhibitors bind to a site other than the substrate-binding site on the enzyme and enzyme–substrate complex so that they do not affect the combination of the substrate with the enzyme, but affect only the enzyme reaction velocity. While an uncompetitive inhibitor can bind only to a substrate–enzyme complex and decrease the maximum enzyme activity, so that it takes longer for the substrate or product to leave the active site. Some food protein derived peptides were reported to be noncompetitive inhibitors [27] or uncompetitive inhibitors [28]. The dissociation constant (Ki) for the enzyme–inhibitor complex was determined from the secondary plot of the slope of the Lineweaver–Burk plot against [I_0] and the resulting Ki was found at 8.85 μM. A large value of Ki indicates a low affinity between enzyme and inhibitor [29]. In this study, the inhibition type of IW-11 towards ACE was further classified using the pre-incubation method. By comparison of IC_{50} values with and without pre-incubation, the inhibition mechanism can be divided into three types: (1) IC_{50} value without significant change indicates that the peptide is a true inhibitor which can resist the cleavage of ACE; (2) increased IC_{50} value means that the peptide is a real substrate which will be transformed into fragments with inactive or reduced activity by ACE; (3) decreased IC_{50} represents that the peptide is a pro-drug that can be converted into more active fragments by ACE. After ACE pre-incubation, the IC_{50} of IW-11 was not changed significantly (from 4.39 μM to 4.68 μM), which indicates that IW-11 is a true inhibitor instead of a substrate-type inhibitor. This conclusion

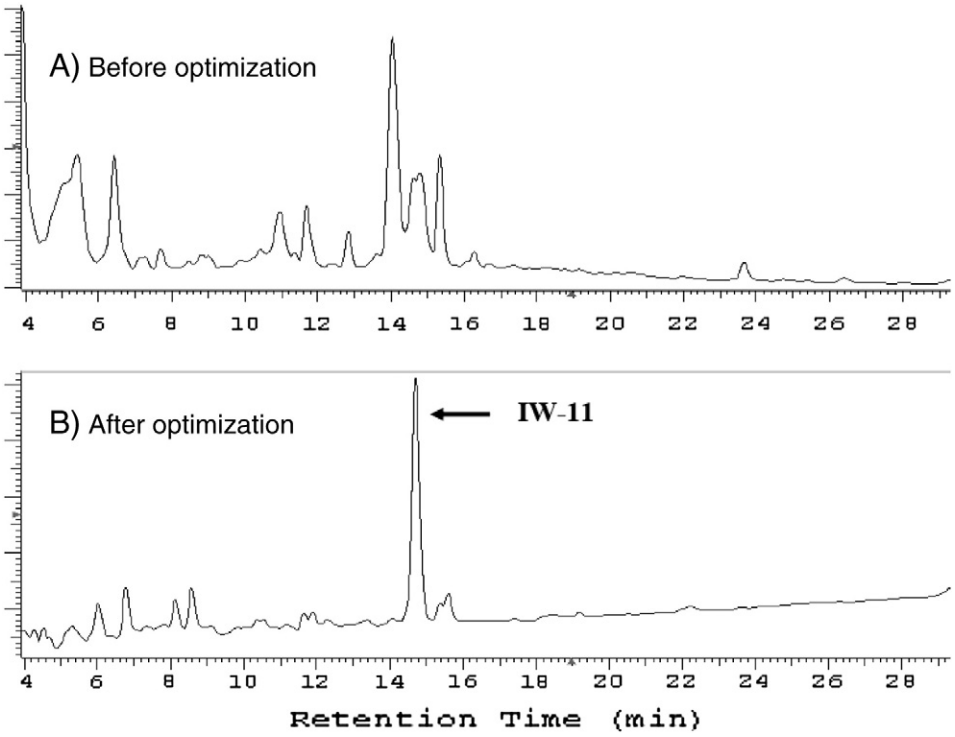

Fig. 2 – Comparison of RP-HPLC chromatogram of SSTEW hydrolysate before and after being optimized. The optimized hydrolysate was produced using E/S ratio of 1:200 (w/w), incubated for 3 h at 60 °C, and ultrafiltration by MWCO 3 kD.

	No inhibitor	IW-11 (10 μM)	IW-11 (20 μM)
Km (mM)	0.34	0.64	1.04
Vmax (mM)	0.0456	0.0444	0.0448

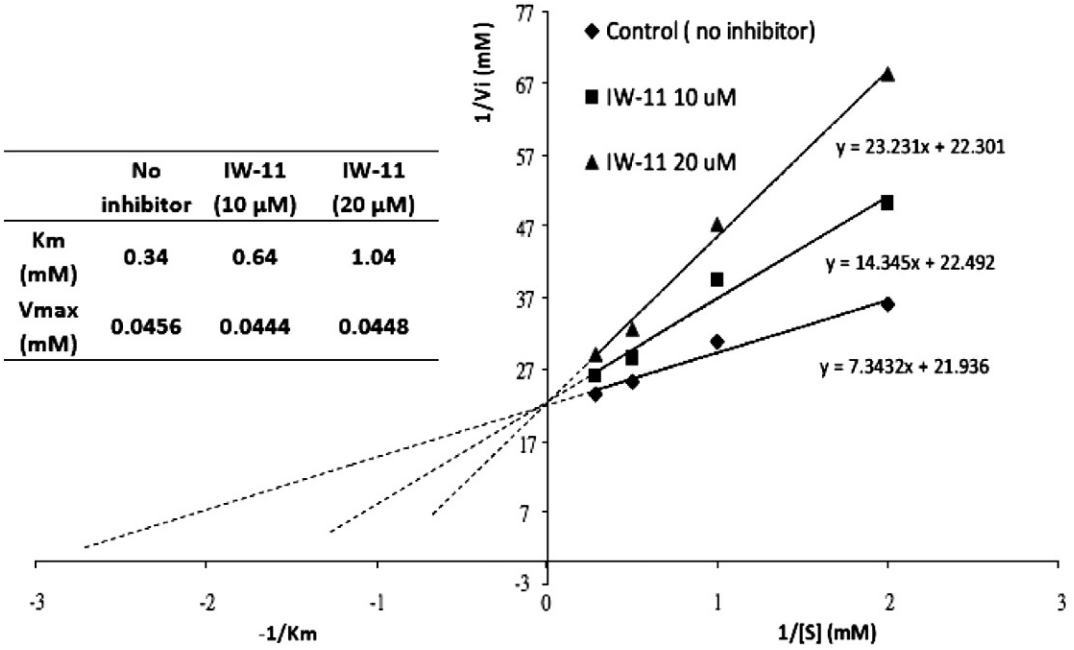

Fig. 3 – Lineweaver–Burk plots of ACE-inhibition by IW-11. The ACE inhibitory activities were measured in the presence or absence of synthetic peptide. 1/[S] and 1/Vi represent the reciprocal substrate concentration and velocity, respectively.

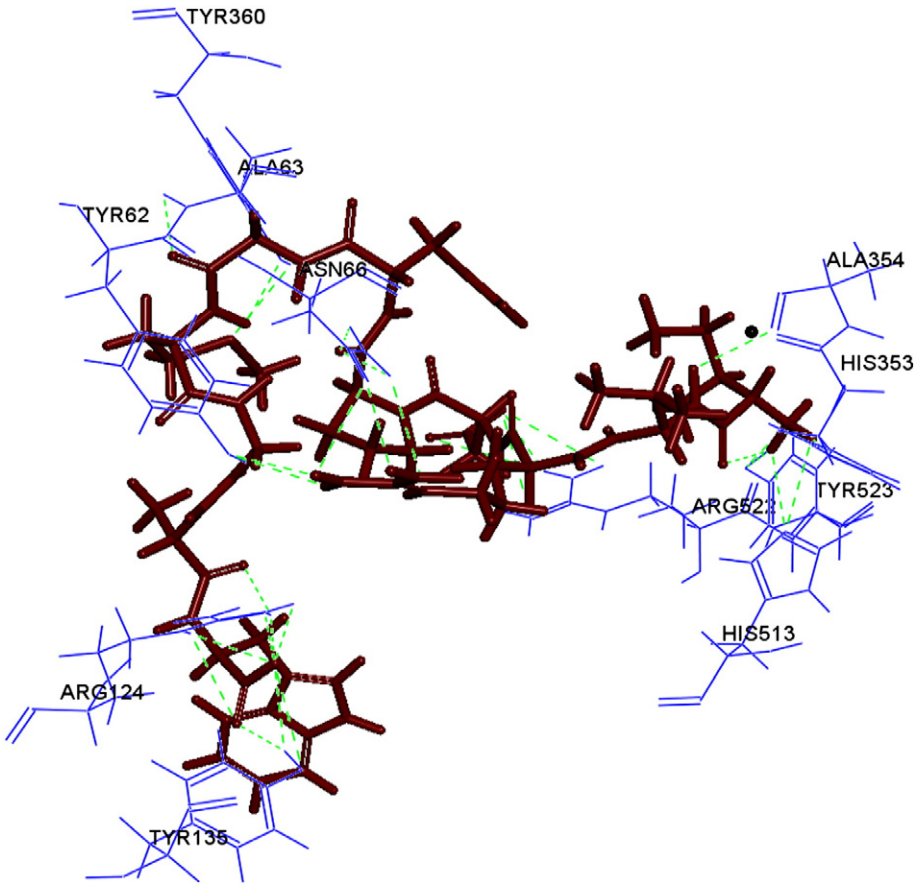

Fig. 4 – Predicted binding mode between ACE and IVRDPNGMGAW after docking at the ACE active site. Peptides are shown in red stick model. ACE residues, Zn (II), and hydrogen bond interaction are represented in blue, black circle, and green dashed lines, respectively (Image obtained with Accelrys Discover Studio Visualizer).

was also confirmed by the integral peptide without any detectable fragment of IW-11 in the LC–MS chromatogram after 3 h pre-incubation in the medium of ACE.

In order to study the resistance of the active peptide to gastrointestinal enzymes, the peptide was subjected to a two-stage hydrolysis process (pepsin then trypsin) which simulates physiological digestion. IW-11 survived the incubation with pepsin but partly hydrolyzed (approx. 30% of the initial peptide was hydrolyzed) by the action of trypsin as pancreatic enzyme. After two-stage pre-incubation with pepsin and trypsin, the IC_{50} value slightly decreased from 4.39 ± 0.3 to 2.42 ± 0.09 μM, which implied that IW-11 is still active under gastrointestinal digestion condition.

3.5. *Molecular docking simulation between IW-11 and ACE*

The docking simulation of the peptide IW-11 at the ACE active site has shown the best returned pose (03) with affinity energy of −19.74 kJ/mol. The best pose for the docking simulation is shown in Fig. 4. The interaction of IW-11 to ACE as given by the best pose showed a different binding mode to those observed in the interaction of ACE with drugs. The molecular docking study revealed that IW-11 establishes hydrogen bonds with the S1 pocket (Ala354 and Tyr523) and the S2′ region (His513 and His353) of ACE. The amine group of the isoleucine residue establishes H-bond interaction with a histidine residue (His353 NE2) and another interaction with Tyr523 OH. Another H-bonding interaction was observed between carbonyl group at N-terminal residue with His513 NE2 and Ala354 O. Those four amino acids are important residues at ACE which interact with lisinopril, a commercial antihypertensive drug [17]. Moreover, the H-bonds to those amino acid residues are much stronger due to the shorter distances (as shown in Table 3). It was previously reported that a significant and solitary inhibitor registration for lisinopril is described by the H-bonding interaction between the C-terminal carboxylate and Tyr520 and Lys511 of ACE, while the carboxylic group of the C-terminal tryptophan in IVRDPNGMGAW establishes hydrogen bonding with Tyr135 and Arg124 instead of Tyr520 and Lys511. Besides, no direct coordination of the catalytic Zn (II) and the carbonyl oxygen of the peptide bond were observed in this simulation. The absence of H-bond interaction of IVRDPNGMGAW with those two residues and no direct zinc coordination probably explain the higher IC_{50} of IW-11 when compared to lisinopril. It is plausible that IW-11 has limitation in size to enter the deep narrow channel of ACE and consequently the ACE-inhibition is lower when compared with the lisinopril.

Table 3 – Hydrogen bonds observed between drug (lisinopril) and ACE and between the best peptides poses obtained from docking simulation and ACE (√ represents existence of hydrogen bonding; x represents nonexistence of hydrogen bonding).

ACE residues in H-bonding	Lisinopril	Distance (Å)	IVRDPNGMGAW (03 pose)	Distance (Å)
Glu 162 OE2	√	3.4	x	–
His 353 NE2	√	2.8	√	2.1
Ala 354 O	√	2.9	√	2.6
Glu 384 OE2	√	2.7	x	–
Lys 511 NZ	√	2.9	x	–
His 513 NE2	√	3.1	√	2.6
Tyr 520 OH	√	2.6	x	–
Tyr 523 OH	√	2.8	√	2.9

4. Conclusions

A novel active peptide isolated from thermolysin hydrolysate of SSTEW proteins showed remarkable activity on the angiotensin-I converting enzyme inhibition with the IC_{50} value at 4.39 ± 0.31 μM. The active peptide was identified as IVRDPNGMGAW and derived from lysozyme C, the major protein in SSTEW. To the best of our knowledge, the present work is the first study focused on screening of ACEI-active peptides from SSTEW proteins, and IW-11 is the first ACEI-active peptide with well-characterized sequence discovered from SSTEW. Moreover, the target peptide IW-11 with more than 80% purity can be readily obtained (15% yield) just by using the optimized digestion condition under 1/200 E/S ratio at 60 °C for 3 h, which implies that practical production of this ACEI peptide from SSTEW is promising. IW-11 has been further demonstrated a competitive inhibitory property which is able to bind to the ACE active site and found to be a true inhibitor against ACE. The molecular docking studies revealed that the ACEI activity of IW-11 is mainly attributed to the formation of hydrogen bonds between IW-11 and the S1 pocket (Ala354 and Tyr523) and the S2′ region (His513 and His353) of ACE. An in vivo study will be further undertaken to prove its antihypertensive activity.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2013.10.006.

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