Innovative Food Science and Emerging Technologies 18 (2013) 212–219

Contents lists available at SciVerse ScienceDirect

Innovative Food Science and Emerging Technologies

journal homepage: www.elsevier.com/locate/ifset

Identification of a novel angiotensin-I converting enzyme inhibitory peptide from ostrich egg white and studying its interactions with the enzyme

Hamid Tanzadehpanah [a], Ahmad Asoodeh [b,d,*], Mohammad Reza Saberi [c], Jamshidkhan Chamani [a]

[a] Department of Biology, Faculty of Sciences, Mashhad Branch, Islamic Azad University, Mashhad, Iran
[b] Department of Chemistry, Faculty of Sciences, Ferdowsi University of Mashhad, Mashhad, Iran
[c] Department of Medical Chemistry, School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran
[d] Cellular and Molecular Research Group, Institute of Biotechnology, Ferdowsi University of Mashhad, Mashhad, Iran

ARTICLE INFO

Article history:
Received 6 September 2012
Accepted 5 February 2013

Editor Proof Receive Date 6 March 2013

Keywords:
Ostrich egg white
Peptide
ACE
Fluorescence spectroscopy
Molecular modeling

ABSTRACT

Ostrich egg white (OEW) proteins were hydrolyzed by trypsin to identify inhibitory peptides of angiotensin I-converting enzyme (ACE). The most active hydrolysate was obtained after 4 h of hydrolysis. It was further consecutively fractionized by ultrafiltration membrane and then was separated into nine fractions by reversed-phase high performance liquid chromatography (RP-HPLC). Among the fractions, the F_3 fraction with amino acid sequence of Ala–Phe–Lys–Asp–Glu–Asp–Thr–Glu–Glu–Val–Pro–Phe–Arg (MW: 1582.74 Da) and IC_{50}: 80.2 μM exhibited the highest ACE inhibitory activity. Kinetic studies revealed that the F_3 peptide acts as a non-competitive inhibitor against ACE. The interaction between the F_3 peptide and ACE was further scrutinized by fluorescence spectroscopy and molecular modeling techniques. The binding of the F_3 peptide to ACE was observed to occur via two classes of binding sites and F3 had more affinity to N-domain than C-domain.

Industrial relevance: Angiotensin converting enzyme (ACE) can increase blood pressure by catalyzing the conversion of the inactive angiotensin-I to the strong vasoconstrictor angiotensin-II. Inhibition of ACE by decreasing the concentration of angiotensin II is of great importance. In this study, a thirteen-amino acid peptide was identified from Ostrich egg white (OEW) hydrolysates which can potently inhibit ACE. Thus, the identified peptide could be considered as a worthwhile peptide to control hypertension via using as a supplement for special food products. Furthermore, the results can be used as a model for studying the interaction of inhibitory peptides with ACE.

1. Introduction

Many people perennially suffer from high blood pressure requiring medical attention. Hypertension is related to the incidence of coronary heart disease and generated by various factors, such as aging, overweight, genetic and environmental factors (Pihlanto, Virtanen, & Korhonen, 2010). Angiotensin converting enzyme (ACE) is an important enzyme in rennin–angiotensin system which increases blood pressure by catalyzing the conversion of the inactive decaptide angiotensin-I to the strong vasoconstrictor angiotensin-II (Liu et al., 2010). Inhibition of ACE by decreasing the concentration of angiotensin II is of great importance. Some of the synthetic drugs such as enalapril, lisinopril and captopril are produced to inhibit ACE thereby treating hypertension. However, the use of synthetic drugs affords potential risks to human health (Lee, Hong, Jeon, Kim, & Byun, 2009). Nowadays, peptides and protein hydrolysates have been found to possess many physiological functions, for example antioxidant (You, Zhao, Cui, Zhao, & Yang, 2009), antimicrobial (Memarpoor-Yazdi, Asoodeh, & Chamani, 2012), antithrombotic (Shimizu et al., 2008) and antihypertensive activities (Asoodeh, Memarpoor-Yazdi, & Chamani, 2012), and also, several peptides have been identified to possess multifunctional properties (Meisel & FitzGerald, 2003). By the use of proteolytic hydrolysis, microorganisms and fermentation methods, bioactive peptides containing 2–20 amino acids in the sequence are produced (Bernardini et al., 2011). A molecular modeling study has been reported the interactions of human testicular ACE with captopril, Ala–Val–Phe and Val–Phe peptides (Vercruysse et al., 2010).

In this study, the ACE inhibitory properties of OEW protein hydrolysates and peptides were evaluated. Containing a large amount of egg proteins, such as ovalbumin, ovotransferrin, ovoinhibitor, lysozyme, ovomacroglobulin, apoprotein and ovomucin, OEW could be considered as a valuable source to obtain bioactive peptides with ACE inhibitory activity (Osuga & Feeney, 1968). To obtain ACE inhibitory peptide, OEW was hydrolyzed by trypsin. The ACE inhibitory activity of peptide fractions was determined by UV-spectroscopy, and then the interaction between the most active peptide and ACE was further studied by fluorescence spectroscopy and molecular modeling. Molecular modeling of rabbit lung ACE with peptides more than three residues in length has not been recorded so far.

* Corresponding author at: Department of Chemistry, Faculty of Sciences, Ferdowsi University of Mashhad, Mashhad, Iran. Tel./fax: +98 5118795457.
E-mail address: Asoodeh@um.ac.ir (A. Asoodeh).

1466-8564/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.ifset.2013.02.002

2. Material and methods

2.1. Material

Fresh ostrich eggs (*Struthio camelus*) were purchased from a local market in Mashhad, Iran. Egg whites were homogenized for enzymatic hydrolysis. Trypsin (from bovine pancreas, type II), trifluoroacetic acid (TFA), angiotensin converting enzyme (ACE) from rabbit lung and (N-(3-[2-furylacryloyl-Phe–Gly–Gly])) FAPGG, were purchased from Sigma. (Louis, MO, USA). Ultrafiltration membrane with a 3 kDa cut-off was procured from Millipore (Bedford, MA, USA). All other chemicals used were of analytical grade.

2.2. Preparation of egg white protein hydrolysates

Ostrich egg white (200 ml) was mixed with 800 ml of 50 mM sodium phosphate buffer (pH 8) and homogenized for about 10 min. To prepare egg white hydrolysates, enzymatic hydrolysis was performed using trypsin protease at 37 °C. The ratio of protein substrate to protease was 20:1. The hydrolysis reaction was performed at different times (0.5–10 h). After hydrolysis, the enzyme was inactivated by incubating the samples in boiling water for 15 min. The hydrolysate was then centrifuged at 8000 ×*g* for 10 min at 4 °C. The supernatant was passed through a membrane with a molecular weight cut-off (MWCO) of 3 kDa to obtain fractions with molecular weights of <3 kDa. Then, the hydrolysates were lyophilized and used for subsequent studies.

2.3. Degree of hydrolysis

The degree of hydrolysis (DH) of OEW protein hydrolysates was determined using Nalinanon, Benjakul, Kishimura, and Shahidi (2011) method with slight modification. DH is defined as the percent ratio of the number of peptide bonds broken to the total number of bonds per unit weight. Free amino groups obtained from the hydrolysis reaction were measured by trinitrobenzenesulfonic acid (TNBS). Briefly, 150 μl of sample was mixed with 2.0 ml of 0.2 M phosphate buffer (pH 8.2) and 1.0 ml of 0.1% TNBS solution, followed by incubation in a 50 °C water bath for 30 min in the dark. The reaction was terminated by adding 2.0 ml of 0.1 M sodium sulphite. After 15 min, the absorbance was measured at 420 nm and α-amino acid was expressed in terms of L-leucine. The total numbers of amino groups were determined in 6 M HCl at 100 °C for 24 h. DH was calculated according to the equation:

$$DH = [(L_t - L_0)/(L_{max} - L_0)] \times 100$$

where L_t is the amount of α-amino acid released at time t. L_0 is the amount of α-amino acid in the original acid-solubilized protein substrate and L_{max} is the total numbers of amino groups.

2.4. Chromatographic separation

The hydrolysates were dissolved in distilled water and then injected into a chromatography column and ACE inhibitory fractions were separated by HPLC on a semi-preparative column (10×250 mm manufactured by Macherey–Nagel GmbH & Co. Düren, Germany). A linear gradient of acetonitrile (5–45%, v/v, 50 min) containing 0.1% trifluoroacetic acid (TFA) was used to elute bound peptides at a flow rate of 2.0 ml/min. Fractions corresponding to each peak were collected by measuring the absorbance at 214 nm. The peptide fractions were then freeze dried and stored at −20 °C.

2.5. Determination of ACE-inhibitory activity

The ACE-inhibition assay was performed as described by Asoodeh et al. (2012). Inhibitory activity (%) was calculated according to the equation:

$$\text{ACE inhibition } (\%) = \left[1 - \left(\Delta A.\min^{-1}_{(\text{sample})} / \Delta A.\min^{-1}_{(\text{control})}\right)\right] \times 100$$

$\Delta A.\ \min^{-1}{}_{(\text{sample})}$ and $\Delta A.\ \min^{-1}{}_{(\text{control})}$ are the reaction rates in the presence and absence of peptide, respectively. Furthermore, ACE inhibition was investigated using five different concentrations (0.062-, 0.125-, 0.25-, 0.50- and 1 mg/ml) of peptide fractions. IC_{50} values of ACE inhibition were calculated by plotting the % ACE inhibition against different concentrations of the peptide.

2.6. Identification of amino acid sequence of the most active peptide

Amino acid sequence and molecular mass of the purified peptide from ostrich egg white (OEW) were determined by MALDI-TOF-TOF mass spectrometer using a 5800 Proteomics Analyzer [Applied Biosystems Proteomics International Pty Ltd., Nedlands, Western Australia]. The peptide samples were diluted in ratios of 1:10 and 1:100 with 2% acetonitrile containing 0.1% TFA. MS/MS spectra were analyzed using PEAKS Studio Version 4.5 SP2 [Bioinformatics Solutions].

2.7. Determination of kinetic parameters of ACE inhibition

The kinetic parameters of ACE in the presence of the most active peptide were determined by Lineweaver–Burk plot. Enzyme activity was measured at different concentrations of FAPGG as substrate (0.5-, 1.0-, 1.5- and 2 mM). On the other hand, the enzyme activities were measured in the presence of three concentrations (0.075, 0.150 and 0.300 mM) of the best inhibitory fraction. The type of ACE inhibition was estimated by comparing the resulted curves in the absence and presence of the inhibitor. The kinetic parameters K_m (Michaeles constant) and V_{max} (maximum reaction velocity) were estimated. Moreover, the inhibition constant (K_i) of a selected inhibitory fraction was obtained from the secondary plot of Lineweaver–Burk plots.

2.8. Fluorescence quenching spectra

All fluorescence emission spectra were measured on a Hitachi spectrofluorometer Model F-2500 equipped with a xenon lamp. A quantitative analysis of the potential interaction between the most active peptide and ACE was performed. In a typical fluorescence measurement, 2 ml of ACE with the concentration of 50 μg/ml was titrated by successive additions of peptide solution (0.0- and 0.65 mM). The fluorescence emission spectra were measured at 298 K in the wavelength rang of 300 to 500 nm with exciting wavelength at 280 nm. Fluorescence spectra were the average of three scans with the baseline corrected by ACE buffer as the control.

2.9. Molecular modeling

The complete three-dimensional structure of rabbit ACE has not been resolved yet, therefore it was modeled based on homology modeling. The amino acid sequence of ACE was retrieved from http://www.uniprot.org/ (Entry code: P12822). Then, the similarity search using Psi-Blast was performed against available PDB structures in order to find proteins with resolved structure and high identity as candidate templates. The resultant structures were N-domain of human somatic ACE (PDB ID: 2C6N) which was used for modeling the N-domain (35–645 residues) of the ACE and human testis ACE (PDB ID: 2OC2) which was used for modeling the C-domain (649–1232 residues) of the ACE (rabbit) molecule. Modeling was carried out by CPH

models 3.2 server (http://www.cbs.dtu.dk/services/CPHmodels/) as well as SWISS-MODEL (http://swissmodel.expasy.org/?pid=smh01) where the SWISS-MODEL based modeling seemed more reliable regarding the assessment tests. The same domains were tested by different softwares including ERRAT (http://nihserver.mbi.ucla.edu/ERRATv2/), and the Ramachandran plots (http://nihserver.mbi.ucla.edu/SAVES/). The best structures were used for further analyses. The structure of the selected peptide was also built in HyperChem program version 7 (http://www.hyper.com), and the geometry of the peptide was subsequently optimized to minimal energy using the same software. applying the following methods and parameters: semi-empirical method; AM1, spin paring was closed-shell restricted Hartree–Fock (RHF); total charge = 0; spin multiplicity = 1; in SCF accuracy of wave function, convergence limit = 0.01 and iteration limit = 50 while convergence was set as accelerated; polarizability and configuration interaction (CI) were set null (i.e. none); in geometry optimization, algorithm was set on Polak–Ribiere and RMS was set at 0.1 kcal/mol. ACE-peptide docking study was performed using the AutoDock 4.0 program package on a Slax Linux workstation to locate possible peptide binding sites on the two domains of ACE. The conformations with the lowest energy and K_i (delta G = −RTlnKi; where delta G is the change in free energy, R is the gas constant, T is the absolute temperature, and K is the equilibrium constant of interest) were used for the final analyses with Swiss-pdb viewer (http://www.expasy.org/spdbv) and Discovery Studio Visualizer 2.5.5. Moreover, the interaction mode between ACE and peptide was determined by Molecular Operating Environment 2008.10 (MOE) software.

3. Results and discussion

3.1. ACE inhibitory activity of ostrich egg white hydrolysates

Trypsin protease was used for the production of ostrich egg white hydrolysate. The ACE inhibitory activity of the obtained hydrolysate was measured during a ten-hour period. The results showed that all hydrolysates demonstrated ACE-inhibitory activity at different levels. In the initial stage (0–4 h), the ACE inhibition by hydrolysate increased to reach its maximum after 4 h of hydrolysis. The inhibitory activity of the hydrolysates varied from approximately 28 to 57%, and then it gradually decreased to 45% after 10 h. Whereas, DH values increased with increasing the incubation time and the highest DH value was 42% after 10 h (Fig. 1a). It seems that the hydrolysis time has a critical role in the production of more active peptides from OEW proteins. During hydrolysis, peptides with a wide variety of sizes were generated. The activities of protein hydrolysates depend on the protein substrate, proteolytic enzyme, time and temperature of hydrolysis as well as amino acid composition and sequence (Bougatef et al., 2008; Lee et al., 2009). Our finding suggests that the hydrolysate obtained after 4 h could be considered as a collection of bioactive peptides with potential ACE inhibitory. These results are consistent with those reported previously such as, protein isolate from pumpkin oil which was hydrolyzed by alcalase and exhibited 53% degree of hydrolysis after 60 min and 71% ACE inhibitory activity (Vaštag, Popovic, Popovic, Krimer, & Pericina, 2010). Wu, Aluko, and Muir (2009) reported a 14.8% ACE inhibitory activity at 70 μg/ml of canola meal hydrolysate. Furthermore, egg white protein hydrolysates obtained from alcalase catalysis exhibited a 58% ACE inhibitory activity after a three-hour hydrolysis (Liu et al., 2010) which accommodates our results.

3.2. Purification of ACE inhibitory peptide

The prepared hydrolysate after four-hour incubation was fractionized by RP-HPLC and nine fractions were collected (Fig. 1b). Using several RP-HPLC runs, sufficient amounts of different factions (2–5 mg) were gathered. The RP-HPLC results showed that the purity of the peptides was higher than 97% (inside of Fig. 1b). Afterward, ACE inhibitory activity was measured and compared with captopril as positive control.

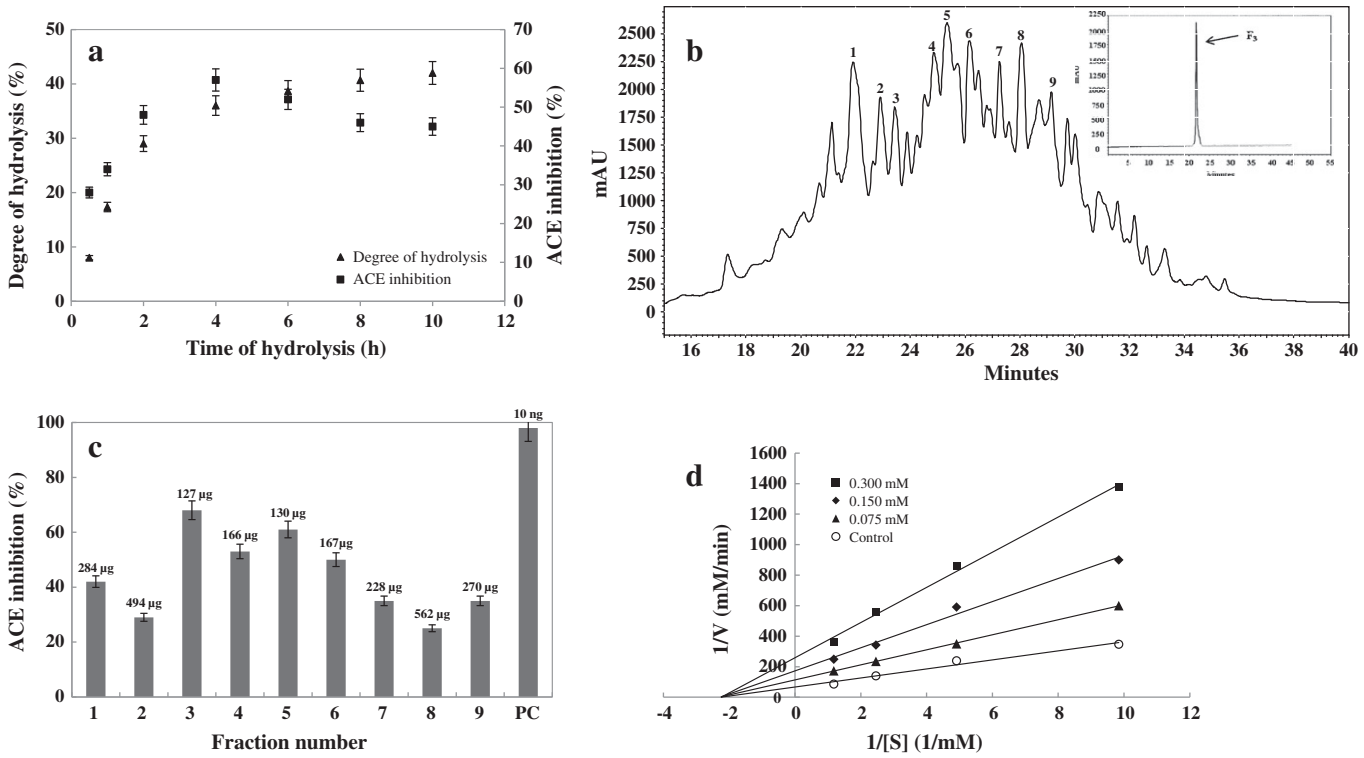

Fig. 1. (a) Degree of hydrolysis and ACE inhibitory activity of ostrich egg white protein hydrolysates during the incubation time. (b) Separation of ACE inhibitory peptides from OEW trypsin hydrolysate (4 h) by RP-HPLC system. Major peaks were numbered sequentially. (c) ACE inhibitory activities of peptide fractions (1–9) from OEW protein hydrolysates. Positive control (PC) is captopril. IC_{50} values of the peptide fractions for ACE inhibition are shown above each column. (d) Lineweaver–Burk plot for determination of inhibitory mode of OEW purified peptide (F_3) on ACE. The ACE activities were measured in the absence (control) or presence of the F_3 peptide.

All fractions displayed ACE inhibitory activity. Among them, the F_3 fraction exhibited the highest level of ACE inhibitory activity (68% at 155 μg/ml). The IC_{50} value was defined as the concentration of inhibitor (protein hydrolysate) required to inhibit 50% of the ACE activity. The IC_{50} values of nine fractions were determined via logarithmic linearization. The results showed that the F_3 fraction with IC_{50} value of 127 μg/ml exhibited the lowest IC_{50} value among other fractions indicating the highest ACE inhibitory activity (Fig. 1c). The ACE inhibitory activity of the peptide fractions was compared with other sources. The peptide fractions with molecular weights of 300 to 1000 from *Undaria pinnatifida* have shown an IC_{50} value of 234 μg/ml (Suetsuna, Maekawa, & Chen, 2004). The ACE inhibitory activity of *Sardinella aurita* by-product fraction was 810 μg/ml (Bougatef et al., 2008). An active fraction from the edible mushroom *Tricholoma giganteum* with IC_{50} value of 400 μg/ml was also reported (Lee, Kim, Park, Choi, & Lee, 2004).

3.3. Amino acid sequence of the most active peptide

The F_3 peptide showed the lowest IC_{50}, which confirms that it is one of most effective inhibitor of ACE activity. To identify the F_3 fraction, it was first undergone further purification as illustrated in Fig. 1a and then was further analyzed by a MALDI-TOF-TOF mass spectrometer. The peptide sequence was found to be Ala–Phe–Lys–Asp–Glu–Asp–Thr–Glu–Glu–Val–Pro–Phe–Arg with a molecular weight of 1582.74 Da (Fig. 2). The resultant amino acid sequence for the F_3 peptide was searched using BLASTp program (http://blast.ncbi.nlm.nih.gov/). The results showed that the identified sequence corresponds to the fragment 188–200 of egg white ovalbumin from *Dromaius novaehollandiae* (92%), *Gallus gallus* (77%), *Meleagris gallopavo* (77%), *Coturnix coturnix* (69%) and *Taeniopygia guttata* (69%). (Table 1).

Several peptides with inhibitory activity against ACE have been identified, including Asp–Asp–Thr–Gly–His–Asp–Phe–Glu–Asp–Thr–Gly–Glu–Ala–Met with IC_{50} value 9.64 μM from *Brachionus rotundiformis* (Lee et al., 2009), and Gly–Asp–Leu–Gly–Lys–Thr–Thr–Thr–Val–Ser–Asn–Trp–Ser–Pro–Pro–Lys–Try–Lys–Asp–Thr–Pro with IC_{50} value of 11.28 μM from tuna frame protein hydrolysates (Lee, Qian, & Kim, 2010). The amino acid composition of ACE inhibitory peptides from fish and shellfish was rich in Asp, Glu, Arg, Pro, Ile and Lys (with a total percentage of 41.20%) at IC_{50} value of 130 μg/ml (Zhang, Wang, & Xu, 2009). Having a positively charged amino acid (Arg or Lys) at the C-terminus of peptide has an important role in the binding to ACE (Cheung, Wang, Ondetti, Sabo, & Cushman, 1980). Since, the positive charge of Arg at the C-terminus of the F_3 peptide may play a critical role in ACE inhibition. Some antihypertensive peptides derived from egg white proteins, including Arg–Val–Pro–Ser–Leu–Met, Thr–Pro–Ser–Pro–Arg, Asp–Leu–Gln–Gly–Lys, Ala–Gly–Leu–Ala–Pro–Tyr and Arg–Val–Pro–Ser–Leu have been reported to match 328–333, 356–360, 127–131, 86–91 and 328–332 residues of the ovotransferrin, respectively (Liu et al., 2010) Additionally, Phe–Arg–Ala–Asp–His–Pro–Phe–Leu (ovokinin) and Arg–Ala–Asp–His–Pro–Phe (ovokinin 2–7) have been found to correspond to the fragments 358–365 and 359–364 of egg albumin (Miguel & Aleixandre, 2006). Furthermore, three peptides including Arg–Val–Pro–Ser–Leu, Gln–Ile–Gly–Leu–Phe, and Thr–Asn–Gly–Ile–Ile–Arg with the IC_{50} values of 20, 75 and 70 μM, respectively, were identified from egg white protein (Yu et al., 2012) Recently, an ACE inhibitory peptide from hen egg white lysozyme (34–45) with the sequence of Phe–Glu–Ser–Asn–Phe–Asn–Thr–Gln–Ala–Thr–Asn–Arg has been reported (IC_{50} = 0.03 mg/ml) (Asoodeh et al., 2012).

Table 1
Amino acid sequences with high homology to the sequence of the F_3 peptide.

Species	Amino acid sequence	Different amino acid(s)	Accession number
Struthio camelus	AFKDEDTEEVPFR	–	This study
Dromaius novaehollandiae	AFKDEDTQEVPFR	Q	E2RVI8.1
Gallus gallus	AFKDEDTQAMPFR	Q, A and M	P01012
Meleagris gallopavo	AFKDEDTQAIPFR	Q, A and I	O73860.3
Coturnix coturnix	AFKAEDTQTIPFR	A, Q, T and I	Q6V115.3
Taeniopygia guttata	AFNEEDTQTVPFR	N, E, Q and T	XP_002199443.1

3.4. Determination of ACE inhibition pattern of OEW purified peptide

The ACE inhibition pattern explains how the F_3 peptide binds to ACE molecule and inhibits its activity. The K_m and V_{max} values at different concentrations of the purified peptide were determined by Lineweaver–Burk plot. The results showed that the inhibition mode of the purified peptide was non-competitive (Fig. 1d). This means that the peptides could bind

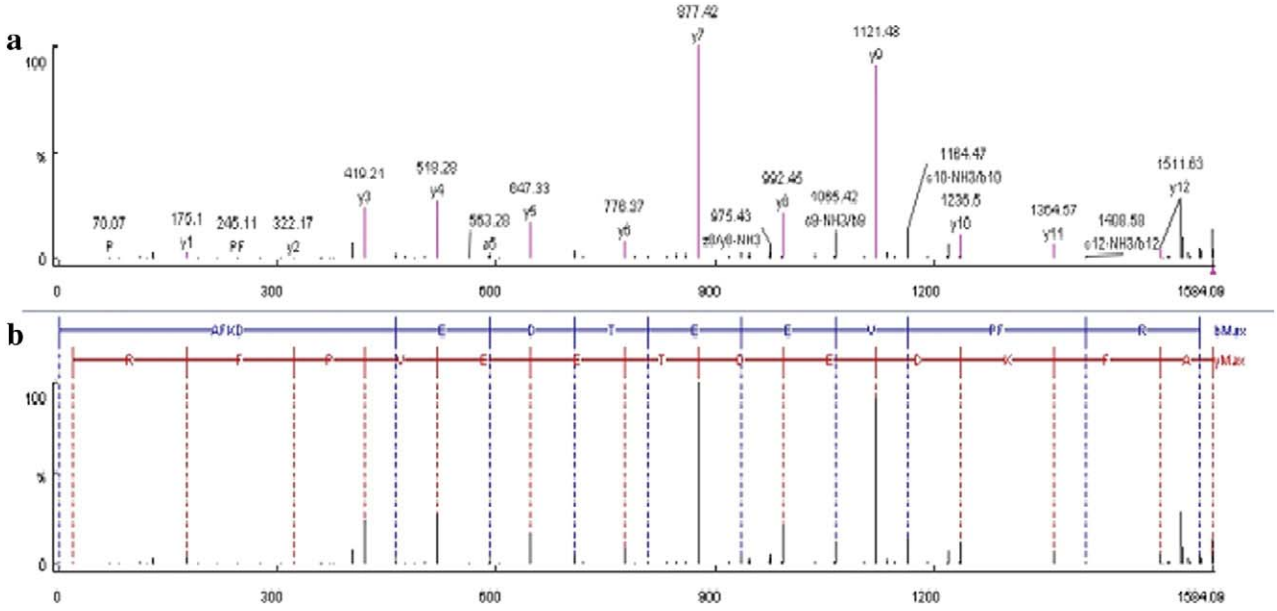

Fig. 2. Identification of the molecular mass and amino acid sequence of the purified peptide fraction (F3) from OEW protein hydrolysate using MALDL-TOF-TOF mass spectrometer. (a) MS/MS spectra of the F_3 fraction separated by RP-HPLC system and (b) the interpretation of the obtained spectra.

ACE another sites different from the FAPGG binding site, which might affect the conformation change of the active site resulting inloss of activity. The K_m, V_{max} and K_i values for F3 were 0.45 mM, 0.015 mM·min^{-1} and 0.104 mM, respectively. Recently, non-competitive ACE inhibitors have been reported Met–Ala–Trp, Val–Tyr–Ala–Pro and Val–Ile–Ile–Phe from *Sepia officinalis* (Balti, Nedjar-Arroume, Bougatef, Guillochon, & Nasri, 2010), Val–Glu–Cys–Tyr–Gly–Pro–Asn–Arg–Pro–Gln–Phe from algae protein waste (Sheih, Fang, & Wu, 2009), Arg–Tyr–Pro–Ser–Tyr–Gly and Asp–Glu–Arg–Phe from bovine casein (Jiang, Tian, Brodkorb, & Huo, 2010) Moreover, competitive ACE inhibitory peptides have been published, for example, Asp–Asp–Thr–Gly–His–Asp–Phe–Glu–Asp–Thr–Gly–Glu–Ala–Met, from *B. rotundiformis* (Lee et al., 2009), Leu–Gly–Phe–Pro–Thr–Thr–Lys–Thr–Tyr–Phe–Pro–His–Phe and Val–Val–Tyr–Tyr–Pro–Trp–Thr, from porcine hemoglobin (Yu et al., 2006).

3.5. Fluorescence quenching of ACE by purified peptide

The fraction F_3 was further studied to evaluate its interaction with ACE using intrinsic fluorescence spectrometry. The fluorescence technique can be used to investigate the binding information of the peptide to ACE molecule, such as the binding mechanism, binding constants and the number of binding sites (Sarzehi & Chamani, 2010). ACE can emit intrinsic fluorescence mainly due to tryptophan, tyrosine and phenylalanine residues. ACE showed strong fluorescence emission peak at 308 nm with an excitation wavelength at 280 nm. The fluorescence intensity of ACE was gradually decreased by increasing the concentration of F_3 (Fig. 3a), indicating the interaction between the peptide and ACE. Furthermore, there was a red shift at the maximum wavelength of ACE (from 308 to 314 nm) after the addition of F_3. The red shift indicates that the fluorescing aromatic residues buried in nonpolar hydrophobic cavities are moved to a more hydrophilic environment (Sarzehi & Chamani, 2010). The fraction F_3 displayed a maximum fluorescence emission peak at 348 nm corresponding to the presence of two phenylalanine residues in its sequence.

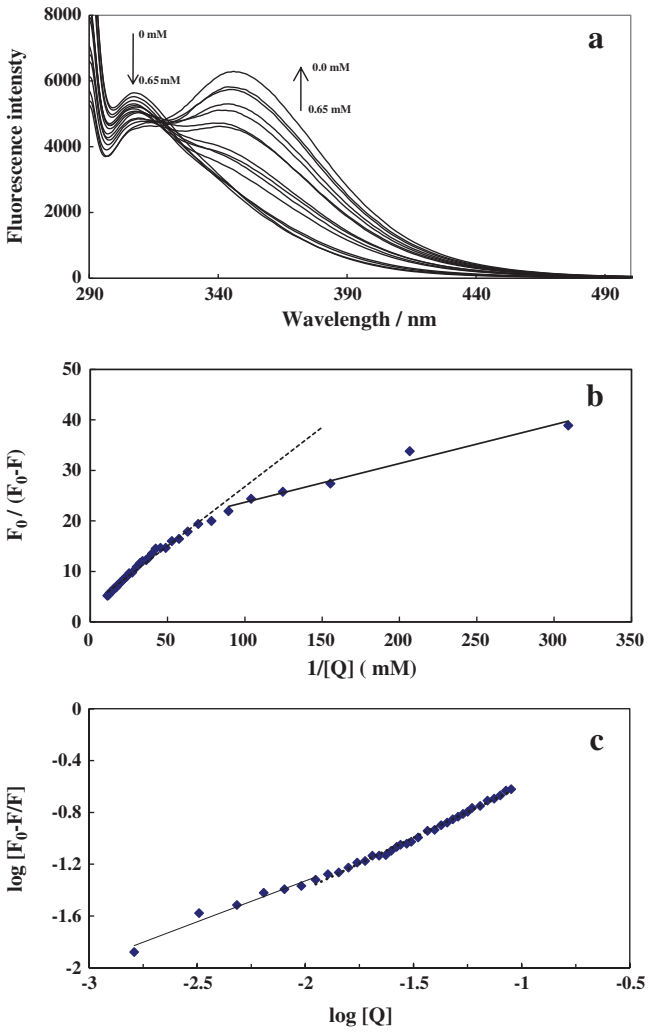

Fig. 3. (a) Fluorescence emission spectra of ACE at varying concentrations of the F_3 peptide at an excitation wavelength of 280 nm. (b) Modified Stern–Volmer curves of fluorescence quenching of ACE–F3. (c) The plots of log(F0-F)/F versus log[Q] for the ACE–F3 system.

3.6. Quenching mechanism analysis

The protein quenching results were analyzed by the modified Stern–Volmer equation (Tang, Luan, & Chen, 2006).

$$F_0/(F_0-F) = 1/(fK[Q]) + 1/f \quad (1)$$

where F_0 is the fluorescence intensity in the absence of the quencher and F is the fluorescence intensity in the presence of the quencher, ΔF is the difference between the fluorescence intensity of ACE in the absence and presence of quencher (F_3 peptide) at concentration [Q], f is the fraction of the initial fluorescence which is accessible to quencher and K is the Stern–Volmer quenching constant (the effective quenching constant for the accessible fluorophore). It can be seen that the plots of $F_0/\Delta F$ versus 1/[Q] concave towards the x-axis at higher [Q], thus illustrating non–linear hyperbolic curves with a negative deviation. These results suggest the additional binding site for the peptide on the ACE. Furthermore, the plot exhibits good linear correlation in two phases (larger than 0.988). Therefore, there are two different slopes (K_1 and K_2) depending on the peptide concentration. In this interaction, K_1 and K_2 were $(1.4\pm0.03)\times10^4$ and $(1.2\pm0.03)\times10^5$ M^{-1}, respectively (Fig. 3b).

Moreover, the value of kq (bimolecular quenching constant) was deduced by the equation as follows:

$$K = kq\tau_0 \quad (2)$$

τ_0 is the lifetime of the biomolecule in the absence of the quencher equal to 10^{-8} s (Hamed-AkbariTousi, Saberi, & Chamani, 2010). The maximum scatter collision-quenching constant of various kinds of quenchers to biopolymer is reported to be 2.0×10^{10} M^{-1} s^{-1}. The kq values in both binding sites were much greater than kq in biopolymers. Therefore, the static quenching could be the main mechanism of the fluorescence quenching of ACE by the peptide. In static quenching interaction, small molecules bind independently to a set of equivalent sites on a macromolecule; whereas in dynamic quenching, the quencher binds to the fluorophore during the lifetime of the excited state.

Another finding deciphered from the fluorescence spectra is that small molecules bind independently to a set of equivalent sites on a macromolecule. The number of binding sites (n) can be determined by the following equation (Hamed-AkbariTousi et al., 2010).

$$\mathrm{Log}[(F_0 - F)/F] = \log K_a + n\log[Q]. \quad (3)$$

The results showed that there were two classes of peptide binding sites on ACE and the n_1 and n_2 values were 0.63 and 0.79, respectively (Fig. 3c). The obtained values indicated that two F3 molecules bind to an ACE molecule which almost corresponds to the difference in binding site affinity. This result was in agreement with the K values obtained from the modified Stern–Volmer equation. Whereas, K is a measure of the affinity of the macromolecule to the ligand, suggesting that the K value for ACE-peptide complex

in the n_2 is higher than n_1. Briefly, the results of fluorescence spectroscopy showed that the interaction between ACE and peptide could be the principal cause of ACE inhibitory activity by the peptide. The fluorescence quenching technique is thus expected to be a promising tool for studying the interaction of peptides and ACE.

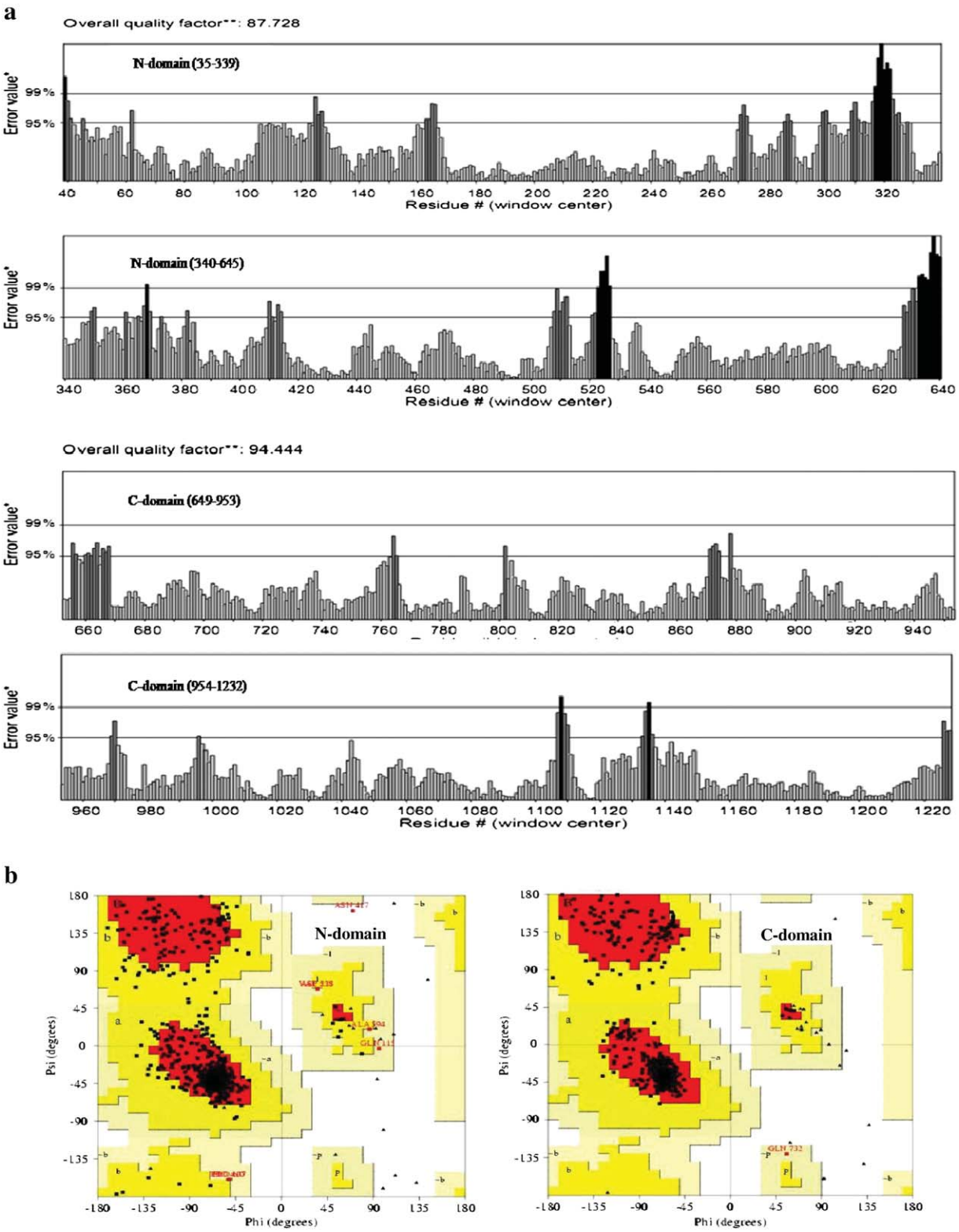

Fig. 4. (a) An ERRAT plot of rabbit ACE molecule. On the error axis, two lines are drawn to indicate the confidence with which it is possible to reject regions exceeding the error value. Since, most areas are within the accepted range for the atomic environment (gray columns). Some black columns represent problematic areas. (b) Ramachandran plot for the rabbit ACE model. The red, yellow, and beige areas represent the favored, allowed, and outlying regions, respectively. (c) Molecular modeling of the interaction between F3 peptide fraction and ACE. The F3 peptide and ACE are shown as CPK (yellow) and surface mode, respectively. Distances between the active site of ACE and F3 were calculated. Only residues with distance less than 4.5 nm to F3 were displayed. Hydrophobic, polar and acidic residues of ACE are represented by green, violet and red rings, respectively.

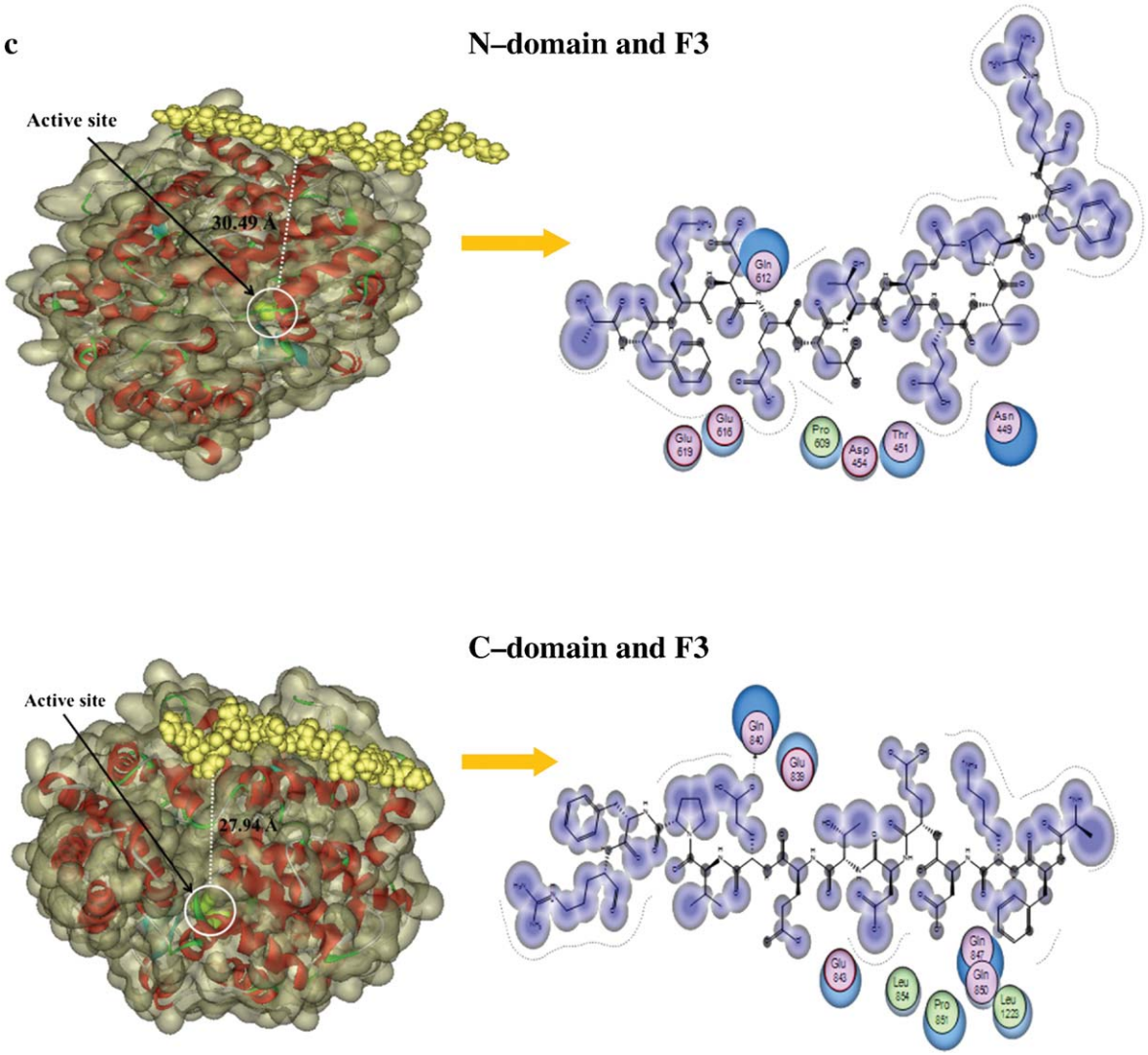

Fig. 4 (*continued*).

3.7. Molecular modeling

The values of overall quality factor of ERRAT test for N- and C-domain were 87.72 and 94.44, respectively which is in an acceptable range especially for the C-domain in which it is over 90% (Fig. 4a). ERRAT is a protein structure verification algorithm which is especially well-suited for evaluating the progress of crystallographic model building and refinement. The program works by analyzing the statistics of non-bonded interactions between different atom types (http://nihserver.mbi.ucla.edu/ERRATv2/). The figure also displays a Ramachandran plot. According to the results, the number of residues for N-domain in favored region, in allowed region and in outlier region was 448 (83.4%), 84 (15.6%) and 4 (0.7%), respectively. Even though the protein suffers from a bit lower percent of amino acids in the favored region (above 95% as mentioned on SAVES site) they are still in the allowed aria (15.6%) which is common in modeled proteins. Furthermore, for C-domain 489 (94.2%) residues were in the favored region. The number of residues in the allowed and outlying regions of C-domain was 29 (5.6%) and only 1 (0.2%), respectively (Fig. 4b). In the previous study, three-dimensional structure of human serum transferrin (hTf) was built based on the same method of homology modeling and then, cyclophosphamide was docked to HSA and hTf to determine the preferred binding site (Hamed-AkbariTousi et al., 2010) The results were all in acceptable ranges.

ACE is predominantly built up from extended α-helices associated with a few short β-strands and has 1310 amino acids and two domains in the regions of 35–634 and 635–1236 joined by an inter-domain linker. Furthermore, it contains two active centers in the residues 396 and 993 in the N- and C-domains, respectively (http://www.uniprot.org/). The active site is located around the central groove (Vercruysse et al., 2010). Multiple amino acid sequence alignments were carried out with ClustalX, and the results of our protein modeling exhibited 43% identity between N-domain and C-domain. Both domains can increase blood pressure by converting the inactive decapeptide angiotensin-I to the potent vasoconstrictor angiotensin-II and, also catalyze bradykinin degradation (a vasodilating nona-peptide), but their affinities to other substrates and inhibitors are different (Corradi, Schwager, Nchinda, Sturrock, & Acharya, 2006). As a consequence, in this study, F3 was docked with both domains distinctly. The best docking results of the interaction between F3 and ACE in all runs (10) of docking procedure are shown in Fig. 4c. Obviously, the F_3 peptide has a greater affinity to the non-common active site in both domains. The results of docking for N-domain showed that the F_3 peptide is located in the ACE by some polar residues (Asn 449, Thr 451 and Gln 612). Gln 612 can contribute in the interaction by forming a stable hydrogen bond. Worth mentioning, the interactions between the F_3 peptide and ACE were not exclusively with hydrophilic residues, because there were a number of specific electrostatic interactions and amino acids, such as Asp 454, Glu 616 and Glu

Table 2
Estimated values of the inhibitory constant (Ki), binding energy, the intermolecular energy, the electrostatic energy and hydrogen bond for ACE with F_3.

System	Ki (μM)	Binding energy (kcal/mol)	Intermolecular energy (kcal/mol)	Electrostatic energy (kcal/mol)	Hydrogen bond
F3- N-domain	21.77	−6.36	−8.06	−2.05	Gln 612
F3- C-domain	39.94	−6.00	−7.80	−1.35	Gln 840

Ki = dissociation constant of the enzyme-inhibitor complex. Evaluation of various energies was conducted using AutoDock 4.

619 in the regions. In addition, F3 participates in a hydrophobic interaction with Pro 609. C-domain was thought to majorly fill with polar amino acids. The acting forces between the F_3 peptide and C-domain are hydrophilic (Gln 840, Gln 847 and Gln 850), hydrophobic (Pro 851, Leu 854 and Leu 1223), electrostatic interactions (Glu 839 and Glu 843) and hydrogen bond (Gln 840) in which the hydrophilic and hydrophobic interactions are dominated. As shown in Table 2, the inhibiting constant for the F3- C-domain is smaller than that of the F3- N-domain complex suggesting that the F_3 peptide has more affinity binding to N-domain with binding energy of −6.36 kcal/mol. Moreover, the distance between F_3 and active site of N- and C-domains was about 30.49 and 27.94 Å, respectively. These results suggested that the interaction between the F_3 peptide and ACE is non-competitive corroborating our result about ACE inhibition pattern in the Lineweaver–Burk plot.

4. Conclusion

In this study, we have isolated ACE inhibitory fractions from OEW protein hydrolysates. A peptide having a high ACE inhibitory activity with the sequence of AFKDEDTEEVPFR was identified by mass spectrometry. Some properties such as inhibitory kinetic parameters (non-competitive), number of binding sites (two classes) and the affinity of the F_3 peptide to N- and C-domains were determined by Lineweaver–Burk plots, fluorescence quenching and molecular modeling, respectively. In conclusion, OEW protein is considered as a source of potential bioactive peptides against hypertension. These results can be used as a model for studying the interaction of inhibitory peptides with ACE.

Acknowledgment

This work was financially supported by Mashhad Branch, Islamic Azad University and Ferdowsi University of Mashhad, Mashhad, Iran. The authors gratefully acknowledge Institute of Biotechnology, Ferdowsi University of Mashhad, Mashhad, Iran for beneficial support.

References

Asoodeh, A., Memarpoor-Yazdi, M., & Chamani, J. (2012). Purification and characterisation of angiotensin I converting enzyme inhibitory peptides from lysozyme hydrolysates. *Food Chemistry*, *131*, 291–295.

Balti, R., Nedjar-Arroume, N., Bougatef, A., Guillochon, D., & Nasri, M. (2010). Three novel angiotensin I-converting enzyme (ACE) inhibitory peptides from cuttlefish (*Sepia officinalis*) using digestive proteases. *Food Research International*, *43*, 1136–1143.

Bernardini, R. D., Harnedy, P., Bolton, D., Kerry, J., O'Neill, E., Mullen, A. M., et al. (2011). Antioxidant and antimicrobial peptidic hydrolysates from muscle protein sources and by-products. *Food Chemistry*, *124*, 1296–1307.

Bougatef, A., Nedjar-Arroume, N., Ravallec-Plé, R., Leroy, Y., Guillochon, D., Barkia, A., et al. (2008). Angiotensin I-converting enzyme (ACE) inhibitory activities of sardinelle (*Sardinella aurita*) by-products protein hydrolysates obtained by treatment with microbial and visceral fish serine proteases. *Food Chemistry*, *111*, 350–356.

Cheung, H. S., Wang, F. L., Ondetti, M. A., Sabo, E. F., & Cushman, D. W. (1980). Binding of peptide substrates and inhibitors of angiotensin converting enzyme. *The Journal of Biological Chemistry*, *255*, 401–407.

Corradi, H. R., Schwager, S. L. U., Nchinda, A. T., Sturrock, E. D., & Acharya, K. R. (2006). Crystal structure of the N domain of human somatic angiotensin I-converting enzyme provides a structural basis for domain-specific inhibitor design. *Journal of Molecular Biology*, *357*, 964–974.

Hamed-AkbariTousi, S., Saberi, M. R., & Chamani, J. (2010). Comparing the interaction of cyclophosphamide monohydrate to human serum albumin as opposed to holo-transferrin by spectroscopic and molecular modeling methods: Evidence for allocating the binding site. *Protein and Peptide Letters*, *17*, 1524–1535.

Jiang, Z., Tian, B., Brodkorb, A., & Huo, G. (2010). Production, analysis and in vivo evaluation of novel angiotensin-I-converting enzyme inhibitory peptides from bovine casein. *Food Chemistry*, *123*, 779–786.

Lee, J. K., Hong, S., Jeon, J. K., Kim, S. K., & Byun, H. G. (2009). Purification and characterization of angiotensin I converting enzyme inhibitory peptides from the rotifer, *Brachionus rotundiformis*. *Bioresource Technology*, *100*, 5255–5259.

Lee, D. H., Kim, J. H., Park, J. S., Choi, Y. J., & Lee, J. S. (2004). Isolation and characterization of a novel angiotensin I-converting enzyme inhibitory peptide derived from the edible mushroom *Tricholoma giganteum*. *Peptides*, *25*, 621–627.

Lee, S. H., Qian, Z. J., & Kim, S. K. (2010). A novel angiotensin I converting enzyme inhibitory peptide from tuna frame protein hydrolysate and its antihypertensive effect in spontaneously hypertensive rats. *Food Chemistry*, *118*, 96–102.

Liu, J., Yu, Z., Zhao, W., Lin, S., Wang, E., Zhang, Y., et al. (2010). Isolation and identification of angiotensin-converting enzyme inhibitory peptides from egg white protein hydrolysates. *Food Chemistry*, *122*, 1159–1163.

Meisel, H., & FitzGerald, R. J. (2003). Biofunctional peptides from milk proteins: mineral binding and cytomodulatory effects. *Current Pharmaceutical Design*, *9*, 1289–1295.

Memarpoor-Yazdi, M., Asoodeh, A., & Chamani, J. (2012). A novel antioxidant and antimicrobial peptide from hen egg white lysozyme hydrolysates. *Journal of Functional Foods*, *4*, 278–286.

Miguel, M., & Aleixandre, A. (2006). Antihypertensive peptides derived from egg proteins. *The Journal of Nutrition*, *136*, 1457–1460.

Nalinanon, S., Benjakul, S., Kishimura, H., & Shahidi, F. (2011). Functionalities and antioxidant properties of protein hydrolysates from the muscle of ornate threadfin bream treated with pepsin from skipjack tuna. *Food Chemistry*, *124*, 1354–1362.

Osuga, D. T., & Feeney, R. E. (1968). Biochemistry of the egg-white proteins of the ratite group. *Archives of Biochemistry and Biophysics*, *124*, 560–574.

Pihlanto, A., Virtanen, T., & Korhonen, H. (2010). Angiotensin I converting enzyme (ACE) inhibitory activity and antihypertensive effect of fermented milk. *International Dairy Journal*, *20*, 3–10.

Sarzehi, S., & Chamani, J. (2010). Investigation on the interaction between tamoxifen and human holo-transferrin: Determination of the binding mechanism by fluorescence quenching, resonance light scattering and circular dichroism methods. *International Journal of Biological Macromolecules*, *47*, 558–569.

Sheih, I. C., Fang, T. J., & Wu, T. K. (2009). Isolation and characterisation of a novel angiotensin I-converting enzyme (ACE) inhibitory peptide from the algae protein waste. *Food Chemistry*, *115*, 279–284.

Shimizu, M., Sawashita, N., Morimatsu, F., Ichikawa, J., Taguchi, Y., Ijiri, Y., et al. (2008). Antithrombotic papain-hydrolyzed peptides isolated from pork meat. *Thrombosis Research*, *123*(5), 753–757.

Suetsuna, K., Maekawa, K., & Chen, J. R. (2004). Antihypertensive effects of *Undaria pinnatifida* (wakame) peptide on blood pressure in spontaneously hypertensive rats. *The Journal of Nutritional Biochemistry*, *15*, 267–272.

Tang, J., Luan, F., & Chen, X. (2006). Binding analysis of glycyrrhetinic acid to human serum albumin: Fluorescence spectroscopy, FTIR, and molecular modeling. *Bioorganic & Medicinal Chemistry*, *14*, 3210–3217.

Vaštag, Ž., Popovic, L., Popovic, S., Krimer, V., & Pericina, D. (2010). Production of enzymatic hydrolysates with antioxidant and angiotensin-i converting enzyme inhibitory activity from pumpkin oil cake protein isolate. *Food Chemistry*, *124*, 1316–1321.

Vercruysse, L., Camp, J. V., Morel, N., Rouge, P., Herregods, G., & Smagghe, G. (2010). Ala–Val–Phe and Val–Phe: ACE inhibitory peptides derived from insect protein with antihypertensive activity in spontaneously hypertensive rats. *Peptides*, *31*, 482–488.

Wu, J., Aluko, R. E., & Muir, A. D. (2009). Production of angiotensin I-converting enzyme inhibitory peptides from defatted canola meal. *Bioresource Technology*, *100*, 5283–5287.

You, L., Zhao, M., Cui, C., Zhao, H., & Yang, B. (2009). Effect of degree of hydrolysis on the antioxidant activity of loach (*Misgurnus anguillicaudatus*) protein hydrolysates. *Innovative Food Science and Emerging Technologies*, *10*, 235–240.

Yu, Y., Hu, J., Miyaguchi, Y., Bai, X., Du, Y., & Lin, B. (2006). Isolation and characterization of angiotensin I-converting enzyme inhibitory peptides derived from porcine hemoglobin. *Peptides*, *27*, 2950–2956.

Yu, Z., Liu, B., Zhao, W., Yin, Y., Liu, J., & Chen, F. (2012). Primary and secondary structure of novel ACE-inhibitory peptides from egg white protein. *Food Chemistry*, *133*, 315–322.

Zhang, F., Wang, Z., & Xu, S. (2009). Macroporous resin purification of grass carp fish (*Ctenopharyngodon idella*) scale peptides with in vitro angiotensin-I converting enzyme (ACE) inhibitory ability. *Food Chemistry*, *117*, 387–392.