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In vitro and *in silico* studies of novel synthetic ACE-inhibitory peptides derived from *Saccharomyces cerevisiae* protein hydrolysate

Mahta Mirzaei[a,*,1], Saeed Mirdamadi[b,*,1], Maliheh Safavi[b], Mahnaz Hadizadeh[b]

[a] Department of Food Science and Technology, Shahr-e-Qods Branch, Islamic Azad University, Tehran, Iran
[b] Department of Biotechnology, Iranian Research Organization for Science & Technology (IROST), Tehran, Iran

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ABSTRACT

The structure-function relation of YR-10 (YGKPVAVPAR) was investigated by synthesizing four structural analogs of that including YHR-10 (YGKHVAVHAR), GA-8 (GKPVAVPA), GHA-8 (GKHVAVHA), and PAR-3 (PAR). GA-8 (GKPVAVPA) was synthesized on the basis of simulated enzymatic gastrointestinal digestion performed by bioinformatics tools (expasy-peptide cutter). This study explains the molecular mechanisms for the interaction of synthetic peptides with ACE. The IC_{50} values of each were 139.554 ± 2.3, 61.91 ± 1.2, 463.230 ± 3.56, 135.135 ± 2.1, 514.024 ± 5.86 μM, respectively. Results indicated that Pro replacement with His in YR-10 and GA-8 increased ACE inhibitory activity respectively, by 55.63% and 70.82%. Removal of Tyr and Arg from respectively N and C terminal positions of YR-10, following in silico simulated gastrointestinal digestion caused the 3.31 fold decrease in ACE inhibitory activity. YHR-10 showed the best docking poses, and GHA-8 exhibited interaction with Zn^{2+}. Lineweaver–Burk plots of most active peptides suggest that they act as noncompetitive inhibitors against ACE.

1. Introduction

Hypertension is a global problem that affects ranges from 15 to 20 percent of the adult's population in the world [1]. These are the most commonly encountered severe health problems and high-risk factors for stroke, cardiovascular disease (CHD), kidney dysfunction, disability, and death [2]. Angiotensin I-converting enzyme (ACE) plays an important role in regulating blood pressure. ACE converts inactive angiotensin I to active angiotensin II, which causes high blood pressure and inactivate bradykinin, which includes blood-pressure control [3].

The search for natural substances in foods with potential therapeutic effects has been considered by researchers and bioactive peptides are used to help treat high blood pressure.

Different protein sources have been considered for the production of biological peptides with ACE inhibitor activity [2,4]. Although, in a limited number of studies, the activity of synthetic peptides has been reported [5,6]. The exact mode of molecular mechanisms and interaction between peptides and ACE is not understood, and there is a lack of information regarding the structure-function relationship of bioactive peptides.

In our last research, a new ACE inhibitory peptide (YR-10, IC_{50} = 0.42 ± 0.02 mg/ml) was purified from *Saccharomyces cerevisiae* protein hydrolysate. Its sequence was identified as YGKPVAVPAR. To extend our earlier experience and studying the structure-function relation of synthetic peptides, four different peptide fragments or analogs were synthesized to compare their activities by the original peptide. YHR-10 (YGKHVAVHAR), was designed based on replacing Pro with His, to compare their role in ACE inhibitory activity. GA-8 (GKPVAVPA), was designed using bioinformatics tools (www.expasy.org/tools/) and theoretically hydrolyzing YR-10 peptide by two low specificity chymotrypsin and pepsin (pH > 2). Furthermore, GHA (GKHVAVHA) was designed to consider the effect of replacing Pro with His in GA-8 sequence. To investigate the role of the three end C-terminal amino acids in the antihypertensive activity of the primary peptide and to answer the question of whether this sequence is active outside the structure of the primary peptide, PAR-3 (PAR) was also synthesized.

We considered the ACE inhibitory activity of all synthetic peptides and also, attempt to find how YR-10, YHR-10, GA-8, GHA-8, and PAR-3 show their antihypertensive effects. This was done through molecular docking study and also with respect to the kinetics of inhibition.

* Corresponding authors.
E-mail addresses: mahtam86@qodsiau.ac.ir (M. Mirzaei), mirdamadi@irost.ir (S. Mirdamadi).
[1] They contribute to this work equally.

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2. Material and methods

2.1. Peptide synthesis

The origin peptide sequence YGKPVAVPAR (YR-10) and different peptide analogs including YGKHVAVHAR (YHR-10), GKPVAVPA (GA-8), GKHVAVHAR (GHA-8), PAR (PAR-3) were synthesized chemically by solid phase method (GL-Biochem, China). The purity (> 90%) of synthesized peptides were determined by HPLC and sequences were verified by analytical HPLC-MS/MS.

2.2. In vitro analysis of ACE-inhibitory activity

The ACE inhibitory activity was evaluated according to Vermeirssen, Van Camp [7]. The rabbit lung powder was prepared as it was described by Lossow et al. [8] with modifications. Rabbit lung acetone powder (100 mg) was dissolved in 1 ml Tris-HCl (50 mM, pH 8.3, containing 5% v/v glycerol), stored at 4 °C for 24 h and centrifuged at 14000 × g for 20 min to obtain lung extract. FAPGG as substrate (5 mM) in Tris- HCl buffer (50 mM, pH 8.3, containing 400 mM NaCl) was mixed with peptides or water (25 µl), and after 20 min incubation at 37 °C, 10 µl of ACE extract was added to each well of ELIZA plate. The IC_{50} value defined as the concentration of the sample required to prevent ACE by 50%.

2.3. Analysis of the physicochemical characteristics of synthetic peptides

The physicochemical characteristics of the four novel synthetic ACEI peptide were predicted by online tools. The Isoelectric point (pI) and water solubility were estimated using peptide property calculator available on https://pepcalc.com/. The PepDraw tool (http://www.tulane.edu/~biochem/WW/PepDraw/), was used to analyze the net charge and hydrophobicity of the peptides and estimate the molecular weight (Da). The instability index of peptides and grand average of hydropathicity (GRAVY) were predicted with the aim of the ProtParam tool (http://web.expasy.org/protparam/).

2.4. Molecular docking of synthetic peptides on the ACE active site

The crystal structure of human ACE (PDB: 1O8A) as a template for docking studies was obtained from the RCSB Protein Data Bank.

Hyperchem program and HADDOCK software were used for respectively, generation of the peptide structure and molecular docking studies [9]. The docking scores and binding energy values were the criteria for the best-ranked docking pose of purified peptides in the active site of ACE. The hydrogen bonds, electrostatic, hydrophilic, and hydrophobic interactions between peptides and amino acids at the active site of ACE were identified by Discovery studio 2016 software. The active site of ACE was identified based on experimental reports. The Zn^{2+} ion is also an important factor for the binding power between ACE and peptides. The ligplot viewer was used to consider the impact of peptides on the tetrahedral coordination of Zn^{2+}.

2.5. Determination of kinetic parameters of ACE inhibition

The Lineweaver–Burk plot determined the kinetic parameters of ACE in the presence of the active peptides (YR-10, YHR-10, and GHA-8). Various FAPGG concentration (0.5–5 mM) as substrate and peptide concentrations (500 and 1000 µM) were incubated with the ACE solution. The ACE inhibition type was estimated by comparing the curves in the presence and absence of inhibitors [10].

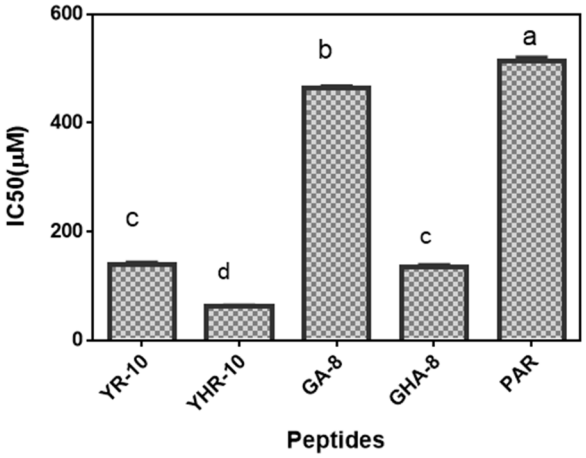

Fig. 1. The ACE-inhibitory activity of chemically synthesized peptides including YR-10 (YGKPVAVPAR), YHR-10 (YGKHVAVHAR), GA-8 (GKPVAVPA), GHA-8 (GKHVAVHA) and PAR-3 (PAR). The results are mean values of experiments carried out in triplicate. Different small letters indicate significant differences at $P < 0.05$.

3. Results and discussion

3.1. Physicochemical characteristics and structures of the synthetic ACE inhibitory peptides

To analyze the interaction mechanism between ACE and the peptides, the physicochemical characteristics of five synthesized peptides (YR-10, YHR-10, GA-8, GHA-8, PAR-3) were considered. The pI and molecular weight of the peptides are presented in Table 2. To evaluate the protein hydrophobicity, GRAVY score that is defined by the amounts of all amino acids hydropathy divided by peptide length was used [11]. A positive GRAVY index obtained for a hydrophobic composition and a negative GRAVY index indicates hydrophilic compounds. The aliphatic index of a protein is defined as the relative volume occupied by the aliphatic amino acids such as Ala, Val, Ile, and Leu. The higher aliphatic index is an indication of higher heat stability [12,13].

The instability index provides an estimate of peptide stability in a test tube. A peptide whose instability index is smaller than 40 is expected to be stable, the value of > 40 predicts that the protein may be unstable [14]. Solubility in water is an estimation, based on the number of charged residues, the isoelectric point (pI), and the peptide length.

3.2. The ACE inhibitory activity of synthetic peptides

The ACE inhibitory activity of the synthesized peptides was evaluated, and results expressed as the IC_{50} value. The IC_{50} value of YR-10 was measured as 139.554 ± 2.30 µM. All other synthetic peptides exhibited the IC_{50} values between 61.91 ± 1.2 and 514.024 ± 5.86 µM. The highest activity was measured for YHR-10.

The results presented in Fig. 1 showed that Pro replacement with His in the sequence of YR-10 peptide led to 55.63% increase in YHR-10 peptide activity. Although it has been previously reported that the presence of hydrophobic Pro residues at one or more positions in the C-terminal tripeptide region positively influence a peptide's ACE inhibitory activity [15], the structure-activity relationship of His-containing peptides has not clearly understood. Our results indicated the more importance of His compare to Pro in YR-10 peptide sequence. This observation may be due to the Zn-chelating activity of imidazole ring in histidine [5]. ACE is a zinc metallopeptidase, and researches indicated that the interactions between the ACE inhibitors and the Zn^{2+} at the ACE active site usually play very important roles in ACE inhibitory activity [16].

In order to investigate the effect of the gastrointestinal digestive

Table 1
Physicochemical characteristics of YR-10 and its fragments including YHR-10, GA-8, GHA-8, and PAR-3.

Peptide	Molecular weight	Net charge	Theoretical pI	Instability index	Aliphatic index	GRAVY index	Water solubility
YR-10	1057.26	+2	10.41	42.26 (unstable)	78.00	−0.130	Good
YHR-10	1137.31	+2	10.41	−7.98 (stable)	78.00	−0.450	Good
GA-8	737.90	+1	10.2	60.94 (unstable)	97.5	0.562	Poor
PAR-3	342.40	+1	11.29	–	–	–	Good
GHA-8	817.45	+1	10.12	−1.86 (stable)	97.5	0.163	Poor

Table 2
Scores and binding energy values for the best-ranked docking pose of five synthetic peptides in the active site of ACE, obtained from docking studies performed using HADDOCK software.

Peptides	E_{vdw} (Kcal/mol)	Electrostatic energy(Kcal/mol)	$_{desolvation}$ (Kcal/mol)	Buried surface area	Haddock score	Binding affinity	Total energy (Kcal/mol)
YR-10	−48	−175	−4.6	1387.7	−48.5	−87.6	−227.6
YHR- 10	−48.6	−187	−2.2	1442.2	−47.4	−88.19	−233.4
PAR-3	−39.5	−83.5	−4.1	936.3	−50.9	−60.3	−118.9
GA-8	−32.5	−143.3	−3.2	973.3	−25.4	−64.4	−172.1
GHA-10	−40.1	−141.8	−4.7	1086.0	−30.2	−72.9	−186.6

Haddock score = (0.2 * Elect) + (1 * vdw) + (1*E desolvation) + (0.1*E Air). Where E_{elec} is the electrostatic energy, E_{vdw} is the van der waals energy, $E_{desolvation}$ is the desolvation energy. Total energy = $E_{Van\ der\ waals} + E_{electrostatic} + E_{desolvation}$, and Binding affinity = Haddock score-(0.1*restraint energy).

system on the ACE inhibitory activity of YR-10, the peptide sequence GA-8 was designed based on the information obtained from bioinformatics tools (www.expasy.org/tools/) that indicated removal of Tyr and Arg residues from respectively, N and C terminal positions of YR-10 after enzymatic digestion. Due to the removal of these two amino acid residues, the ACE-inhibitory activity of peptide reduced 3.31 times. Structure-activity data from different researches suggest that the positive charge on the guanidine of C-terminal Arg contribute substantially to ACE-inhibitory potency [17,18] and our results confirmed it. Also, Cheung et al. [19] reported the importance of Tyr in the C-terminal position of ACE-inhibitory peptides. However, according to our knowledge, there is no report to show its significant role when located in the *N*-terminal position of ACE-inhibitory peptides. The reduction in ACE-inhibitory activity of YR-10 can mostly be contributed to Arg removing.

Besides due to the removal of Tyr and Arg, the amino acid in the penultimate position changed from Ala in YR-10 to Pro in GA-8, and the amino acid in ultimate position changed from Arg in YR-10 to Ala in GA-8. According to previous reports, in addition of the critical role of the tripeptide sequence at the C-terminus of peptides, the amino acids in the penultimate and ultimate positions are important in interaction with ACE. The most ACE-inhibitory peptides include basic (Arg), aromatic (Tyr-Phe), and aliphatic (Val-Ile-Ala) residues in their penultimate position and positively charged amino acid at their ultimate position of C-terminal [20,21]. Our results confirmed the importance of Ala and Arg respectively at penultimate and ultimate positions of YR-10.

As it was observed for YR-10 peptide sequence, the Pro replacement with His in the GA-8 peptide, also increased its activity by 70.82%. The results confirmed the importance of His in the sequence of ACE-inhibitory peptides. It is also worth mentioning that the role of His in penultimate position of GHA was more significant compared to its position in the YHR-10 peptide sequence.

Peptide PAR-3 containing just three C-terminal amino acids of YR-10 exhibited an activity of 3.68 fold less than the initial peptide. It confirms that despite the importance of the three amino acids in the C-terminal position of original peptide, the entire structure and stereo properties of the peptide are also decisive in the binding and enzyme inhibition activity [22].

Based on the GRAVY indexes presented in Table 1, GA-8 is the most hydrophobic peptide, and after that, respectively, GHA-8, YR-10, and YHR-10 exhibited the most hydrophobicity, but there was no direct relation between hydrophobicity and ACE-inhibitory activities of our synthetic peptides. Due to the hydrophobic nature of the C-terminal domain of ACE, a common feature of ACE inhibitory peptides is the hydrophobic nature of the *N*-terminal end and total hydrophobicity of peptides [23,24]. Results indicated that the inhibition mechanisms of our peptides might not be the result of strong hydrophobic interaction between the enzyme and peptide and blocking the *N*-terminal of ACE, as reported for competitive inhibitors [25]. The ACE inhibition mechanism may involve interactions with subsites that are not occupied by substrates or to a binding site different from the catalytic site of the enzyme [23,26].

Also, our results showed that more positively charged peptides (YR-10 and YHR-10) exhibited more ACE-inhibitory activity. This observation was in accordance with the previous report that the positive charge of side-chain groups in amino acids contributes to ACE inhibitory activity [27].

3.3. Docking study of the interaction between synthetic peptides and ACE

While there are various reports of enzyme inhibitory activity of purified and synthetic peptides, there is limited information on the interaction between ACE and peptides. Molecular docking studies are effective to find the relationships between the structure and function of peptides.

In this study, five synthetic peptides were subjected to in silico study in order to predict their interaction with human ACE by computational modelling.

The interaction scores between five synthetic peptides and ACE obtained from docking results are presented in Table 2. The binding values indicated the different potential of all synthetic peptides to bind the ACE. The binding affinity is a good parameter to identify the best ligand for a particular receptor[28]. Particularly, the lowest total energies (van der Waals, electrostatic and desolvation energy) were recorded in YHR-10 (−233.4 Kcal/mol) and YR-10 (−227.6 Kcal/mol) and followed by GHA-8 (−186.6 Kcal/mol), GA-8 (−172.1 Kcal/mol), and PAR-3 (−118.6 Kcal/mol). The buried surface area between peptide was measured as 1442.2, 1387.7, 1086.0, 973.3 and 936.3 respectively for YHR-10, YR-10, GHA-8, GA-8, PAR-3. These data suggest that these peptides could interact effectively with ACE and there was a good correlation between these parameters and ACE-inhibitory activity of five synthetic peptides.

Moreover, 2D diagrams reported in Fig. 2(A–E) revealed that the

five synthesized peptides could bind to ACE through a network of van der Waals, salt bridge and hydrogen bonds. It is suggested that the hydrogen bonds potentially played the most significant role in binding of the inhibitors to ACE [29,30]. However, it is known that covalent, noncovalent, hydrophobic and electrostatic interactions play essential roles in the inhibitory activity [31]. The predicted number of hydrogen bonds between peptides and ACE were also determined and presented in Table 3. The best pose of YHR-10, YR-10, GHA-8, GA-8, PAR-3 with minimal total interaction energy were stable respectively by7, 5, 4 and four hydrogen-bonds. The amino acid residues involved in interactions of ACE and peptides are also presented in Table 3. The highest number of Hydrogen bonds was observed between ACE and YHR-10. According to Chaudhary et al.[32], this observation indicates the effective interaction between this peptide and ACE and thus explaining its stronger inhibition activity presented in Fig. 1.

As reported by Wu et al. [33] ACE has three active pockets, S1, S2, and Ś1. S1 contains Ala354, Glu384, and Tyr523, S2 contains Gln281, His353, Lys511, His513, and Tyr520, while Ś1 contains the residue Glu162. As it is shown in Fig. 2 and Table 3, the H-bond interaction with Glu-162 is present in the docking of YR-10, YHR-10, GA-8 and PAR-3 at the ACE active site (Ś1 pocket) but none of the important amino acids in the active site were involved in interaction with GHA-8. Although, Lisinopril, as a synthetic antihypertensive drug, share interactions at His 353, Ala 354 and Glu 384 of ACE [33]. These findings suggest the non-competitive inhibitory activity of synthetic peptides. Although ACE inhibitory peptides have frequently been reported to act as competitive inhibitors of ACE [4,34,35], in recent years, some non-competitive ACE-inhibitory peptides are reported that interact to ACE and cause conformational change [2,36].

The observed ACE-inhibitory activity of GHA (Fig. 1) was more than what was expected based on the number of interaction H-bonds, and the residues of ACE involved in interaction compare to other peptide

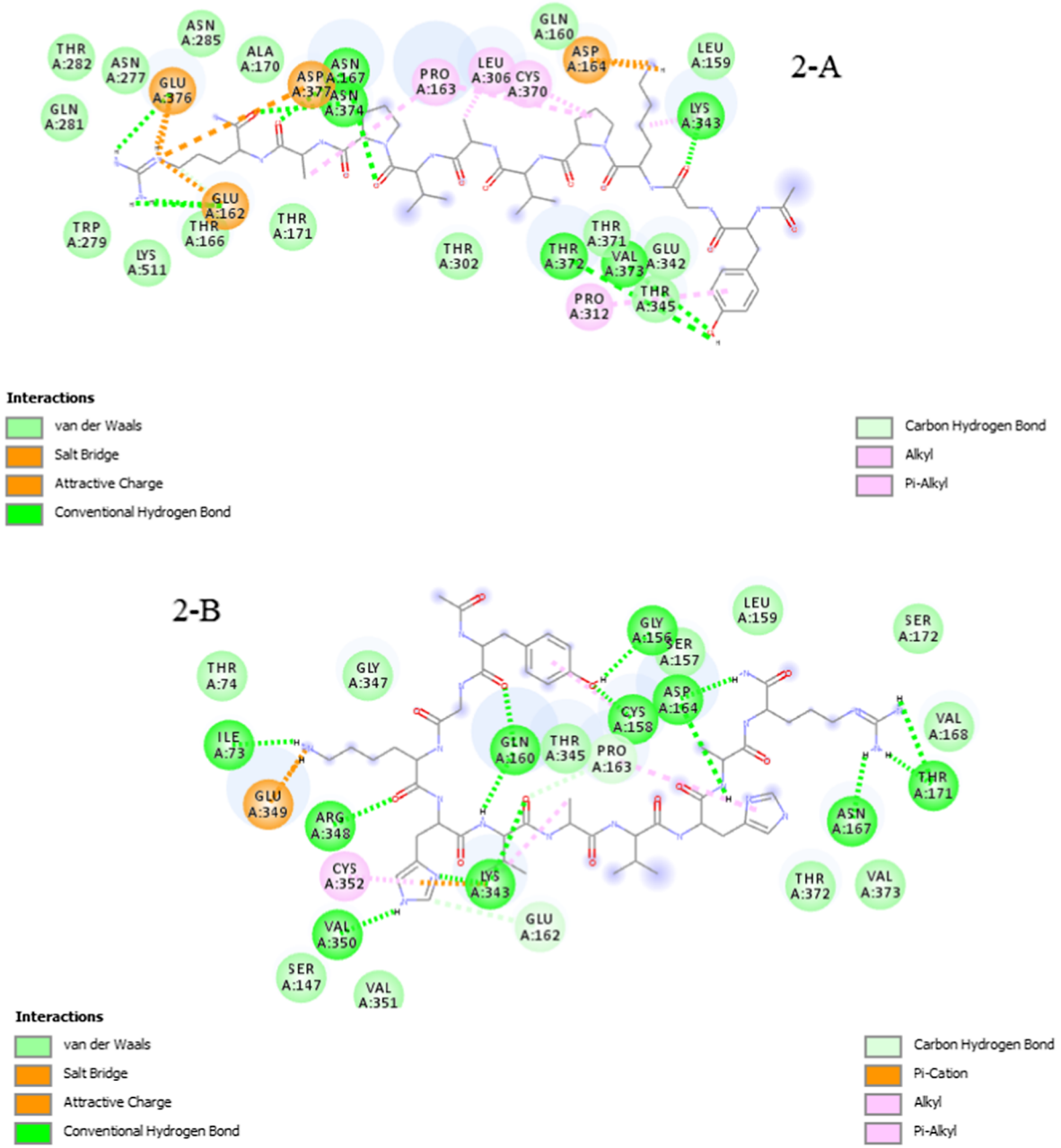

Fig. 2. Details of the interaction between ACE and YR-10 (A), YHR-10 (B), GA-8 (C), GHA-8 (D), PAR-3 (E) after automated docking of the peptides at the ACE-active site (image obtained with Accelrys DS Visualizer software).

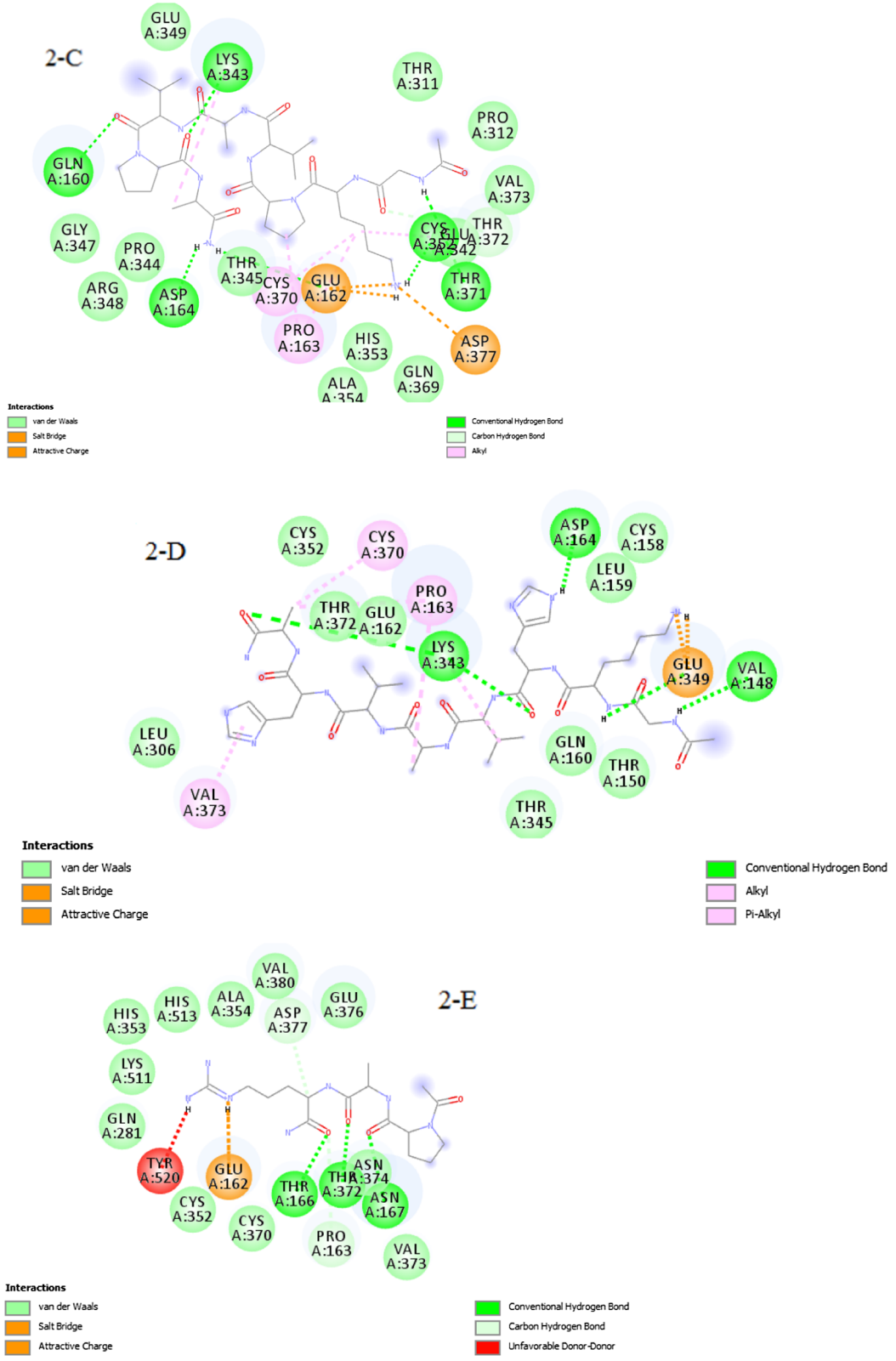

Fig. 2. (*continued*)

sequences. The reason for this observation was considered by investigation the tetrahedral coordination of zinc at the enzyme site, after peptides docking. The results of this study are presented in Fig. 3. ACE consists of two N and C domains. The Zn^{2+} ion in the binding active site of each domain is bound to three ligands (His383, Glu411, and His387) and show tetrahedral coordination. The hydrophobic and H-bond interactions between peptides and zinc are effective in enzyme activity, and metal chelating agents can inhibit ACE [37-39]. As it is represented in Fig. 3, YR-10, YHR-10, GA-8, and PAR-3 exhibited no effect on Zn^{2+} coordination in the active site of the enzyme, but GHA-8 pose exhibited the ability to coordinate with the zinc ion and change the conformation of the active site. This model supports the non-competitive inhibition

Table 3
Hydrogen bonds observed between ACE and the best pose of five synthetic peptides (YR-10, YHR-10, GA-8, GHA-8, PAR-3) obtained from docking results and Lig plus viewer.

YR-10	Tyr			Gly	Lys			Pro	Val	Ala	Val		Pro	Ala	Arg									
ACE	Vla-373			×	Asp-164			×	×	×	Asp-167		×	Asp-167	Glu-162		Glu-376		Asn-374					
YHR-10	Tyr			Gly	Lys		His			Val				Ala	Val	His	Ala	Arg						
ACE	Gly-156	Cys-158	Gln-160	×	Ile-73	Glu-349	Val-350	Lys-343	Glu-162	Gln-160	Lys-343			×	×	×	×	Asn-167	Thr-171					
GA-8				Gly	Lys			Pro		Val	Ala		Val		Pro		Ala							
ACE					Glu-162	Cys-352		×		×	×		Gln-160		Lys-343		×							
GHA-8				Gly			Lys		His				Val	Ala	Val		His	Ala						
ACE				Val-148	Thr-150		Glu-349		Asp-164		Lys-343		×	×	×		×	×						
PAR-3													Pro	Ala				Arg						
ACE													Asp 167	Thr-372			Asn-167	Glu-162						

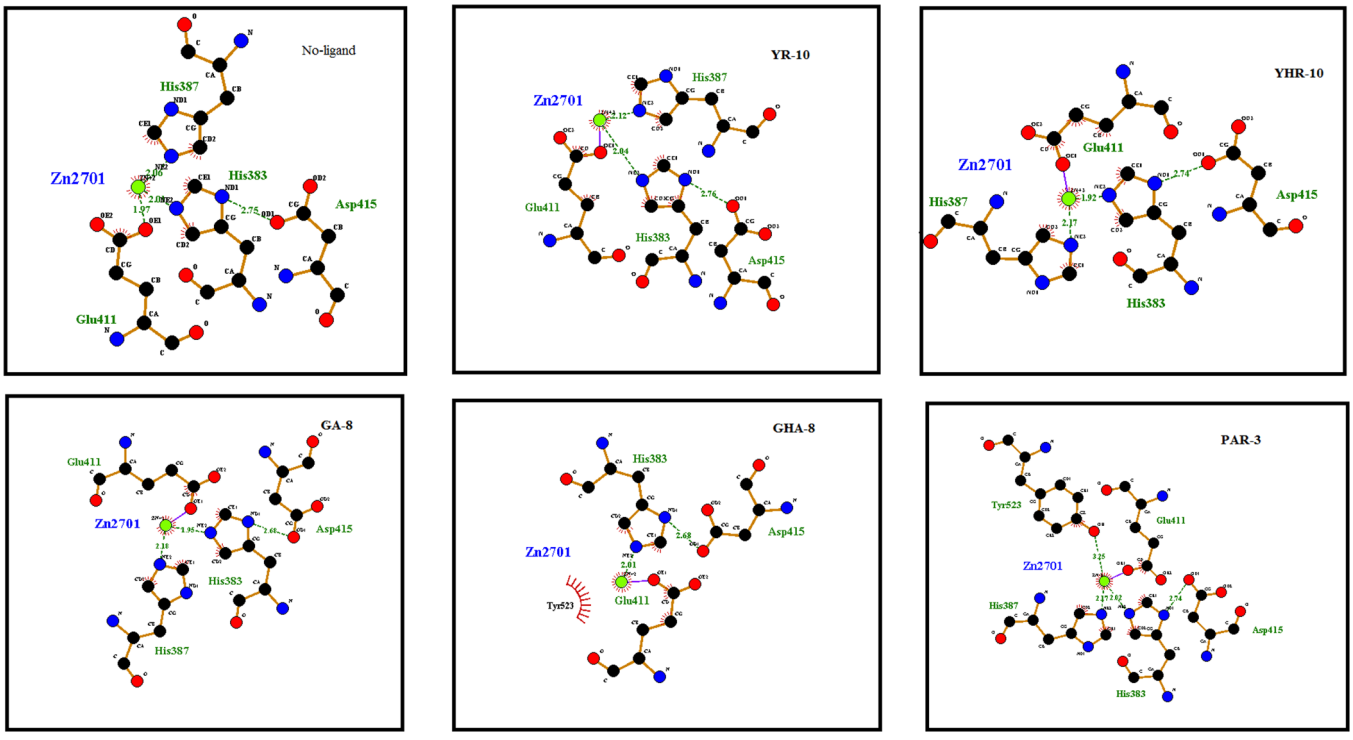

Fig. 3. Schematic diagram showing Zn^{2+} (green) coordination with ACE residues before docking (No-ligand), after docking with YR-10, YHR-10, GA-8, GHA-8 and PAR-3. Green dotted, Blue and brown lines indicate respectively, hydrogen, ligand, and non-ligand bonds formation. Image obtained with Ligplot version v.1.4.5 software. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

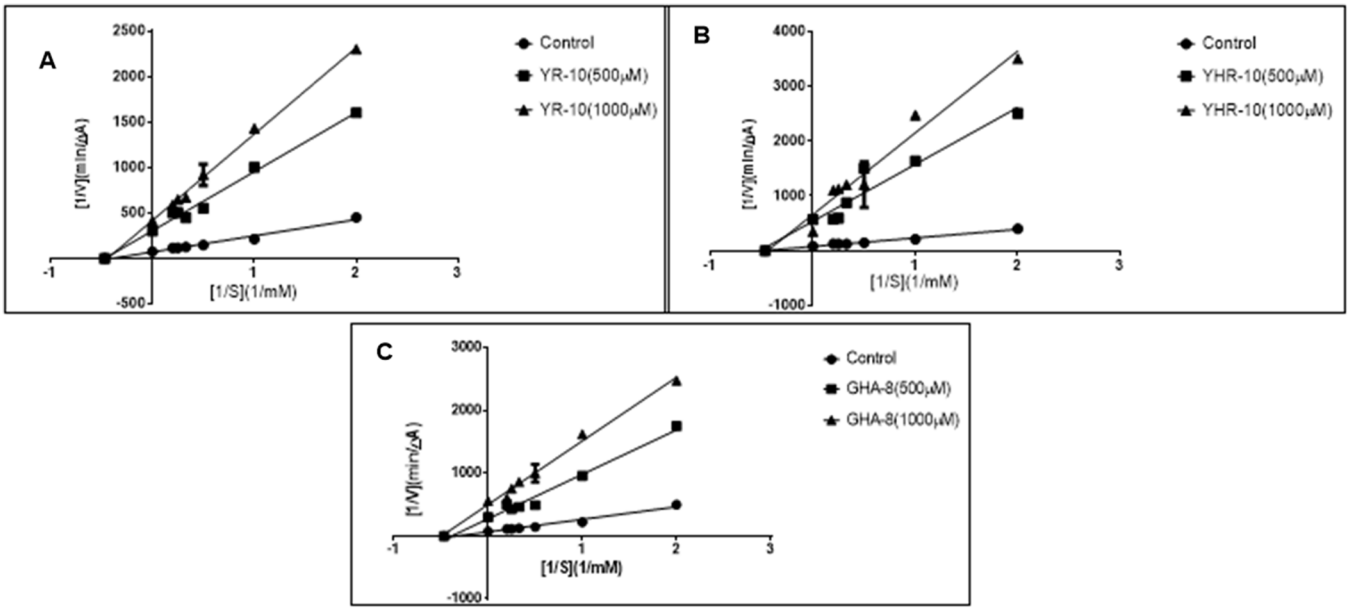

Fig. 4. Lineweaver–Burk plot of ACE activity in the absence and presence of YR-10 (A), YHR-10 (B)and GHA-8 (C) in tris-HCl buffer 50 mM, pH 7.8 at room temperature.

mechanism of peptides. It means that despite the binding of FAPGG onto the active site of the enzyme, it could not be catalyzed to FAP because of the change that is made in the Zn^{2+} coordination [36]. This observation may be due to the chelating activity of histidine imidazole group present in the sequence of GHA-8 [18] and could justify the greater activity of peptide GHA-8 than what was expected compared to other peptide sequences. Many studies indicated the interaction between lisinopril and the Zn^{2+} at the active site of ACE, besides its interaction with the S1 and S2 pockets of the enzyme [16,18].

3.4. Inhibitory kinetics of synthesized peptides

We also attempt to determine the inhibition pattern of peptides (competitive or non-competitive) on ACE and elucidate how they exert their ACE-inhibitory activity, by Lineweaver–Burk plots. The velocity of ACE at different concentrations of FAPGG as the substrate in the presence of two fixed concentrations (500 and 1000 μM) of peptides were analyzed. Fig. 4 demonstrates the Lineweaver–Burk plots of peptides. The variation in K_m and/or V_{max} of ACE in the presence of peptide was also investigated to clarify the inhibition mechanism of YR-10, YHR-10 and GHA-8. According to the results presented in Table 4 and Fig. 4, in the presence of inhibitors, The V_{max} decreased, and K_m did not change. So, the inhibition patterns of the peptides were found to be non-competitive [36].

The non-competitive enzyme inhibitory is a condition in which its inhibitor molecule complicates both free enzymes and the enzyme-substrate complex and indirectly cause conformational changes in the active site of the enzyme [40]. This result was consistent with the previous study of three ACE inhibitory peptide purified from Cuttlefish [2] and hexapeptide (TPTQQS) purified from yeast that had non-competitive inhibition pattern [36].

Besides, peptides were characterized by inhibitor constant (K_i), and it was measured 0.22, 0.0009, and 0.247 mM respectively, for YR-10, YHR-10 and GHA-8 (Table 4). Results showed the highest affinity of YHR-10 to ACE and confirmed its highest ability to inhibit ACE.

Table 4
The kinetic parameters (K_m, V_{max}, and K_i) of ACE in presence and absence of synthetic peptides (YR-10, YHR-10 and GHA-8).

	Peptide concentration (mM)	V_{max} (mM/min)	Km	K_i (mM)
Control	0	0.013	2.141	–
YR-10	0.5	0.003		0.22
	1	0.0024		
YHR-10	0.5	0.0017		0.0009
	1	0.0009		
GHA-8	0.5	0.0033		0.247
	1	0.0018		

4. Conclusion

In conclusion, all synthetic peptides exhibited the ACE-inhibitory activity, and among them, YHR-10 had the most activity. After structure–activity relationship study, it was concluded that Pro replacement with His in both peptides, YR-10 and GA-8, increased enzyme inhibitory activity and removal of Tyr and Arg from respectively, N and C terminal position of YR-10 significantly decreased the ACE-inhibitory activity. Despite the importance of three C-terminal residues in binding to ACE, this sequence was not equally active outside the structure of the primary peptide.

Results from the docking simulation suggested that peptide sequences were able to bind the ACE through H-bonds, hydrophobic, hydrophilic, and electrostatic interactions. YHR-10 exhibited the most number of H-bonds interactions, and this observation confirmed its higher ACE-inhibitory activity. GHA-8 pose showed the ability to coordinate with the Zn^{2+} ion besides its interaction with residues of ACE. Inhibition kinetics study confirmed the non-competitive inhibition manner of YR-10, YHR-10, and GHA-8.

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