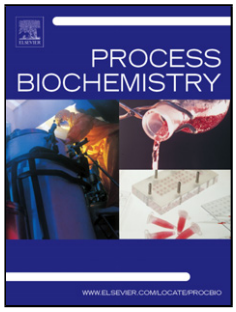
Accepted Manuscript



Title: Angiotensin- I- converting enzyme (ACE) inhibitory peptides from Pacific cod skin gelatin using ultrafiltration membranes

Author: Dai-Hung Ngo Thanh-Sang Vo BoMi Ryu Se-Kwon Kim

PII: S1359-5113(16)30249-5
DOI: http://dx.doi.org/doi:10.1016/j.procbio.2016.07.006
Reference: PRBI 10737

To appear in: *Process Biochemistry*

Received date: 12-2-2016
Revised date: 14-6-2016
Accepted date: 6-7-2016

Please cite this article as: Ngo Dai-Hung, Vo Thanh-Sang, Ryu BoMi, Kim Se-Kwon.Angiotensin- I- converting enzyme (ACE) inhibitory peptides from Pacific cod skin gelatin using ultrafiltration membranes.*Process Biochemistry* http://dx.doi.org/10.1016/j.procbio.2016.07.006

Angiotensin- I- converting enzyme (ACE) inhibitory peptides from Pacific cod skin gelatin using ultrafiltration membranes

Dai-Hung Ngo[a1], Thanh-Sang Vo[b1], BoMi Ryu[c], and Se-Kwon Kim[d,e*]

[a]*Faculty of Resources and Environment, Thu Dau Mot University, Binh Duong, Vietnam*

[b]*Pharmacotechnology and Biopharmacy Lab, Nguyen Tat Thanh Institute of Hi-Technology, Nguyen Tat Thanh University, Ho Chi Minh City, Vietnam*

[c]*School of Pharmacy, The University of Queensland, Brisbane, Qld 4072, Australia*

[d]*Marine Bioprocess Research Center, Pukyong National University, Busan 608-739, Republic of Korea*

[e]*Department of Marine-Bio Convergence Science, Specialized Graduate School Science and Technology Convergence, Pukyong National University, Busan 608-739, Republic of Korea*

[1]*These authors contributed equally to this work.*

[*]Corresponding author:

Se-Kwon Kim

Director, Marine Bioprocess Research Center;

Department of Marine-Bio Convergence Science,

Specialized Graduate School Science and Technology Convergence,

Pukyong National University,

Busan 608-739,

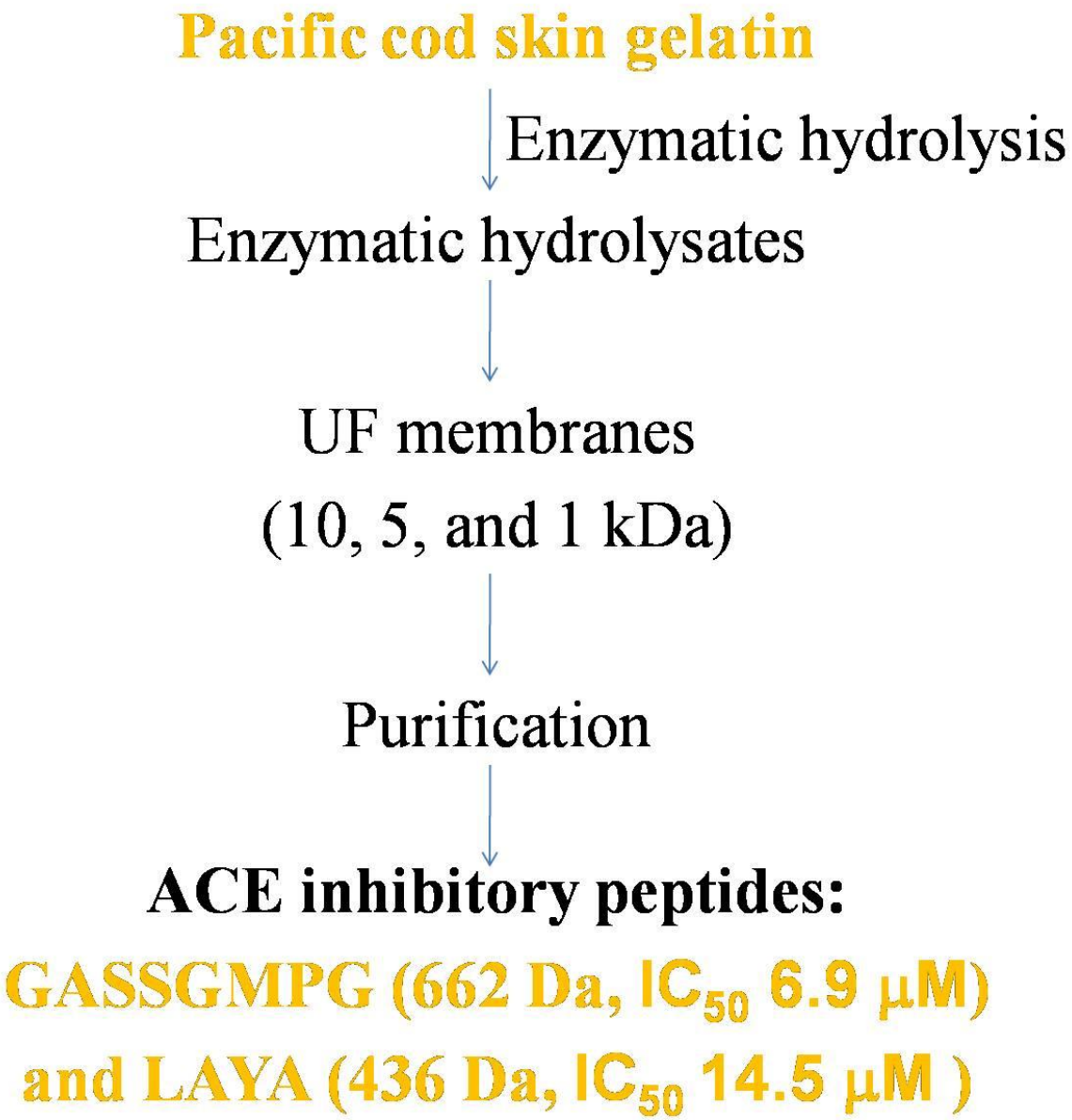
Republic of Korea.

Tel.: +82-51-629-7096;

Fax: +82-51-629-7099.

E-mail*:* sknkim@pknu.ac.kr

Graphical Abstract



Highlights

- Gelatin was isolated from Pacific cod skin, seafood processing byproduct.
- Two bioactive peptides GASSGMPG (662 Da) and LAYA (436 Da) were purified.
- Peptides showed potent ACE inhibition with IC_{50} values of 6.9 and 14.5 μM.
- Molecular mechanism of peptides and ACE was conducted by computational docking.
- Peptides could be used as functional ingredients for improving cardiovascular health.

ABSTRACT

Angiotensin- I- converting enzyme (ACE) is crucial in the control of hypertension and the development of type- 2 diabetes and other diseases associated with metabolic syndrome. The aim of this work was to utilize Pacific cod skin to purify ACE inhibitory peptides. First, gelatin was extracted from Pacific cod skin and hydrolyzed with several enzymes (pepsin, papain, α-chymotrypsin, trypsin, neutrase, and alcalase). The pepsin hydrolysate showed the strongest ACE inhibitory effect and was further fractionated into different ranges of molecular weight (<1, 1–5, 5–10, and >10 kDa) using ultrafiltration (UF) membranes. The peptic hydrolysate below 1 kDa resulted in two potent ACE inhibitory peptides, GASSGMPG (662 Da) and LAYA (436 Da), with IC_{50} values (concentration required to decrease the ACE activity by 50%) of 6.9 and 14.5 μM, respectively. Moreover, to explore the interaction between the peptides and ACE molecule, the tertiary structure of ACE and docking simulation to the peptides were predicted using Docking Server. Pacific cod peptides can be used as functional food ingredients to prevent hypertension and its related diseases.

Keywords: Antihypertension; bioactive peptides; functional ingredients; molecular docking; Pacific cod skin; ultrafiltration membrane.

1. Introduction

The global burden of chronic diseases such as cardiovascular diseases (CVDs), diabetes, obesity, and cancer is increasing rapidly. High blood pressure is an independent risk factor for CVDs and is responsible for most preventable deaths worldwide [1,2]. Human angiotensin- I- converting enzyme (ACE) is crucial in the control of hypertension and electrolyte homeostasis by converting angiotensin I to angiotensin II (vasoconstrictor) and by annulling the potent vasodilator bradykinin to its inactive fragments [3,4]. Synthetic ACE inhibitors such as captopril, lisinopril, and enalapril, although used extensively, are responsible for adverse side effects such as coughing, taste disturbances, skin rashes, dizziness, headache and angioedema [5]. Therefore, it is necessary to discover ACE inhibitors from naturally available sources without side effects.

The seafood processing industry produces a large amount of by-products that usually consist of bioactive materials such as proteins, enzymes and fatty acids. Ultrafiltration (UF) membrane bioreactors can effectively produce bioactive components of desirable molecular weight (MW) such as bioactive peptides from seafood processing by-products [6,7]. Marine bioactive peptides exhibit biological activities such as antihypertensive, antioxidant, antimicrobial, anticancer, mineral-binding, antithrombotic and hypocholesterolemic effects [8-10]. ACE inhibitory peptides can prevent hypertension by binding to the ACE molecule.

In recent years, computational (*in silico*) docking has minimized the time-consuming process of molecular analyses for selecting a suitable ligand, and it has been used to predict the interaction between protein and small molecules such as bioactive peptides [11,12]. Therefore, computational approaches can be used for studying inhibitory mechanisms as an assistant tool and designing novel enzyme

inhibitors. Increasingly more studies have focused on the quantitative structure- activity relationship and the mechanism of peptide binding with ACE using computational simulation, and current software include Discovery Studio, AutoDock and Docking Server [13,14].

In the present study, ACE inhibitory peptides were purified via enzymatic digestion of Pacific cod (*Gadus macrocephalus*) skin gelatin using UF membranes (10, 5, and 1 kDa), fast protein liquid chromatography (FPLC, anion-exchange column and gel filtration column), reversed-phased high-performance liquid chromatography (RP-HPLC) and quadrupole-time-of-flight (TOF) liquid chromatography (LC)/mass spectroscopy (MS) /MS mass spectrometer. Moreover, the tertiary structure of the ACE molecule and docking simulation to the peptides were predicted using Docking Server to explore the binding mechanism including estimation of the free energy of binding, estimation of the inhibition constant, Van der Waals interaction force, hydrogen bonds, polar interaction, hydrophobic interaction, electrostatic interaction force, total intermolecular energy, frequency and interaction surface between the peptides and ACE molecule.

2. Materials and methods

2.1. Materials

Pacific cod (*G. macrocephalus*) skin was collected from the Jagalchi fish market, Busan, South Korea. Captopril, papain, α-chymotrypsin, pepsin, trypsin, ACE (from rabbit lung), and hippuryl-histidyl-leucine (HHL) were provided by Sigma Chemical Co.

(St. Louis, MO, USA). Alcalase and neutrase were obtained from Novozymes Co. (Bagsvaerd, Denmark). UF membranes were procured from GE Healthcare Bio-Sciences Corp. (Westborough, MA, USA). All other chemicals used in the experiments were of analytical grade.

2.2. Gelatin extraction and hydrolysis

Gelatin was extracted as described in Ref. [15]. The collected gelatin was separately hydrolyzed with pepsin (pH 2, 37 °C), papain (pH 6, 37 °C), α-chymotrypsin (pH 8, 37 °C), trypsin (pH 8, 37 °C), neutrase (pH 8, 50 °C), and alcalase (pH 7, 50 °C) [16]. For each enzyme, the enzyme/substrate ratio was 1/100. The resulting mixture was stirred for 4 h and then heated at 100 °C for 10 min to inactivate the enzyme. The pepsin hydrolysate was desalted and separated into four ranges of MW (>10, 5–10, 1–5, and <1 kDa) using UF membranes of 10, 5, and 1 kDa, respectively (GE Healthcare Bio-Sciences Corp, Westborough, MA, USA). All fractions were desalted and lyophilized in a freeze dry system. The bioactive peptide was isolated from the peptic hydrolysate below 1 kDa via FPLC (AKTA, Amersham Bioscience Co., Uppsala, Sweden) on a HiPrep 16/10 diethylaminoethyl fast-flow (DEAE FF) anion-exchange column (16 × 100 mm, Amersham Biosciences, Piscataway, NJ, USA) and a GE Healthcare Superdex™ Peptide 10/300 GL gel filtration column (10 × 300 mm). The purified peptide was desalted and then subjected to amino acid sequencing.

2.3. Measurement of ACE inhibitory activity

The ACE inhibitory effect was measured by measuring the release of hippuric acid from the substrate HHL using the method proposed by Jimsheena and Gowda [15,17] with slight modifications.

2.4. Computational docking

The PDB files of human ACE metalloprotease (1O8A) and captopril were downloaded from RCSB (www.rcsb.org). In addition, the peptides were drawn and converted to PDB file format using the ChemBioOffice 2010 tool. The docking of the target protein and the ligands was simulated using Docking Server (http://www.dockingserver.com/web) [18].

Essential hydrogen atoms, Kollman united atom-type charges, and solvation parameters were added with the aid of AutoDock tools. Affinity (grid) maps of XXÅ grid points and 0.375 Å spacing were generated using the Autogrid program [19].

Docking simulations were performed using the Lamarckian genetic algorithm (LGA) and the Solis and Wets local search method [20]. The initial position, orientation, and torsions of the ligand molecules were set randomly. All rotatable torsions were released during docking. Each docking experiment was derived from 10 different runs that were set to terminate after a maximum of 250,000 energy evaluations. The population size was set to 150. During the search, a translational step of 0.2 Å, and quaternion and torsion steps of five were applied.

2.5. Statistical analysis

The results of the analyses were presented as mean ± standard deviation from three independent experiments. Statistical comparisons between different treatments were done using Student's *t*-test. Differences with a value of $p < 0.05$ were considered significant.

3. Results and discussion

3.1. ACE inhibitory activity of enzymatic hydrolysates using UF membranes

To release ACE inhibitory peptides, gelatin was separately hydrolyzed using different enzymes such as pepsin, α-chymotrypsin, trypsin, papain, alcalase, and neutrase. Among them, at 1 mg/ml concentration, the pepsin- hydrolysate showed the strongest ACE inhibitory effect of about 91% (**Fig. 1A**). The ACE inhibitory activity of the hydrolysate varied with the MW distribution, and the MW distribution of the desired functional peptide can be controlled by using a UF membrane bioreactor system [21,22]. Therefore, the peptic hydrolysate was further fractionated into different MWs using UF membranes of 10, 5, and 1 kDa. Four fractions with different MWs of >10, 5–10, 1–5, and <1 kDa were obtained. As shown in Fig. 1B, VW, a commercial drug, was used as the positive control, which showed the strongest ACE inhibitory activity. Meanwhile, the peptic hydrolysate <1 kDa showed the strongest ACE inhibitory effect of 70% at 500 μg/ml (**Fig. 1B**). This result is consistent with previous studies of ACE inhibitory peptides, in which the low-MW peptides had more potent ACE inhibitory activity than the high-MW peptides. Ko et al. [23] found that the marine *ellipsoidea* protein hydrolysate can be fractionated into three fractions (>10, 5–10, and <5 kDa) by UF according to MWs, and the

fraction below 5 kDa showed the strongest ACE inhibitory activity. Ngo et al. [24] separated skate (*Okamejei kenojei*) skin gelatin hydrolysate using an UF membrane (1 kDa), and the fraction below 1 kDa exhibited the highest ACE inhibitory activity. Thus, the peptic hydrolysate below 1 kDa was selected for further purification.

3.2. Purification of ACE inhibitory peptides

To obtain active peptides with ACE inhibitory activity, the peptic hydrolysate below 1 kDa was further separated into three fractions using the FPLC technique (**Fig. 1C**), and the resultant fractions were also analyzed for their ability to inhibit ACE. At 250 μg/ml concentration, fraction 1 exhibited a strong effect on the ACE inhibitory property of about 81% (**Fig. 1C**). Therefore, fraction 1 was further purified.

FPLC fraction 1 was subjected to a GE Healthcare Superdex™ Peptide 10/300 GL gel filtration column (10 × 300 mm) analysis, and five fractions were obtained (**Fig. 1D**). All five fractions were analyzed for their potency in inhibiting ACE. As shown in **Fig. 1D**, at 100 μg/ml concentration, fraction 1 (FI-1) showed a strong effect on the ACE inhibitory activity of about 96%. The purity of FI-1 was further confirmed by re-chromatographing using RP-HPLC (data not shown).

The peptides were collected and identified as GASSGMPG **(Fig. 2A)** and LAYA **(Fig. 2C)** using a hybrid Q-TOF LC/MS/MS mass spectrometer (AB Sciex Instruments, Redwood City, CA, USA) coupled with an electrospray ionization (ESI) source. The structure of GASSGMPG is composed of eight amino acids (three glycine, two serine, one alanine, one methionine, and one proline)

with MW 662 Da, isoelectric point (p*I*) 5.60, net charge 0, hydrophobicity +12.24 kcal/mol, extinction coefficient[1] 0 M^{-1}/cm, and extinction coefficient[2] 0 M^{-1}/cm (**Fig. 2B)**. The structure of LAYA is composed of four amino acids (two alanine, one leucine and one tyrosine) with MW 436 Da, isoelectric point (p*I*) 5.57, net charge 0, hydrophobicity +6.94 kcal/mol, extinction coefficient[1] 1490 M^{-1}/cm, and extinction coefficient[2] 1490 M^{-1}/cm (**Fig. 2D)**. GASSGMPG, LAYA, and captopril exhibited ACE inhibitory effect with IC_{50} values (the concentration required to decrease the ACE activity by 50%) of 6.9, 14.5, and 0.38 μM, respectively. Most of the reported ACE inhibitory peptides are usually low-MW peptides that can cross the intestinal barrier [25]. The ACE inhibitory activity of GASSGMPG (662 Da, IC_{50} 6.9 μM) was higher than that of ASL (289 Da, IC_{50} 102.15 μM) from silkworm pupa (*Bombyx mori*) protein [26], PVNNPQIH (919 Da, IC_{50} 206.7 μM) from small red bean *Phaseolus vulgaris* [27], GDLGKTTTVSNWSPPKYKDTP (2482 Da, IC_{50} 11.28 μM) from tuna frame protein [28], and AHEPVK (679 Da, IC_{50} 63 μM) from edible mushroom *Agaricus bisporus* [29]. Indeed, the present study showed that the ACE inhibitory peptides GASSGMPG (662 Da) and LAYA (436 Da) have MW of <1 kDa. Therefore, it can be assumed that purified peptides are easily absorbed in the digestive tract and exert biological effects. Both peptides can be incorporated into functional foods as novel ACE inhibitors.

3.3. Molecular docking of the purified peptide on the ACE binding site

In order to explore the molecular mechanism of the interactions between the peptides and ACE molecule, the docking simulation was performed using Docking Server. The docking simulation of the ACE–ligand complexes was well-performed between ACE and the peptides or captopril. The interaction energy of captopril and the peptides binding to ACE is presented in **Table 1A**.

Molecular docking of ACE with GASSGMPG was investigated. According to the results **(Table 1)**, GASSGMPG bound to ACE with estimation free energy of binding –5.16 kcal/mol, estimation inhibition constant (K_i) 166.21 μM, hydrogen bond energy –4.67 kcal/mol, electrostatic energy –1.38 kcal/mol, total intermolecular energy –6.05 kcal/mol, frequency 50% and interaction surface 667.21. The inhibitor combined with the residues of ACE via the main interaction forces of hydrogen bonds, polar, hydrophobic interactions, and Van der Waals and electrostatic forces. The interaction force of hydrogen bonds was crucial [30,31]. The most stabilized pose of GASSGMPG bonding with ACE was determined, and its three-dimensional (3D) and two-dimensional (2D) structures are shown in **Fig. 3a** (supplementary document) and **Fig. 3b** (supplementary document), respectively. The interaction between GASSGMPG and ACE was simulated and depicted in a hydrogen bond plot **(Fig. 3c)** (supplementary document). The binding site of GASSGMPG on ACE was observed at residues of Asn72, Thr74, Glu76, Ile80, Gly347, and Glu349, involving three hydrogen bonds, six polar, three hydrophobic, and 12 other interactions **(Table 1B).**

The molecular docking of ACE with LAYA was also determined. As shown in **Table 1A,** LAYA bound to ACE with binding energy –4.88 kcal/mol, K_i 262.90 μM, hydrogen bond energy –4.67 kcal/mol, electrostatic energy –1.48 kcal/mol, total intermolecular energy –6.16 kcal/mol, frequency 20% and interaction surface 501.331. The most stabilized pose of LAYA bonding with ACE was

obtained, and its 3D and 2D are exhibited in **Fig. 4a** (supplementary document) and **Fig. 4b** (supplementary document), respectively. The interaction of LAYA and ACE was simulated and depicted in a hydrogen bond plot **(Fig. 4c)** (supplementary document). The docking study of the ACE–LAYA interaction revealed the binding site at residues of Asn72, Asp346, and Arg348, involving one hydrogen bond, eight polar, and 22 other interactions **(Table 1C)**.

Molecular docking of ACE with captopril can facilitate good energy level calculations suitable for drug modeling of the ligand. As shown in **Table 1A,** captopril bound to ACE with binding energy –3.81 kcal/mol, K_i 1.61 mM, hydrogen bond energy –4.90 kcal/mol, electrostatic energy –0.02 kcal/mol, total intermolecular energy –4.92 kcal/mol, frequency 50% and interaction surface 436.008. The most stabilized pose of captopril bonding with ACE was obtained, and its 3D and 2D structures were shown in **Fig. 5a** (supplementary document) and **Fig. 5b** (supplementary document), respectively. The molecular docking of captopril on the ACE-binding site revealed that captopril was enveloped by a hydrophobic pocket that was formed by the electron cloud of hydrophobic interactions [32,33]. The interaction of captopril and ACE was simulated and depicted in a hydrogen bond plot **(Fig. 5c)** (supplementary document). The ACE–captopril interaction study showed interaction among six amino acid residues (Trp67, Asn68, Thr71, Asn72, Met340, and Arg348), involving two polar, four hydrophobic, and 14 other interactions **(Table 1D)**.

The binding sites of GASSGMPG and captopril on ACE were observed to be the same at the Asn72 residue. The binding sites of LAYA and captopril on ACE were also observed to be almost the same at residues including Asn72 and Arg348.

The signaling cascade molecules in hypertension must be analyzed computationally to determine better ligands. Molecular docking is an ideal tool for drug design at initial stages. In the present study, the simulation of the protein–ligand chemistry, binding and dissociation energy were the focus. The energy and interaction details were developed using Docking Server. The free energy of ACE–GASSGMPG, ACE–LAYA and ACE–captopril bindings are –5.16, –4.88, and –3.81 kcal/mol, respectively, which is in good agreement with the physiological protein–ligand (hormones, and enzymes) interaction range of –2.00 to –6.00 kcal/mol [34]. In addition, the K_i values of the ACE–GASSGMPG, ACE–LAYA, and ACE–captopril complexes are 166.21 μM, 262.90 μM, and 1.61 mM, respectively. These results suggest the potential candidates for ACE–ligand interaction, which can lead to the development of novel ACE inhibitors.

Other factors such as Van der Waals force and hydrogen bonds of molecules also stabilize the ligand–protein interaction in docking studies, which indicates good protein–drug interaction. The binding site of GASSGMPG on ACE at the six residues of Asn72, Thr74, Glu76, Ile80, Gly347, and Glu349 shows precise conformity. Three hydrogen bonds and the electrostatic force obtained in the results are significant enough for strong bonding in the case of ACE–GASSGMPG interaction. Furthermore, the abundance of polar, hydrophobic, and other interactions in the docking study of ACE–GASSGMPG leads to a further more stable association. The ligand GASSGMPG interacted well with the protein ACE in the docking grid.

The docking results of the ligand LAYA with ACE revealed the binding site at residues of Asn72, Asp346 and Arg348, which also shows precise conformity. Despite the presence of a single hydrogen bond, the electrostatic force is significant enough for strong

bonding in the case of ACE–LAYA interaction. Moreover, the abundance of polar and other interactions greatly contributed to the combinative stabilization. LAYA interacted well with the ACE in the docking grid. These findings indicated that the blockades of the Pacific cod peptides on the ACE molecule might contribute to the development of bioactive food ingredients for preventing hypertension and its related diseases.

Information on the molecular mechanisms of the interactions between the ACE molecule and ACE inhibitory peptides has many potentially favorable consequences for further design and synthesis of their derivatives. Ngo et al. [35] reported that MVGSAPGVL (829 Da, IC_{50} 3.09 μM) and LGPLGHQ (720 Da, IC_{50} 4.22 μM) from skate skin gelatin could interact with ACE residues Asn72, Thr74, Glu76, Thr77, Asp346, and Arg348. Li et al. [36] showed that ACLEP (531 Da, IC_{50} 126 μM) from pistachio hydrolysates could interact with ACE residues His387, Glu384, Arg522, Asp358, Ala356, and Asn70. Jia et al. [37] reported that KHV (382 Da, IC_{50} 12.82 μM) could interact with ACE residues Asn277, Gln281, Thr282, His383, Asp415, Lys454, Ser526, Phe527, and Gln530. The result in this study showed that the binding mode might be different from the previously studied ACE inhibitory peptides. Molecular docking indicated that the purified peptides from Pacific cod skin gelatin could effectively interact with the active site of ACE.

4. Conclusion

Bioactive peptides derived from fish by-products are natural resources that can be used in health and food products. In this study, two peptides, GASSGMPG (662 Da) and LAYA (436 Da), were purified from Pacific cod (*G. macrocephalus*) skin gelatin by pepsin hydrolysis. Both bioactive peptides showed potent ACE inhibitory activity. Furthermore, molecular docking of ACE with ligands GASSGMPG and LAYA when conducting docking analysis using Docking Server, predicted an *in silico* result with free energy of bindings. This result was in line with the physiological range for protein–ligand interaction, indicating the potential of these peptides as ACE inhibitors. Accordingly, ACE inhibitory peptides from Pacific cod skin gelatin can be used in functional food preparations targeted at lowering the blood pressure and reducing the risk of CVDs.

Acknowledgments

This research was supported by a grant from Marine Bioprocess Research Center of the Marine Biotechnology Program (2004-6002) funded by the Ministry of Oceans and Fisheries, Republic of Korea.

References

[1] Kearney PM, Whelton M, Reynolds K, Muntner P, Whelton PK, He J. Global burden of hypertension: analysis of worldwide data. Lancet 2005;365:217-23.

[2] Mohamed S. Functional foods against metabolic syndrome (obesity, diabetes, hypertension and dyslipidemia) and cardiovascular disease. Trends Food Sci Technol 2014;35:114-28.

[3] Mayakrishnan V, Kannappan P, Abdullah N, Ahmed ABA. Cardioprotective activity of polysaccharides derived from marine algae: an overview. Trends Food Sci Tech 2013;30:98-104.

[4] Wijesekara I, Kim SK. Angiotensin-I-converting enzyme (ACE) inhibitors from marine resources: prospects in the pharmaceutical industry. Mar Drugs 2010;8:1080-93.

[5] Vermeirssen V, Van CJ, Verstraete W. Optimisation and validation of an angiotensin-converting enzyme inhibition assay for screening of bioactive peptides. J Biochem Biophys Methods 2002;51:75-87.

[6] Kim SK, Mendis E. Bioactive compounds from marine processing byproducts - a review. Food Res Int 2006;39:383-93.

[7] Ngo DH, Kang KH, Jung WK, Byun HG, Kim SK. Protective effects of peptides from skate (*Okamejei kenojei*) skin gelatin against endothelial dysfunction. J Funct Foods 2014;10:243-51.

[8] Betoret E, Betoret N, Vidal D, Fito P. Functional foods development: trends and technologies. Trends Food Sci Tech 2011;22:498-508.

[9] Pihlanto-Leppälä A. Bioactive peptides derived from bovine whey proteins: opioid and ACE-inhibitory peptides. Trends Food Sci Tech 2001;11:347-56.

[10] Ngo DH, Vo TS, Ngo DN, Wijesekara I, Kim SK. Biological activities and potential health benefits of bioactive peptides derived from marine organisms. Int J Biol Macromol 2012;51:378-83.

[11] Goodsell DS. Computational docking of biomolecular complexes with AutoDock. Cold Spring Harb Protoc, 2009;2009:pdb.prot5200.

[12] Muthukumar V, Vanisree AJ. Molecular interaction of Survivin and Piperine by computational docking analyses for neuroblastoma targeting. Ann Neurosci 2011;18:145-7.

[13] Li P, Jia J, Fang M, Zhang L, Guo M, Xie J, Xia Y, Zhou L, Wei D. *In vitro* and *in vivo* ACE inhibitory of pistachio hydrolysates and in silico mechanism of identified peptide binding with ACE. Process Biochem 2014;49:898-904.

[14] Wu Q, Jia J, Yan H, Du J, Gui Z. A novel angiotensin-I converting enzyme (ACE) inhibitory peptide from gastrointestinal protease hydrolysate of silkworm pupa (*Bombyx mori)* protein: biochemical characterization and molecular docking study. Peptides, 2015;68:17-24.

[15] Ngo DH, Ryu BM, Vo TS, Himaya SWA, Wijesekara I, Kim SK. Free radical scavenging and angiotensin-I converting enzyme inhibitory peptides from Pacific cod (*Gadus macrocephalus*) skin gelatin. Int J Biol Macromol 2011;49:1110-6.

[16] Himaya SWA, Ngo DH, Ryu BM, Kim SK. An active peptide purified from gastrointestinal enzyme hydrolysate of Pacific cod skin gelatin attenuates angiotensin-1 converting enzyme (ACE) activity and cellular oxidative stress. Food Chem 2012;132:1872-82.

[17] Jimsheena VK, Gowda LR. Colorimetric, high-throughput assay for screening angiotensin I-converting enzyme inhibitors. Anal Chem 2009;81:9388-94.

[18] Bikadi Z, Hazai E. Application of the PM6 semi-empirical method to modeling proteins enhances docking accuracy of AutoDock. J Cheminform 2009;1:15.

[19] Morris GM, Goodsell DS, Halliday RS, Huey R, Hart WE, Belew RK, Olson AJ. Automated docking using a Lamarckian genetic algorithm and an empirical binding free energy function. J Comput Chem 1998;19:1639-62.

[20] Solis FJ, Wets RJB. Minimization by random search techniques. Math Oper Res 1981;6:19-30.

[21] Shahidi F, Kamil JYVA. Enzymes from fish and aquatic invertebrates and their application in the food industry. Trends Food Sci Technol 2001;12:435-64.

[22] Erdmann K, Cheung BWY, Schroder H. The possible roles of food-derived bioactive peptides in reducing the risk of cardiovascular disease. J Nutr Biochem 2008;19:643-54.

[23] Ko SC, Kang N, Kim EA, Kang MC, Lee SH, Kang SM, Lee JB, Jeon BT, Kim SK, Park SJ, Park PJ, Jung WK, Kim DK, Jeon YJ. A novel angiotensin I-converting enzyme (ACE) inhibitory peptide from a marine Chlorella *ellipsoidea* and its antihypertensive effect in spontaneously hypertensive rats. Process Biochem 2012;47:2005-11.

[24] Ngo DH, Ryu BM, Kim SK. Active peptides from skate (*Okamejei kenojei*) skin gelatin diminish angiotensin-I converting enzyme activity and intracellular free radical-mediated oxidation. Food Chem 2014;143:246-55.

[25] Korhonen H, Pihlanto A. Bioactive peptides: production and functionality. Int Dairy J 2006;16:945-60.

[26] Wu Q, Jia J, Yan H, Du J, Gui Z. A novel angiotensin-I converting enzyme (ACE) inhibitory peptide from gastrointestinal protease hydrolysate of silkworm pupa (*Bombyx mori*) protein: biochemical characterization and molecular docking study. Peptides. 2015;68:17-24.

[27] Rui X, Boye JI, Simpson BK, Prasher SO. Purification and characterization of angiotensin I-converting enzyme inhibitory peptides of small red bean (*Phas olus vulgaris*) hydrolysates. J Funct Foods 2013;5:1116-24.

[28] Lee SH, Qian ZJ, Kim SK. A novel angiotensin I converting enzyme inhibitory peptide from tuna frame protein hydrolysate and its antihypertensive effect in spontaneously hypertensive rats. Food Chem 2010;118:96-102.

[29] Lau CC, Abdullah N, Shuib AS, Aminudin N. Novel angiotensin I-converting enzyme inhibitory peptides derived from edible mushroom *Agaricus bisporus* (JE Lange) Imbach identified by LC–MS/MS. Food Chem 2014;148:396-401.

[30] Patil R, Das S, Stanley A, Yadav L, Sudhakar A, Varma AK. Optimized hydrophobic interactions and hydrogen bonding at the target-ligand interface leads the pathways of drug-designing. PLoS ONE 2010;5:1-10.

[31] Bikadi Z, Demko L, Hazai E, Functional and structural characterization of a protein based on analysis of its hydrogen bonding network by hydrogen bonding plot. Arch Biochem Biophys 2007;461:225-34.

[32] Akif M, Masuyer G, Schwager SLU, Bhuyan BJ, Mugesh G, Isaac RE, Sturrock ED, Acharya KR. Structural characterization of angiotensin I-converting enzyme in complex with a selenium analogue of captopril. FEBS Journal 2011;278:3644-50.

[33] Douglas RG, Sharma RK, Masuyer G, Lubbe L, Zamora I, Acharya KR, Chibale K, Sturrock ED. Fragment-based design for the development of N-domain-selective angiotensin-1-converting enzyme inhibitors. Clin Sci 2014;126:305-13.

[34] Kortemme T, Baker D. A simple physical model for binding energy hot spots in protein-protein complexes. Proc Natl Acad Sci USA 2002;99:14116-21.

[35] Ngo DH, Kang KH, Ryu BM, Vo TS, Jung WK, Byun HG, Kim SK. Angiotensin-I converting enzyme inhibitory peptides from antihypertensive skate (*Okamejei kenojei*) skin gelatin hydrolysate in spontaneously hypertensive rats. Food Chem 2015;174:37-43.

[36] Li P, Jia J, Fang M, Zhang LJ, Guo MR, Xie JL. *Invitro* and *invivo* ACE inhibitory of pistachio hydrolysates and insilico mechanism of identified peptide binding with ACE. Process Biochem 2014;49:898-904.

[37] Jia JQ, Wu QY, Yan H, Gui ZZ. Purification and molecular docking study of a novel angiotensin-I converting enzyme (ACE) inhibitory peptide from alcalase hydrolysate of ultrasonic-pretreated silkworm pupa (*Bombyx mori*) protein. Process Biochem 2015;50:876-83.

Table legends

Table 1: Estimation free energy of binding, estimation inhibition constant, Van der Waals interaction force, hydrogen bonds, polar interaction, hydrophobic interaction, electrostatic interaction force, total intermolecular energy, frequency, and interaction surface between the ACE molecule and the ligands GASSGMPG, LAYA, and captopril **(A)**. Interaction table of residues and atoms between the ACE molecule and the ligands GASSGMPG **(B)**, LAYA **(C)**, and captopril **(D)**.

Figure legends

Figure 1. Purification profiles of novel ACE inhibitory peptide from Pacific cod skin gelatin. **(A)** ACE inhibition of enzymatic hydrolysates (pepsin, papain, alcalase, neutrase, α-chymotrypsin, and trypsin) from Pacific cod skin gelatin. **(B)** ACE inhibition of pepsin hydrolysates (>10, 5–10, 1–5 and <1 kDa) using UF membranes and VW (valine–tryptophan) as the positive control. **(C)** FPLC of pepsin hydrolysate below 1 kDa using a HiPrep™ 16/10 DEAE FF anion-exchange column (upper panel). ACE inhibitory activity of each FPLC fraction is shown in the lower panel. **(D)** FPLC of fraction 1 using a GE Healthcare Superdex™ Peptide 10/300 GL column (upper panel). ACE inhibitory activity of each fraction is shown in the lower panel. Results are the mean ± SD of three independent experiments, and significance was determined at (∗) $0.01 < p < 0.05$, (∗∗) $0.001 < p < 0.01$ and (∗∗∗) $p < 0.001$.

Figure 2. Mass spectrum and amino acid sequence of the purified peptide GASSGMPG **(A)**. The structure and properties of the purified peptide GASSGMPG **(B)** derived from Pacific cod skin gelatin. Mass spectrum and amino acid sequence of the purified peptide LAYA **(C)**. The structure and the properties of the purified peptide LAYA **(D)** derived from Pacific cod skin gelatin**.**

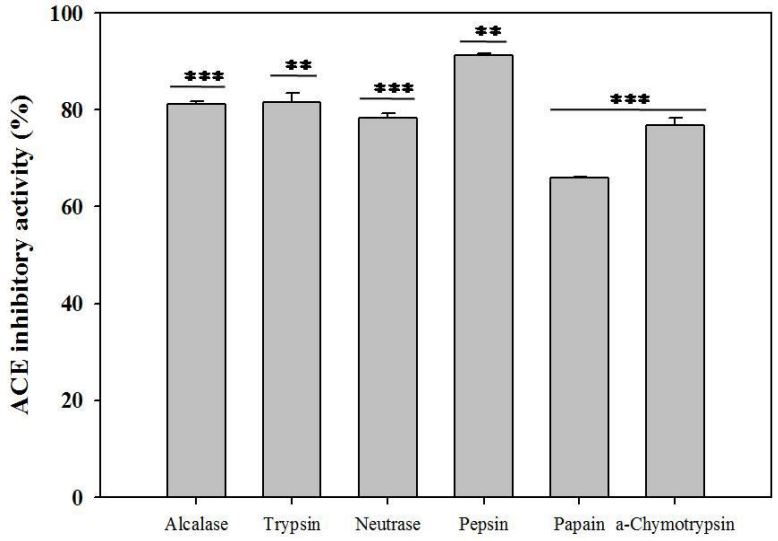


Figure 1A. Ngo et al (2016)

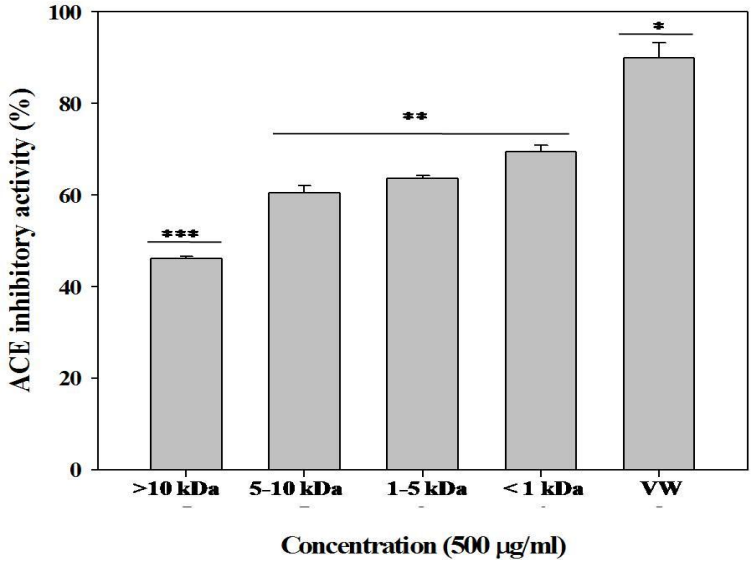
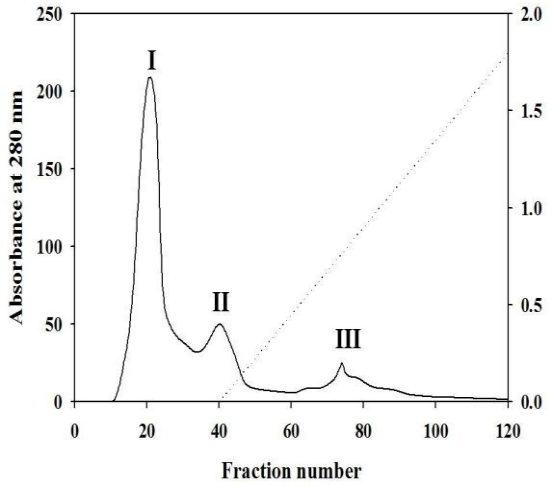


Figure 1B. Ngo et al (2016)



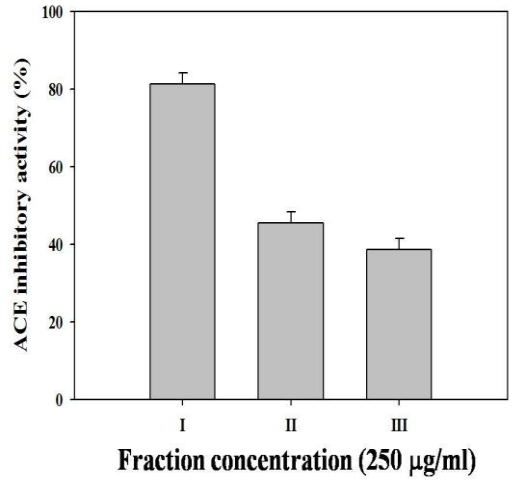
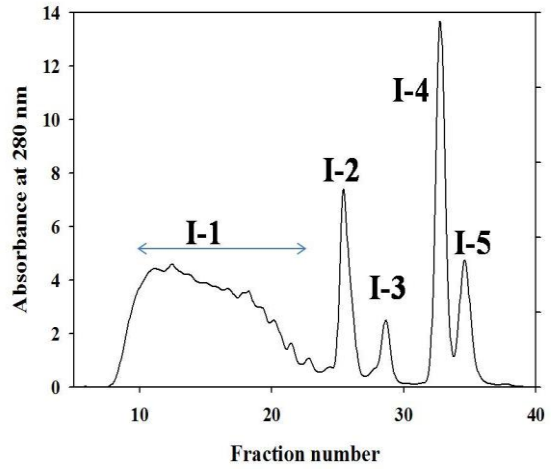


Figure 1C. Ngo et al (2016)



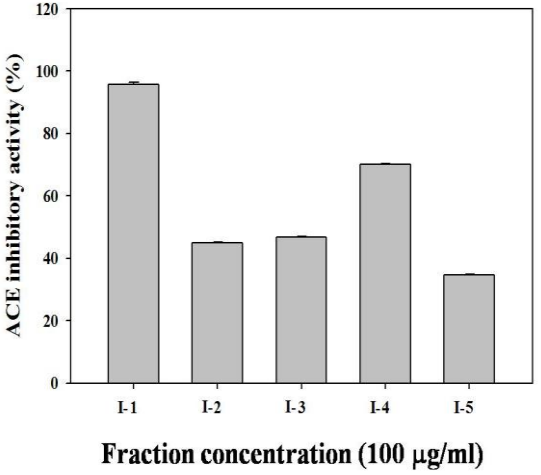


Figure 1D. Ngo et al (2016)

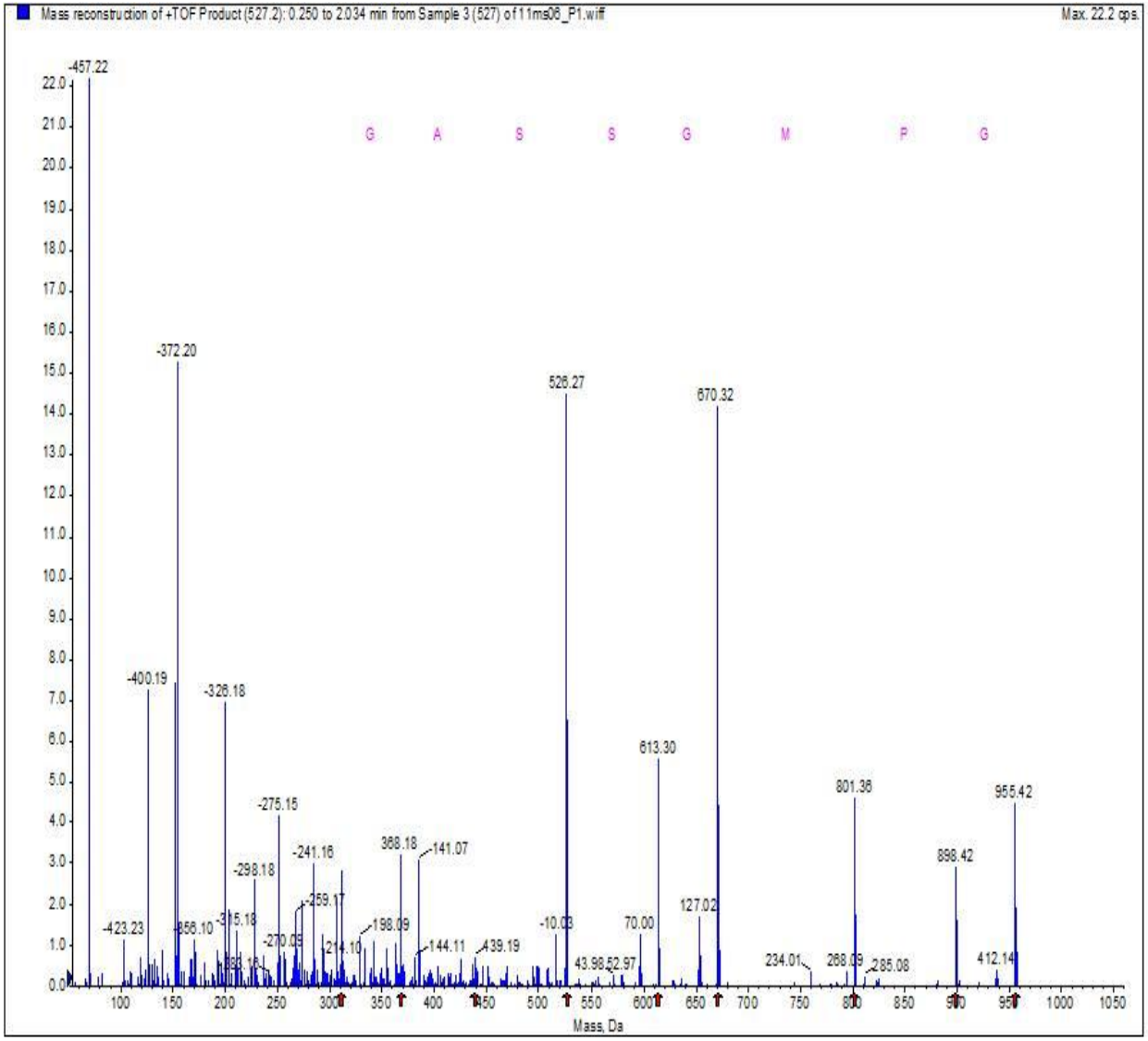
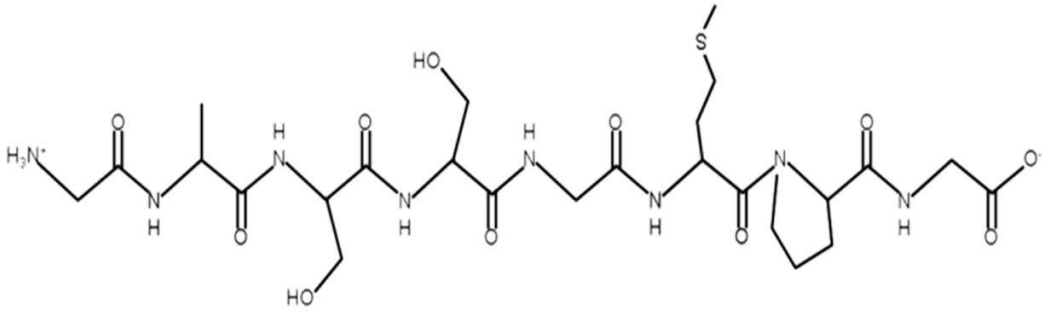


Figure 2A. Ngo et al. (2016)



Properties	
Sequence:	GASSGMPG
Length:	8
Mass:	662.2684 Da
Isoelectric point (pI):	5.60
Net charge:	0
Hydrophobicity:	+12.24 kcal * mol^{-1}
Extinction coefficient[1]:	0 M^{-1} * cm^{-1}
Extinction coefficient[2]:	0 M^{-1} * cm^{-1}

Figure 2B. Ngo et al. (2016)

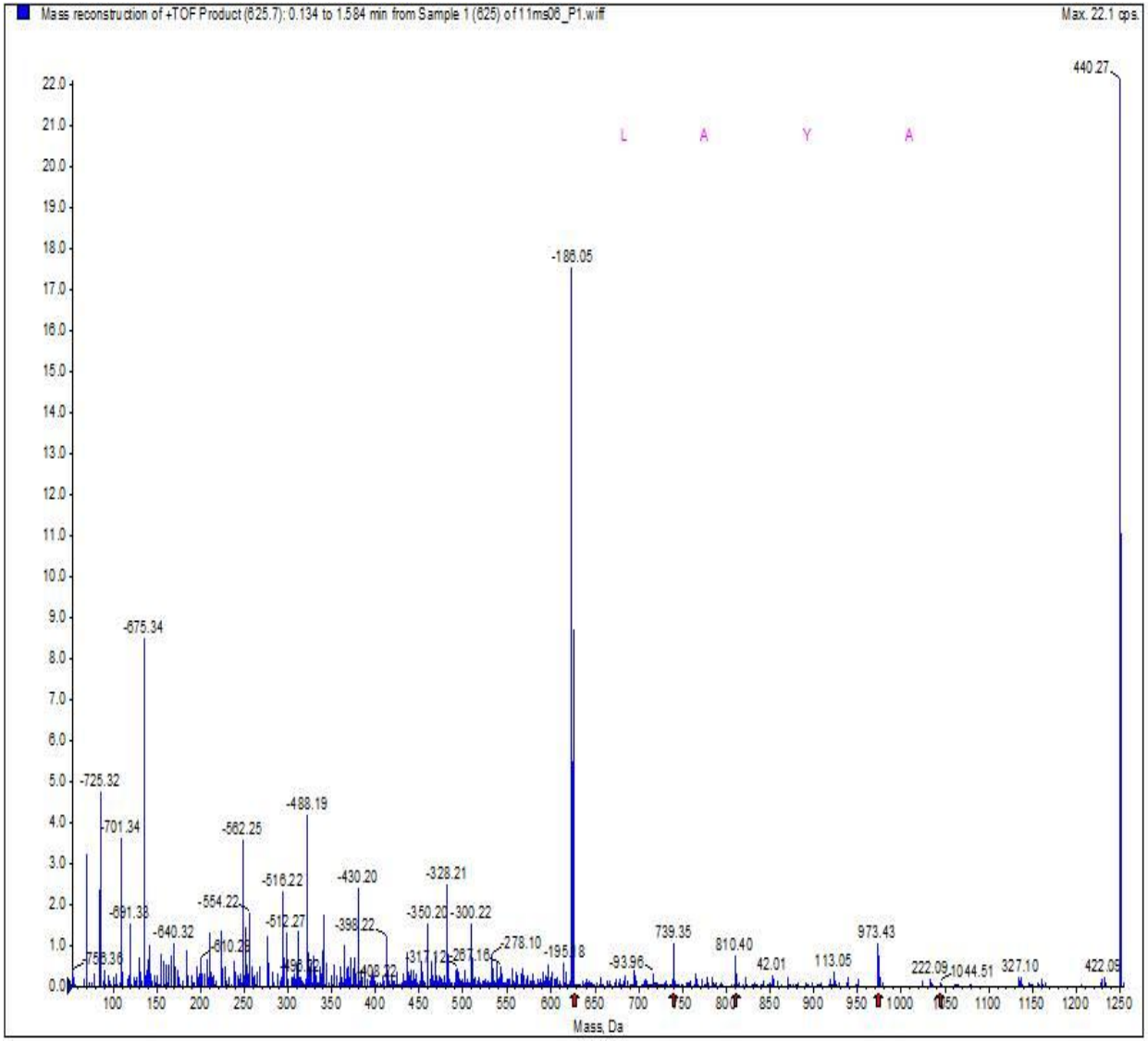
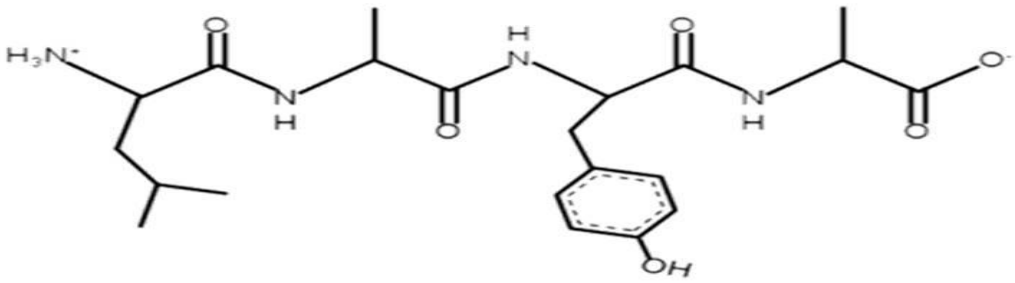


Figure 2C. Ngo et al. (2016)



Properties	
Sequence:	LAYA
Length:	4
Mass:	436.2314
Isoelectric point (pI):	5.57
Net charge:	0
Hydrophobicity:	+6.94 kcal * mol^{-1}
Extinction coefficient¹:	1490 M^{-1} * cm^{-1}
Extinction coefficient²:	1490 M^{-1} * cm^{-1}

Figure 2D.Ngo et al. (2016)

Tables

Table 1A: Ngo et al. (2016)

Ligand	Rank	Est. free energy of binding (kcal/mol)	Est. inhibition constant (*Ki*)	vdW + Hbond + desolv energy (kcal/mol)	Electrostatic energy (kcal/mol)	Total intermolec. energy (kcal/mol)	Frequency (%)	Interact. surface
GASSGMPG	1	-5.16	166.21 µM	-4.67	-1.38	-6.05	50	667.21
GASSGMPG	2	-4.24	782.82 µM	-4.00	-1.77	-5.77	10	540.487
GASSGMPG	3	-4.01	1.15 mM	-3.06	-1.81	-4.88	10	565.22
GASSGMPG	4	-3.93	1.31 mM	-3.54	-1.36	-4.90	10	519.459
GASSGMPG	5	-3.57	2.40 mM	-2.99	-1.35	-4.34	10	472.717
GASSGMPG	6	-3.51	2.69 mM	-3.07	-1.34	-4.41	10	370.465
LAYA	1	-4.88	262.90 µM	-4.67	-1.48	-6.16	20	501.331
LAYA	2	-4.80	300.68 µM	-4.56	-1.38	-5.94	10	513.863
LAYA	3	-4.21	820.25 µM	-4.88	-0.63	-5.51	20	560.199
LAYA	4	-4.12	947.61 µM	-3.76	-1.62	-5.38	10	520.964
LAYA	5	-4.08	1.02 mM	-3.43	-1.83	-5.26	20	425.046
LAYA	6	-3.90	1.38 mM	-3.50	-1.71	-5.21	10	513.763
LAYA	7	-3.34	3.59 mM	-2.98	-1.64	-4.62	10	426.157
Captopril	1	-3.81	1.61 mM	-4.90	-0.02	-4.92	50	436.008
Captopril	2	-3.45	2.98 mM	-3.98	-0.09	-4.07	20	367.300
Captopril	3	-3.05	5.80 mM	-4.07	-0.01	-4.08	10	419.764
Captopril	4	-3.02	6.10 mM	-4.20	-0.06	-4.26	20	391.592

Table 1B: Ngo et al. (2016)

GASSGMPG-ACE interaction table		
Hydrogen bonds	**Polar**	**Hydrophobic**
O () [3.33] – THR74 (CB, CG2, G1)	N () [3.76] – ASN72 (ND2)	CB () [3.34] – ILE80 (CD1)
N () [2.92] – GLY347 (O)	O () [3.22] – GLU76 (OE2)	CA () [3.55] – ILE80 (CD1)
N () [2.57] – GLU349 (CB, CD, OE1)	O () [3.75] – GLU349 (OE1)	C () [3.87] – ILE80 (CD1)
	1H () [2.93] – GLU349 (OE1)	
	2H () [1.67] – GLU349 (OE1, OE2)	
	3H () [2.80] – GLU349 (OE1)	

Table 1C: Ngo et al. (2016)

LAYA-ACE interaction table	
Hydrogen bonds	**Polar**
N () [2.58] – ASP346 (CG, OD1, OD2)	N () [3.41] – ASN72 (ND2)
	OXT () [3.52] – ASN72 (ND2)
	H () [3.10] – ASN72 (ND2)
	1H () [3.57] – ASP346 (OD1)
	2H () [2.25] – ASP346 (OD1, OD2)
	3H () [2.47] – ASP346 (OD1)
	O () [2.99] – ASP346 (OD1, OD2)
	H () [1.98] – ASP346 (OD1, OD2)

Table 1D. Ngo et al. (2016)

Captopril-ACE interaction table					
Polar			**Hydrophobic**		
O2 () [3.84]	–	TRP67 (NE1)	C9 () [3.57]	–	TRP67 (CD1)
H1 () [2.83]	–	THR71 (OG1)	C4 () [3.54]	–	TRP67 (CE2, CZ2)
			C3 () [3.38]	–	TRP67 (CZ2)
			C3 () [3.37]	–	MET340 (CB)