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Searching for Active Compounds with Preventive Effects on Hypertension : A Focus on Angiotensin-Converting Enzyme Inhibitors

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Searching for Active Compounds with Preventive Effects on Hypertension:
A Focus on Angiotensin-Converting Enzyme Inhibitors

Tran Hai Bang

2015

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Thesis Declaration

I, Tran Hai Bang, hereby to declare that all data described in this thesis are results of my own work unless otherwise acknowledged or referenced. This thesis submitted in fulfilment of the requirements for the award of Doctor of Philosophy, in the Faculty of Agriculture at Kyushu University and has not been submitted for qualifications at any other academic institution.

Dedication

For my mother who always cares about me and gives me immense love

For my wife who ignore herself to accept me and support me

For my children who are the source of my motivation

Abstract

From the pioneering discovery of synthetic ACE inhibitor, captopril, many other ACE-targeted inhibitors have been developed and dozen of them are now available for using in hypertensive medication. However, the accompanied side effects such as dry cough, dizziness, headache sometime prevent patient from using of these drugs. Therefore, beside synthetic pathway, scientists have also sought for ACE inhibitors from natural source with aims at increasing the antihypertensive activity while reducing side effects of inhibitors.

In this study a bilateral approach, which both use nature-derived products and synthetic compounds for discovering potential ACE inhibitors, was done. By screening 29 wild-type mushrooms, several potent ACE inhibitor-containing samples were found. Among the potential ones, *Ganoderma lingzhi* was selected for further investigation for ACE inhibitory activity. Results showed that *G. lingzhi* contains many peptides with moderate ACE-inhibitory activity. Among 11 identified ones, 4 peptides showed rather high inhibition against ACE with IC_{50} values ranging from 73.1 µM to 162.7 µM. Beside peptides, lanostane-type triterpenoids contained in this mushroom also showed inhibition effect against the enzyme at some degree. Of all the 32 triterpenoids used in this study, ganoderic acid A showed highest inhibitory activity with IC_{50} was recorded at 100 µM. Structure-activity relationship study also reveals some important structural features of an ACE inhibitor. This information helps us to understand more about structural characteristics for potential inhibitors.

In combination with previous studies, a combined *in silico – in vitro* approach for designing ACE inhibitory tripeptides with drug-like properties was described. By using this method 16 potential ACE inhibitors were found. Among these peptides, 14 were reported for the first time. With low IC_{50} values and high-predicted bioavailability, the peptides identified by our protocol are comparable in terms of ACE-inhibition to those derived from costly and time-consuming wet screening.

This study failed to find out applicable ACE inhibitors that can be used for antihypertensive medication; however it successfully figured out the potential compounds as well as the appropriate approach in seeking to drug-like ACE inhibitors.

Recent studies on ACE structure, which showed ACE has two domains with somewhat difference in biological function, surely re-directs future studies to domain-specific inhibitors. New types of inhibitors may help to improve present anti-ACE medication efficacy and reduce the drug's side effects that is the main barrier preventing patient's use. With the development of screening techniques and *in silico* rational design, recent future may witnesses the development of domain–specific inhibitors which undoubtedly improve the current medication in aspects of safety and effectiveness.

List of abbreviations

AAPH: 2,2'-Azobisisobutyramidinium chloride
ACE: Angiotensin-Converting Enzyme
CVD: Cardiovascular Disease
RAAS: Renin Angiotensin Aldosterone System
ORAC: Oxygen Radical Absorption Capacity
DPPH: 2,2-diphenyl-1-picrylhydrazy
ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
UVB: Ultra Violet B
HPLC: High Performance Liquid Chromatography
LCMS: Liquid Chromatography Mass Spectroscopy
ADR: Auto-digestion Reishi (extract)
HWR: Hot Water Reishi (extract)
ADME: Absorption-Distribution-Metabolism-Excretion
HTS: High Throughput Screening
VS: Virtual Screening
WHO: World Health Organization

Publication arising from studies of this Thesis

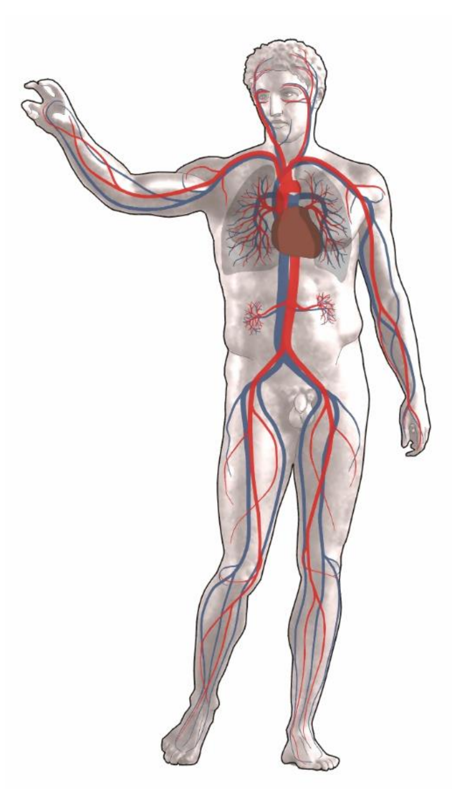
Journal articles

1. Hai-Bang, T., Suhara, H., Doi, K.; Ishikawa, H., Fukami, K., Parajuli, G. P., Katakura, Y., Yamashita, S., Watanabe, K., Adhikari, M. K., Manandhar, H. K., Kondo, R., Shimizu, K., Wild Mushrooms in Nepal: Some Potential Candidates as Antioxidant and ACE-Inhibition Sources. *Evidence Based Complementary Alternative Medicine* (2014), 195305.
2. Hai-Bang, T., Shimizu, K., Potent Angiotensin-Converting Enzyme Inhibitory Tripeptides identified by a Computer-based Approach, *Journal of Molecular Graphics and Modelling* (2014), 53, 206–211.
3. Hai-Bang, T., Yamamoto, A., Matsumoto, S., Ito, H., Igami, K., Miyazaki T., Kondo, R., Shimizu, K., Hypotensive Effects and Angiotensin-Converting Enzyme Inhibitory Peptides of Reishi (*Ganoderma lingzhi*)'s Auto-digested Extract, *Molecules 2014, 19(9), 13473-13485*

Conference Abstracts and Posters

1. Hai-Bang Tran, Kuniyoshi Shimizu, Inhibition of angiotensin converting enzyme by lanostanoids from reishi (*Ganoderma lingzhi*): structure-activity relationship and inhibition pattern, *The 8th JSP-CCTCNM-KSP Joint Symposium on Pharmacognosy*, Fukuoka, Japan 13-14th Sep. 2014.
2. Toshitaka Kohno, Hai-Bang Tran, Kuniyoshi Shimizu, Seiichi Sakamoto, Hiroyuki Tanaka, Satoshi Morimoto, Tubulin-polymerizing activity of Ganoderma triterpenoids and production of their monoclonal antibody, *The 8th JSP-CCTCNM-KSP Joint Symposium on Pharmacognosy*, Fukuoka, Japan 13-14th Sep. 2014.
3. Toshitaka Kohno, Ken Sawai, Hai-Bang Tran, Kuniyoshi Shimizu, Reishi (*Ganoderma lingzhi*) no kōgan kassei hatsugen mekanizumu no kaimei (in Japanese), *The 64th Annual Meeting of the Japan Wood Research Society*, Ehime, Japan 13-15th Mar. 2014.
4. Marina Nishida, Hai-Bang Tran, Hiroto Suhara, Katsumi Doi, Katsuya Fukami, Gopal Prasad Parajuli, Kazuo Watanabe, Mahesh Kumar Adhikari, Hira Kaji Manandhar, Kuniyoshi Shimizu, Search for the bioactive components of wild-type mushroom in Nepal, *The 64th Annual Meeting of the Japan Wood Research Society*, Ehime, Japan 13-15th Mar. 2014.
5. Hai-Bang Tran, Hiroto Suhara, Katsumi Doi, Hiroya Ishikawa, Katsuya Fukami, Kuniyoshi Shimizu, Gopal Prasad Parajuli, Kazuo Watanabe, Mahesh Kumar Adhikari, Hira Kaji Manandhar, Ryuichiro Kondo, Wild Mushroom in Nepal: Some potential Candidates as Antioxidant and ACE Inhibition Sources, *The 63rd Annual Meeting of the Japan Wood Research Society,* Iwate, Japan, 27-29 Mar. 2013
6. Hai-Bang Tran, Hisatomi Ito, Kentaro Igami, Toshitsugu Miyazaki, Ryuichiro Kondo, Kuniyoshi Shimizu, Hypotensive effects of Reishi (*Ganoderma lingzhi*)'s auto-digested extract and it's potent peptides for the inhibition of angiotensin- converting enzyme, *日本きのこ学会第１７回大会*, Hiroshima, Japan, 11-13th Sep. 2013.

Chapter 1
Introduction



Cardiovascular system
(source: *Wikipedia*)

1.1. Cardiovascular diseases and risk factors

Blood, a bodily fluid in animals, delivers nutrients and oxygen to the cells and transports metabolic waste products away from those same cells and; therefore, a good supply of blood is essential for a normal operation of every organ. Any factors affecting the blood-supplying system or the cardiovascular system will surely affect to normal operation of the whole body. According to the World Health Organization (WHO), the term cardiovascular diseases (CVDs) is used to mention to diseases of the heart, brain's vascular and other diseases of blood vessels [1]. WHO classifies CVDs into 2 categories of 7 types. The firs category, *CVDs due to atherosclerosis*, includes ischemic heart

disease or coronary artery disease, cerebrovascular disease of the aorta and arteries, including hypertension and peripheral vascular disease. *The second category or other CVDs* includes congenital heart disease, rheumatic heart disease, cardiomyopathies and cardiac arrhythmias. CVDs and other heart diseases and stroke account for 17.3 million deaths in 2008 and the death toll is estimated to rise to 23 million by 2030. In 2010, 863 billion dollars were spent on treatment of CVDs and the number is about to rise to 1,044 billion by 2030 [2].

There are many factors promoting the development of cardiovascular diseases and the World Heart Federation has divided these factors into 3 groups. The first group is made of modifiable or controllable factors such as: high blood pressure, cholesterol, overweight/obesity, tobacco use, lack of physical activity and diabetes; the second group contains uncontrollable factors including age, gender, family history and ethnic origin and the third group include contributing factors like overconsumption of alcohol. Among the risk factors, hypertension is considered a major concern. In 2008, hypertension alone was responsible for 7.5 million death worldwide or 12.8% of total annual deaths. This raised hypertension to the most deadly risk factor for coronary heart disease and cerebrovascular disease [1]. The other leading risk factors are tobacco exposure, hyperglycemia, physical inactivity, obesity and hypercholesterolemia.

1.2. Blood pressure and hypertension

With size of a fist, however, the human beats more than 100,000 times a day to pump about 2,000 gallons of blood through vessels to delivers nutrients and oxygen to the cells and transports metabolic waste products away from those these cells [3]. Strength of the force when the heart contract and pump blood around body is called blood pressure that is measured in millimeters of mercury (mmHg). The readings are given as two numbers, the systolic pressure as a numerator and the diastolic pressure as a

denominator. Systolic pressure refers to blood pressure when the heart beats and pumps blood to other organ while diastolic pressure refers to the pressure when the heart rests between heart beats. For example, a measure of blood pressure with a result of "120/80" mean the systolic blood pressure is 120 mmHg and the diastolic blood pressure is 80 mmHg. American Heart Association's classifies the blood pressure for people who older than 18 by 6 groups as shown in table 1.

Table 1. Classification of blood pressure for adults who older than 18 years old [4]

Category	systolic, mmHg	diastolic, mmHg
Hypotension	< 90	< 60
Desired	90–119	60–79
Prehypertension	120–139	80–89
Stage 1 hypertension	140–159	90–99
Stage 2 hypertension	160–179	100–109
Hypertensive emergency	≥ 180	≥ 110

To ensure that all parts of the body can maintain their normal operation, blood must be supplied at an adequate pressure. While either low blood pressure (hypotension) or high blood pressure (hypertension) can give negative effect to human, the latter get more consideration due to its harmful effect for a normal human life. There are two types of hypertension include primary (or essential) and secondary (or inessential) hypertension. Primary hypertension, by definition, has no identifiable cause and accounts for about 95% of hypertensive patients [5] while secondary hypertension is mainly caused by endocrine diseases, kidney diseases, tumors and side effect of some medications. Without appropriate treatment, hypertension make the heart strained and may result in hypertensive heart disease and coronary artery disease [6]. Hypertension can also develop heart attacks, strokes, heart and kidney failure, and aneurysm (a deadly rupture of blood vessels) [3].

1.3. Angiotensin-converting enzyme (ACE) and Renin angiotensin aldosterone system (RAAS)

1.3.1. Angiotensin-converting enzyme: structure and function

Angiotensin-converting enzyme (ACE) (EC 3.4.15.1) is a zinc metallopeptidase which converts angiotensin I to angiotensin II, a potent vasoconstrictor. The enzyme also degrades bradykinin and inactivates the activity of this vasodilator. Therefore, ACE acts to increase blood pressure and has been targeted for antihypertensive medication for long time. This dipeptidyl carboxypeptidase, previously named as hypertensin-converting enzyme, was first identified and isolated from horse plasma in 1956 by Skeggs et al. [7] and it took around 50 years from this isolation to structural characterization for the enzyme by the work of Natesh et al. [8]. There are two forms of human ACE that are transcribed from a single gene. The ubiquitous somatic ACE (sACE) is composed of a single, large polypeptide chain of 1,277 amino acids, and the other shorter form known as germinal or testicular ACE (tACE) is made up of a polypeptide chain of 701 amino acids [8]. Both sACE and tACE are mostly extracellular; the intracellular C-terminus part of each is connected with the outside part by a short trans-membrane segment [9]. tACE is an enzyme with one active binding site of a HEXXH zinc-binding motif whereas sACE consists of two, one in N-domain and another in C-domain. The tACE has amino acid sequence similar to that of the C-domain of the sACE except for 36 residues at the N-terminus of the one-domain enzyme. In sACE, 36 amino acids at this N-teminus play role of a linker connecting the C-domain and the N-domain. Figure 1 shows amino acid sequence, secondary structure and tertiary structure of a truncated version of testicular angiotensin converting enzyme (pdb code 1O86) that lacks the O-glycan-rich, N-terminal 36 residues and the trans-membrane domain [8].

Unlike the tACE whose sequence is nearly identical to C-domain of the sACE, the N-domain of the sACE are somewhat different from the C-domain in amino acid sequence although both domain of the sACE contain same binding site motif (in this case is HEMGH, with the two histidines coordinating zinc). The difference may be the main reason resulting in differences in their substrate specificities, inhibitor and physiological functions [10].

Although ACE is a membrane-anchored protein, it can also be found in soluble form which resulted from the enzymatic cleavage of tissue-bound ACE [11]. The secretase that shed ACE out of endothelium is still unidentified; however, cleavage sites of ACEs were established by some previous studies. Ramchandran et at. [12], when studying testicular ACE, determined that the shedding occurs at a monobasic site between Arg-663 and Ser-664 liberating the soluble enzyme and leaving a cell-bound protein 74 of residues; whereas Woodman et al. [13] established that human and porcine sACE were cleaved between Arg1203 and Ser1204 and this cleavage is identical to Arg/Ser motif of tACE. Beside secrectase, elevated serum ACE was also reported in patient with active sarcoidosis [14] and a some pulmonary diseases [15], [16]. The elevation of ACE also appeared in adults with Gaucher's disease [17] and in apoE-deficient mice fed a cholate- containing high-fat diet [17].

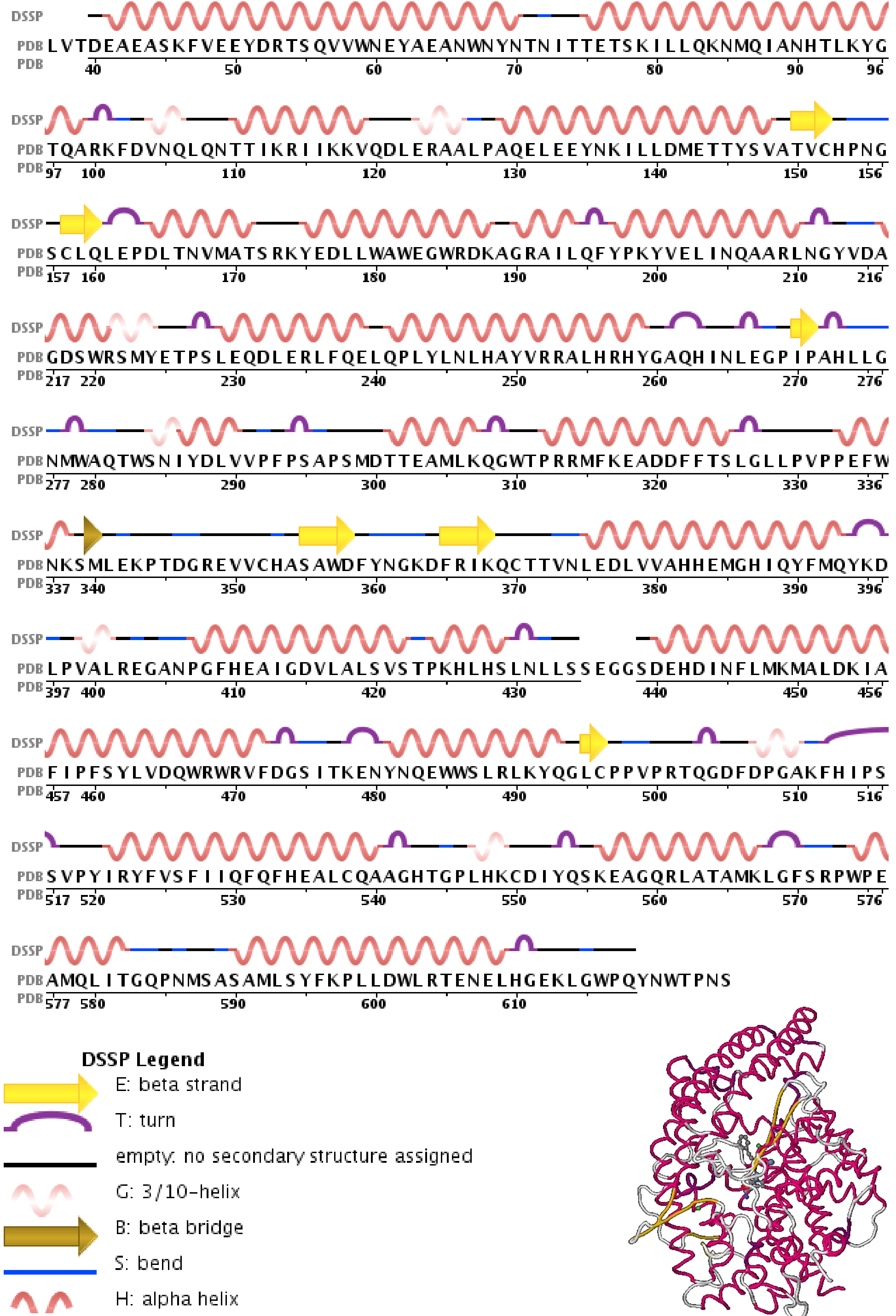


Figure 1.1. Amino acid sequence, secondary structure and tertiary structure of testicular angiotensin-converting enzyme (adapted from http://www.rcsb.org/pdb, pdb code 1O86)

As mentioned above, the primary studies of ACE were directed toward understanding of blood pressure include the degradation of angiotensin I and bradykinin. Besides, many other physiological roles of this enzyme have also been recognized. ACE was reported to affect the development of kidney. Experiment on mice null for ACE showed that they cannot effectively concentrate urine due to a marked expansion of the renal pelvis as a result of an underdevelopment of the renal medulla and papilla [18]. ACE-deficient male mice were also reported with deficiency of reproduced ability [18], [19] but this phenomena was not seen in female mice that lack of the converting enzyme. This defect in male mice is directly attributed to testis ACE. Mice that lack of tACE (but not sACE) showed normal blood pressure level but could not reproduce and this reproduction deficiency could not be restored by the transgenic expression of sACE in the sperm of ACE -/- mice [20] but was restored with a transgenic expression of tACE [21]. These studies suggested the unique function of the tACE in fertile activity and physiological non-equivalence of the two isoforms of ACE. ACE plays an important role for normal development of early hematopoietic progenitors as well as hematopoietic cell development [22] and inhibition of ACE resulted in a reduction of hematopoietic colony-forming cells generation of embryoid bodies [23]. By using a mouse line expressing a truncated form of ACE whose activity in plasma was 34% of normal level, Cole et al. [24] proved that ACE plays indirect role in erythropoiesis by increasing angiotensin II level. ACE-knockout mice, whose plasma levels of angiotensin II is low, are anemic despite having normal renal function. However, administration of angiotensin II for 2 weeks increased hematocrit to near normal levels. Besides erythropoiesis, ACE also appears to affect myelopoiesis. Recent study by Lin et al. using ACE-knockout mice found a myelopoietic abnormality in these mice. The abnormalities characterized by increased bone marrow immature myelocytic cells, as

well as extramedullary myelopoiesis [25]. The study revealed that ACE decreased myeloid cell maturation via angiotensin II and substance P, a peptide that affects bone marrow development, and through the angiotensin II receptor AT1 as well as substance P neurokinin 1 receptors. There are evidences of the inclusion of ACE in stimulating immune responses. Running experiment using mouse model called ACE 10/10 in which ACE was overexpressed in some body parts Shen et al. [26] indicated that those mice showed significant tumor-resistant activity compare to wild-type mice. Histological study revealed that inflammatory response was enhanced and numbers of tumor epitope-specific $CD8^+$ T cells was increased after ACE 10/10 mice were challenged with melanoma or lymphoma; an increased production of interleukin-12 and nitric oxide but reduced interleukin-10 was also reported.

Many studies have shown diverse actions of ACE that far beyond the liberation of angiotensin II from angiotensin I, and the inactivation of bradykinin; and range of functions of this enzyme may be still widened in the future. However, the enzyme hydrolyzing actions, which play critical role in cardiovascular function, is always an important target for discovering newly potent drug used for treatment of hypertension as the structural information of this enzyme has been piled up more and more in recent years. Together with renin and aldosterone, ACE makes an essential hormone system responsible for the homeostasis of blood pressure in mammals, the renin angiotensin aldosterone system (RAAS).

1.3.2. Renin angiotensin aldosterone system

The renin angiotensin aldosterone system (RAAS) is the best-known hormone system that regulates blood pressure and body fluid balance. As indicated by its name, the conventional RAAS is composed of renin, angiotensin-converting enzyme and aldosterone. When a decrease of blood pressure or plasma sodium is detected, the

kidneys will release renin to start a multi-enzyme cascade that controls blood pressure. At first, renin - an aspartyl protease, convert angiotensinogen produced by liver into angiotensin I and this decapeptide is then hydrolyzed to angiotensin II and angiotensin(1-7) under the action of angiotensin-converting enzyme and its homologue. Angiotensin II interacts with several receptor; however, it performs actions of constricting blood vessel, increasing plasma-aldosterone, retaining water and sodium, and enhancing thirst and salt appetite, mainly through the AT_1 receptor [27]. After being secreted aldosterone, in turn, increase sodium (re)absorption and potassium secretion as well as promote water retention in the kidney [28]. In combination, these actions help to maintain fluid and electrolyte homeostasis and raise blood pressure. A general picture of the RAAS is shown in figure 2.

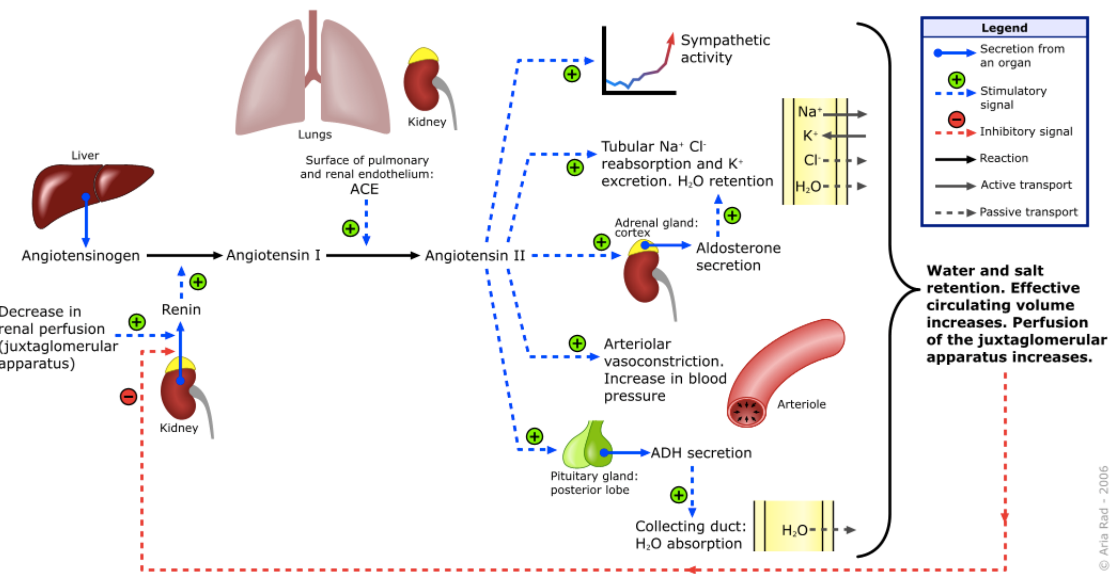


Figure 1.2. The Renin-angiotensin-aldosterone system and it   (adapted from wikipedia.com)

Beyond this classical system which known as circulating RAAS, studies also emerge about existence of local tissue RAAS which can operate independently of the classical one [29]. The local system has been identified in different organs including brain, kidney, heart, adipose tissue, hematopoietic tissue, gastrointestinal tract, liver, endocrine system and blood vessels [30]. The exact function of local tissue RAAS is

still vague, but it may play role in tuning vascular tone and intensifying the effect of circulating system [30]. In circulating RAAS, Angiotensin II is a major effector [31] and this octapeptide is primarily produced under the hydrolyzing action of luminal surface's ACE of endothelial cells. That's the reason why inhibition of ACE is dominant in anti-hypertensive therapies.

1.4. Development of angiotensin converting enzyme inhibitors

Angiotensin-converting enzyme was first recognized in 1954 by Skeggs et al. [32] but it was not until 1967 when John Vane, a consultant at The Squibb Institute for Medical Research, encourage people in the institute to join a group and to study on inhibition this enzyme, inhibition of the enzyme was seriously investigated [33]. By investigation of venom peptide, which shown inhibitory activity against ACE, and the assumption that ACE shared similar catalyzing characteristics to carboxypeptidase A, Cushman and Ondetti [34] successfully produced compounds with high inhibition strength. One of these compounds is D-3-mercapto-2-methylpropanoyl-L-proline that is known as the first truly synthesized antihypertensive drug targeting ACE and this has been then marketed as Captopril. From this pioneering discovery, many other ACE-targeted inhibitors have been developed and dozen of them are now available for using in hypertensive medication. Surprisingly, these inhibitors were developed without knowing about the real structure of human ACE. With the breakthrough discovery of structure of testical ACE in 2003 [8] and somatic ACE's N-domain in 2006 [35], recent future may witnesses the development of domain–specific inhibitors which undoubtedly improve the current medication in aspects of safety and effectiveness [11].

Beside synthetic pathway, searching ACE inhibitors from natural source has also been done for long time with aims at increasing the antihypertensive activity while reducing side effects. The long list of natural ACE inhibitors has been made with milk products

and food proteins. One of the most successful story for nature-derived ACE inhibitors is finding of IPP and VPP from sour milk [36]. However, even the effect of these peptides were shown clinically in placebo-controlled studies [37], [38], they are now mainly supplied in form of supplement rather than a direct medication. Beside peptides, some studies on phenolic compounds [39], triterpenoids [40] and flavonoids [41] also showed that these compounds possess potent inhibitory effect against the enzyme. Problem with these compounds is their low inhibition strength or high IC_{50} value. This limitation encumbers them to jump into clinical trials and requires structural modification for increasing the activity to go further to next step of drug-discovery process.

Recently, development of modeling software together with accumulation of protein's X-ray data have emerged the potential of using computer for designing drugs including ACE inhibitors. This computer-based experiment allows researchers to see and to compute the interaction of target protein and its ligands (promoters or inhibitors) which can be virtually created by software. The *in silico* approach can also significantly reduces budget used in the drug discovery process as the most laborious screening-experiments can be done by computer and only drug-like and lead-like compounds are subjected to *in vitro* tests. There is dozen of successful stories on computer-aided design in drug discovery. Several active agents resulted from virtual screening are now available in the drug market and many of them already entered the phase III of clinical trials [42]. One typical example in this growing area is the development of zanamivir, an inhibitor of influenza virus replication [43]. By investing of neuraminidase 3D structure with computer program, the authors derived conclusion about the enzyme's surface character and made modification to a weak transition state inhibitor and this change led to the discovery of the first neuraminidase inhibitor available in the drug market. This *in silico* approach has also been applied to seek for more effective and less

side effect ACE inhibitors. The following work of Norris el al. [44], Masuyer el al. [45] and Silva et al. [46] are just some examples for enormous effort of scientists in designing better ACE inhibitor using computer as assistant tools.

For thousands of years, nature has been a precious source for medicinal solutions. To date, 50% of drugs in Western medicine are derived from natural products [47]; therefore it is undoubtedly ascertain that nature-derived compounds still play important role in drug discovery process. The development of computational calculation, software technology as well as structural biology the process will be surely accelerated and we can envision the hope of finding better ACE inhibitors in the near future.

Chapter 2

Screening for antioxidants and ACE inhibitors from wild-mushrooms

Some edible mushrooms
(source: mushrooms.ca)

2.1. Introduction

For millennia, mushrooms have been used as a part of the human diet and as medicinal sources. In term of nutrition, mushrooms are recognized as a healthy food as they are low in calories and fat but rich in proteins and dietary fiber [48], [49], while pharmacologically, the potential of medicinal mushrooms is considered enormous but mostly untapped [50]. With their wide variety of components, mushrooms - both edible and medicinal - have a broad spectrum of bioactivities [51], [52]. According to FAOSTAT data [53], the total world production of mushrooms including truffles has sharply increased from 2.0 million metric tons in 1990 to nearly 7.4 million metric tons in 2010 and the market of mushroom-derived dietary supplements is also quickly growing and is valued at more than US $15 billion today [54]. This tendency may

reflect an increase in the recognition of the value of mushrooms as a healthy food and an important source of medicinal compounds.

Oxidative stress, a chronic imbalance between antioxidant ability of biological systems and production of reactive oxygen species (ROS) that involved in many diseases including skin aging and hypertension [55]–[57]. UV exposure is initial step of ROS generation, many of alterations and mutations in skin [58]–[60]. The skin itself has antioxidant defense system used to deactivate ROS but when this system is overwhelmed there is a need of antioxidant supplement through food or treatment therapies [59]–[61]. Abundance of studies have reported about beneficial effect of antioxidant on skin protection against ROS and were thoroughly reviewed in recent reports [59], [62]–[64]. Experimental and clinical studies have also indicated that hypertension occurs after a biological system is exposed to oxidative stress and increased production of $^{\bullet}O_2^-$ and H_2O_2 has also been observed in salt-sensitive and angiotensin II-induced hypertension [57], [65]. Oxidative stress is both cause and effect in hypertension [66]. These findings imply that the lower level of ROS, the lower risk of hypertension, and vice versa. In fact, a high intake of flavonoid-containing fruits and vegetables has been associated with a decrease in blood pressure in humans [67], [68]. Mushrooms, being neither plants nor animals, reside in their own kingdom with their own antioxidant profile and therefore have the ability to complement the benefits of antioxidants found in plant foods [69].

Besides oxidative stress-induced hypertension, a widely accepted signaling pathway of hypertension is through the angiotensin-I-converting enzyme (ACE), which plays an important role in the regulation of blood pressure. The inhibition of ACE is considered a useful therapeutic approach in the development of drugs to control hypertension. Many studies have reported on potential ACE inhibitors from not only well-known

medicinal mushrooms like *Ganoderma lucidum,* and *Lentinus edodes* but also normal edible mushrooms such as *Grifola frondosa*, *Lyophyllum decastes,* and *Tricholoma giganteum* [69]–[72]. Many mushrooms have also shown hypotensive effects on spontaneously hypertensive rats [71], [73], [74] and humans [75]. With around 140,000 species of mushrooms estimated on earth [50], including both known and unknown species, mushrooms remain a untapped medicinal resource. It is likely that we can discover some species that are in treating other diseases as well as hypertension.

Nepal, a small country located between India and Tibet, has dramatic differences in elevation and tremendous variation in climate. With five climate zones, within a 150 km range, one can rapidly move from a typical tropic area to a permanently frozen arctic-like zone [76]. This variation has endowed the country with a diverse phytogeograph and enriched it with economically important mycoflora. Wild mushrooms are diverse and play vital roles in many local communities in Nepal [77] but surprisingly, almost no published research can be found on the pharmacological potential or bioactive components of mushrooms grown in Nepal. Accordingly, the main purpose of this study was to determine the antioxidant and ACE-inhibition activities of wild mushrooms in Nepal, for many of which this is the first report on such activities.

2.2. Materials and Methods

2.2.1. Mushroom collection and identification

Fully matured mushrooms were collected from the forests of Kathmandu, Lalitpur and Bhaktapur of Nepal in August and September, 2011. Species were identified by morphological observation of basidiomata using a stereomicroscope, and by genetic analyses of samples. Morphological observations were carried out using Nikon Eclipse 80i stereomicroscope (Nikon, Tokyo). For microscopic observation, pieces of dried fungal material were mounted in 3% (w/v) KOH or Melzer's reagent [78]. Twenty

measurements were made per element (spore, basidia, cystidia, and other tissue features) for each specimen.

Genetic analysis was carried out on the internal transcribed spacer region (ITS) of ribosomal DNA. Extraction of genome DNAs from the mushroom samples was performed with ISOPLANT II (NIPPON GENE CO., LTD, Tokyo, Japan) with some modifications. DNA samples were kept at −20 °C until used for PCR amplifications. The region between the genes 18S rRNA and 28S rRNA was amplified using ITS1 and ITS4B [79], [80] primers. A 50 μL reaction mixture was prepared with 1 U Tks Gflex DNA Polymerase (Takara Bio, Inc., Shiga, Japan), 0.5 μM of each primer, 1× Gflex PCR buffer with 1 mM $MgCl_2$ and 200 μM dNTPs, and 50 ng of genomic DNA as template according to the manufacturer's instructions. Amplification reaction was performed in a TProfessional Thermocycler (Biometra GmbH, Göttingen, Germany).

An amplified DNA fragment was ligated to the pTA2 plasmid vector (Toyobo, Osaka, Japan). Recombinant plasmid DNA was introduced into *E. coli* DH5α competent cells, and then isolated with an alkaline lysis method. The nucleotide sequence was determined by Applied Biosystems 3130xl Genetic analyzer (Life Technologies Corporation, Carlsbad, CA, USA). A homology search of the determined nucleotide sequence was carried out using a BLAST server [81].

2.2.2. Mushroom extract preparation

Mushroom samples were air-dried and then kept in an air-ventilated oven at 35 °C for 10 hours and at 45 °C for 1 hour. Twenty-nine samples, after being ground into powder, were extracted in 24 hours at room temperature with reverse osmosis water and ethanol (Wako Pure Chemical Industries, Japan), using an orbital shaker for obtaining 58 extracts. Water extracts were lyophilized while ethanol extracts were rotary evaporated

to dryness when preparing samples for assays. The resultant extracts were kept in glass-capped vials sealed with parafilm and stored in a cool place until assayed.

2.2.3. Phenolic content determination

Total phenolic content was determined by a method described by Singleton and Gillespie [82], [83] with some minor modifications. This assay is based on the electrons transferred in alkaline medium from phenolic compounds to blue-colored phosphomolybdic/phosphotungstic acid complexes which have maximum absorbance at 765 nm. Details of the procedures are as follow: 50 μL of sample solution was mixed well with 100 μL of 10% Folin-Ciocalteu solution (a mixture of Na_2WO_4, Na_2MoO_4, Li_2SO_4, HCl and H_3PO_4 with an appropriate ratio) in a 1.5-mL plastic tube. The mixture was equilibrated for several minutes, then, 400 μL of 7.5% Na_2CO_3 was added to the tube and the reaction mixture was incubated at room temperature for 60-90 minutes. After the incubation period, reaction tubes were centrifuged at 6000 rpm for 2 minutes whenever necessary, 200 μL of supernatant of samples (or blank) was transfer to an optically clear 96-well microplate, and the absorbance was measured at 765 nm using Molecular Devices FlexStation 3 Microplate Reader. Data were managed by SoftMax® Pro 5.4.1 software. Gallic acid was used as the standard and was measured in the same conditions as the samples.

2.2.4. Free radical scavenging by the ORAC assay

This assay measures the oxidative degradation of the fluorescence of fluorescein after being mixed with the free radical generator AAPH (2,2'-azobis(2-amidino-propane)dihydrochloride). Heating AAPH is said to produce the peroxyl radical, which damages fluorescein molecules resulting in the loss of fluorescence. Antioxidants suspected to be contained in extracts are considered to protect the fluorescein molecules from this oxidative degeneration. The degree of protection was quantified using a

fluorometer. In this study, both water and ethanol extracts were dissolved in 75-μM phosphate buffer (pH 7.4) for use in the ORAC assays but ethanol extracts were pretreated with a small amount of acetone, final concentration of which in the assay reaction was less than 0.1%. Experiments were conducted in 96-wells plates as described previously [84], [85] with some modification, and the main steps were as follows: First, 20 μL sample, buffer and trolox solutions were added into the sample, blank and control wells, respectively. Second, 200 μL fluorescein solution was added into the same wells. After 10 minutes-incubation at 37 ^{o}C in, 75 μL of 37 ^{o}C pre-incubated AAPH working solution was also injected into the wells. Finally, fluorescence degradation was measured over 90 minutes, 30 second intervals using Molecular Devices FlexStation 3 Microplate Reader; the excited wavelength and emission wavelength were 485 nm and 535 nm, respectively. Data were managed by SoftMax® Pro 5.4.1. The minimum and maximum concentrations of extracts in buffer were 6.25 and 50 μg/mL, respectively. In the control assay 6.25, 12.5, 25, 50 μM trolox solutions were used to make the standard curve. All chemicals used for the ORAC assay were of analytical grade and purchased from Wako Chemical, Osaka, Japan.

2.2.5. Free radical scavenging by DPPH assay

The radical scavenging activity of mushroom extracts against the $DPPH^{\bullet}$ radical (2,2-diphenyl-2-picrylhydrazyl hydrate; Sigma-Aldrich, Steinheim, Germany) was determined by the method of Brand Williams modified by Dudonné [86], [87]. DPPH radicals have an absorption maximum at 515 nm; upon reduction by the antioxidant the solution color fades and the reaction progress is easily monitored by a spectrophotometer (UVmini-1240, Shimadzu, Kyoto, Japan). Determination procedures were as follow: 3 mL of $6{\times}10^{-5}$ M $DPPH^{\bullet}$ solution (prepared daily), was mixed with 100 μL of methanolic solutions of mushroom extracts (maximum dissolved concentration);

after 20 min-incubation for at 37 °C, absorbance decrease of the mixture was monitored at 515 nm (A_s). Blank samples with 100 µL of methanol in the above DPPH$^{\bullet}$ solution were prepared and measured daily at same wavelength (A_b). The experiment was carried out in triplicate. Radical scavenging activity was calculated using the following formula:

$$\text{Inhibition rate (\%)} = [(A_b - A_s)/A_b] \times 100 \qquad (*)$$

2.2.6. Free radical scavenging by ABTS assay

ABTS assay was mostly based on the methods described previously [88] in which ABTS$^{\bullet+}$, the oxidant, was generated by persulfate oxidation 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid). Specifically, to 5 mL of 7-mM ABTS ammonium aqueous solution, 88 µl of 140-mM potassium peroxydisulfate ($K_2S_2O_8$) was added and the resulting mixture was then allowed to stand at room temperature for 12 - 16 hour to yield a dark blue solution. The mixture was then adjusted by 99.5% ethanol so that it gave an absorbance of 0.7±0.02 units at 734 nm for a making working solution. One milliliter of working solution was mixed with 10 µl of mushrooms extract (maximum dissolved concentration) and shaken well for 10 seconds; after 4 minutes of incubation at 30 °C, the absorbance of the reaction mixture was measured at 734 nm (UVmini-1240, Shimadzu, Kyoto, Japan) to give "A_s" values. Ethanol 99.5% was used as a blank (absorbance was "A_b") and the inhibition rates were calculated using (*).

2.2.7. Detection of intracellular UVB-induced H_2O_2

Intracellular H_2O_2 was assessed using immortal human keratinocyte line (HaCaT) as cell model. HaCaT cells (Cell Line Service, Eppelheim, Germany) were cultured in DMEM supplemented with L-glutamine, 10% fetal bovine serum and 1% penicillin/streptomycin antibiotic solution. After being cultured for two days at 37 °C in a 95% air/5% CO_2 atmosphere, cells were removed from culture dish by trypsinization

and seeded at a density of 4×10^5 cells/dish in a 5-cm petri dish. After two days culturing, medium was removed and cells were exposed to 10 mJ/cm^2 UVB (CL-1000 Ultraviolet Crosslinker, UVP, Upland, CA, USA). Soon after UVB irradiation, cells were refilled with cultured medium supplemented with 10 ppm of mushroom extracts. After one more day incubation, cells were transferred to 96-well µClear Fluorescence Black Plate (#655090, Greiner Bio-one, Tokyo, Japan) at a density of 2×10^4 cells/well, and incubated for 24 hours. Nucleus was stained by Hoechst 33342 (Dojindo, Kumamoto, Japan) and the amount of intracellular H_2O_2 was quantified based on the amount of difluorofluorescein (DFF) released from the reaction of H_2O_2 and BES-H_2O_2-Ac (Wako Chemical, Osaka, Japan). The images of each well were acquired from IN Cell Analyzer 1000 (GE Healthcare, Amersham Place, UK) using 360 nm (Hoechst 33342) and 480 nm (BES-H_2O_2-Ac) excitation filters and monitored through 460 nm and 535 nm emission filters, respectively. The images of Hoechst 33342 and BES-H_2O_2-Ac staining were analyzed using Developer software and resulted data were then applied to Spotfire Decision Site Client 8.2 software for visualizing the results. Cells unexposed to UVB irradiation were used as controls; cells exposed to UVB and cultured in the presence or absence of resveratrol (10 ppm of final concentration) were used as positive or negative control, respectively.

2.2.8. Angiotensin-converting enzyme inhibitory assay

Water extracts were dissolved in milli-Q water (Millipore, MA, USA) and those that were difficult to dissolve in water were pre-treated with a small amount of ethanol before being dissolved in milli-Q water (final concentration of organic solvent in enzyme reactions was less than 1%). Both types of extract were subjected to ACE inhibitory assay using Dojindo ACE Kit-WST test kit (Dojindo Laboratories, Kumamoto, Japan). Details of the method’s principle can be found elsewhere [89].

Briefly, the enzymatic reaction was initiated by the ACE and aminoacylase in the mixture containing 3HB-GGG (3-hydroxylbutyrate glycylglycylglycine) and the ACE inhibitor. The mixture was then incubated at 37 °C for 60 min. During this incubation, the substrate, 3HB-GGG, was enzymatically cut into 3HB-G and G-G, and then 3HB and G. The yield of 3HB was monitored indirectly through formazan concentration, which was measured at 450 nm after 10-minute reaction at 25 °C.

Testing procedures were run according to the manufacturer's instructions using a 96-well plate without modification, and the inhibition rate was calculated based on a comparison of the optical absorbance of samples-treated wells (A_s), control wells (A_c) and blank wells (A_b). Absorbance was measured at 450 nm using the microplate reader Biotek-ELX800 (BioTek, Vermont, USA). Inhibition rates were calculated using the following equation.

$$\text{Inhibition rate (\%)} = [(A_c - A_s)/(A_c - A_b)] \times 100$$

Samples were suspected to inhibit the ACE activity, and therefore inhibit the formation of formazan. The more strongly inhibitory the activity of the samples, the less color appeared in the final solution.

2.2.9. Statistical analysis

Each ORAC experiment was repeated four times while the ACE inhibitory assay and phenolic content determination were performed in triplicate. The results are expressed as mean ± SD. The correlation coefficient between phenolic content and antioxidant assays was determined by least-square linear regression analysis using MS Excel 2007.

2.3. Results and Discussion

2.3.1. Mushroom collection and identification

As shown in Table 2.1, 29 mushroom samples, collected from the mountainous area of Nepal were first identified by morphological observation. Some mushrooms for which ITSs were obtained were subjected to a BLAST search via INSDC.

Mushroom samples were collected at mass from 11.6 to 117.7 g in dried weight. Purified genome DNAs were successfully obtained from the mushroom samples. DNA fragments containing the ITS sequence were amplified in the 705 to 894 b.p. range. From BLAST search results, the mushroom listed in Table 2.1 were identified. Six samples (from N001 to N006) were included in the genus *Ganoderma*, and three samples (N016, N018 and N019) belonged to the genus *Phellinus.* Samples N009, N011, N014, N027 and N028 were identified as *Trametes versicolor*.

2.3.2. Antioxidant activities

2.3.2.1. Free radical scavenging by the ORAC assay

Many methods have been developed for measuring antioxidant capacity in vitro. The underlying chemistry, advantages and disadvantages have also been well documented and reviewed [90], [91]. Among these methods, the oxygen radical absorbance capacity (ORAC) method, with some modifications that have been made over time, has been widely used to evaluate the antioxidant activity of many herbal extracts, food additives and even biological samples [92]. The existence of the USDA (U.S. Department of Agriculture) ORAC database, and the recently launched web-based database for this index [93] show the scientific community's estimation of the ORAC assay for measuring antioxidant capacity of herbal samples. In the initial checking for antioxidant activity of mushroom extracts in this study, we ran ORAC experiments in which fluorescein was used as fluorescent probe in a 96-well plate assay as described above.

The results of ORAC assays of samples are shown in Table 2.2. ORAC values (µmol TE/g extract) ranged from 342.8 to 21015.4 for ethanol extracts and from 83.2 to 1196.9

for water extracts. Among the samples *Inonotus andersonii* and *Phellinus gilvus* ethanol extracts showed extremely high activity. This is the first time such high ORAC values have been seen for mushroom extracts. Until now, such high ORAC values have only been reported for extracts of well-known antioxidant spices like cloves, pimento and cinnamon [87], [94]. High antioxidant activities have also been reported for some mushrooms in *Inonotus* species such as *I. hispidus, I obliquus* [95], [96], but we could not find any published report on the antioxidant capacity of the *I. andersonii* mushroom. It is worth to note here that the main anti-oxidative compounds isolated from above-mentioned *Inonotus* mushrooms are hispidin and hispidin moiety-contained compounds such as inonotusin A, B in *I. hispidus* [95], inonoblins and phelligridins in *I obliquus* [97]. From these results, we think that *I. andersonii* may also contain such-like compounds and this mushroom should be a good candidate for future antioxidant researches.

2.3.2.2. Phenolic content, ABTS and DPPH radical scavenging assays

Phenolic compounds are considered one of the major groups of nonessential dietary components which have been suggested to be beneficial for human health and their physiological importance is said to relate to their abilities to chelate metals, inhibit lipoxygenase, and scavenge free radicals [98]. The Folin-Ciocalteu method is often used to estimate the phenolic content of plant extract samples although the reagent used for determining phenolic content does not react exclusively with phenolics and has even been proven to be affected by a variety of compounds such as thiol derivatives, vitamin derivatives, amino acids and metal complexes [99]. Thus, the reagent often overestimates the phenolic contents in samples, but because it is a cheap, simple, convenient and, in some aspects, useful method for determining total phenolic content, Folin-Ciocalteu is still widely used to estimate the total antioxidant capacity of samples.

Many studies on spices, vegetables, fruits and plants extracts have shown a good relationship between phenolic content and antioxidant activity [87], [100]–[102]. It is also generally accepted that the main antioxidants in mushrooms are phenolics, mainly phenolic acids [103]. To confirm the relationship of phenolic compounds in mushrooms and their antioxidant activities we selected 10 mushroom samples which had the highest ORAC values and carried out experiments for determining phenolic content and other radical scavenging activities. The correlation coefficients between phenolic content and antioxidant activity resulting from different assays were also calculated and the results were shown in Table 2.3.

Experimental results showed good relationships between phenolic content and antioxidant activities in which the correlation coefficient R of phenolics and ORAC, ABTS and DPPH activities were 0.923, 0.936 and 0.986, respectively. The close correlation between phenolic content and ABTS inhibition is not surprising since the methods used to determine phenolics and ABTS inhibition rates are both based on the electron transfer ability of the sample's components. However, while the ORAC assay is based on hydrogen atom transfer reactions, we still could see a good correlation between phenolic content and ORAC values. This may come from the fact that phenolic compounds are not only a rich electron source but the phenolic hydroxy group can also act as a hydrogen donor supplying hydrogen atom to wipe out peroxy radicals by forming stabilized phenoxyl radicals in the ORAC assay. In this context, phenolic compounds can be both electron and hydrogen atom donors, and therefore can be in good correlation with both the ORAC and ABTS assays. The very high correlation found between DPPH assays and total phenolic content with R equal to 0.986 indicates a close relationship between phenolic compound concentration in mushroom extracts and their nitrogen-radical scavenging capacities.

Despite the fact that there have been many studies referring to the antioxidant activity of mushroom, almost no report has mentioned a correlation between mushroom genus (or family) and antioxidant activity. Our present results suggest such a relationship. For example, all studied *Ganoderma* (Ganodermataceae) samples had a medium antioxidant activity while *Phellinus* and *Inonotus* samples (Hymenochaetaceae) showed quite high activity. Some previous discrete studies [97], [104]–[108] have also shown the high antioxidant capacity of many mushrooms in the *Inonotus* and *Phellinus* genera. This consistency across different studies results can be used to consolidate and direct future research on antioxidant activity. Assuming that this genus bioactivity relationship can be further established, we think that these genera could be good candidates for studies of mushroom's antioxidants properties in the future.

2.3.2.3. Intracellular UVB-induced H_2O_2

Ultraviolet-induced ROS cause chemical modification, oxidative stress and play an important role in photoaging. After being created, ROS activate many cell surface cytokines and growth factor receptors which stimulate transcriptions of matrix metalloproteinases that significantly contribute to the skin aging process [109], [110]. Many antioxidants such as vitamin C, vitamin E, carotenoids and, especially polyphenols have been reported with ability of enhancing resistance to oxidative stress and preventing skin aging [59], [61], [62], [64]. In this study 10 mushroom's extracts having highest antioxidant activity (highest phenolic contents) were subjected to the UVB-induced H_2O_2 generation assay to check the ability of samples on anti-oxidative stress effects using HaCaT cells as cellular model. The results were shown in Table 2.4.

Four among ten selected samples showed good protection effect against UVB-induced H_2O_2 generation. The levels of intracellular H_2O_2 in cells treated with these samples were as low as in control. Two highest phenolics-containing samples *I. andersonii* and

P. gilvus also belonged to group of these 4 samples. Considerably, cells treated with *P. gilvus* even showed lower level of H_2O_2 than control's implying the ability of this sample on scavenging intracellular generated H_2O_2 or other types of ROS. In previous parts, we proposed the use of this mushroom for further investigation on antioxidant activity and this result confirmed our proposal about targeting the mushroom as a potent candidate for future studies as anti-oxidative stress agents.

Surprisingly, resveratrol not only failed to show protection effects against UVB-induced H_2O_2 but also performed a stimulation of generation of this compound. This result was different from previous reports of Park [111] on the protective activity of resveratrol against H_2O_2-induced oxidative stress. However, while Park incubated HaCaT cells with resveratrol before applying UVB, in this study we applied UVB before treating cells with resveratrol. UVB-exposure has been reported to decrease the catalase activity [112] while pretreatment of cells with resveratrol has been reported to increase the expression of SOD and glutathione peroxidase [113] and catalase [114], major enzymes responsible for the inactivation of ROS. The differences of our results and previous report's could result from the difference in resveratrol-treatment methods.

2.3.3. ACE inhibitory assay

Water and ethanol extracts of 29 mushrooms samples were used for screening the ACE inhibitory effect using Dojindo ACE test kits. Each test was repeated three times and inhibition rates were calculated based on a comparison of blank and control samples. Results are shown in Table 2.5.

High blood pressure is one of the major independent risk factors for cardiovascular diseases and is considered a worldwide health problem. Angiotensin-I-converting enzyme (EC 3.4.15.1; ACE) plays a crucial role in blood-pressure regulation by converting angiotensin I to angiotensin II, a potent vasoconstrictor. Therefore, the

inhibition of ACE activity is a major target in the prevention of hypertension [115]. Until now, ACE inhibitors have been mainly sourced from food protein, especially milk protein. Many milk protein-derived peptides have demonstrated inhibitory effects on ACE *in vitro* [36], [116], [117] and on antihypertension in *vivo* [38], [118], [119]. Recently, mushrooms have also been considered as good candidate sources of hypotensive agents. Several peptides and proteins extracted from mushrooms have been shown to have an ACE-inhibitory effect. Many mushroom extracts have been screened for this activity [72], [120]–[122], and most of the time, the dominant ACE inhibition extracts have been aqueous. Consistently with previous results, our study also showed a predominance of water extracts for ACE inhibition. While there were 15 water extracts which showed rather high inhibitory effect with inhibition rates higher than 50% at 100 µg/mL, only one ethanol extract showed more than 50% inhibition at this concentration. Besides traditionally well-known mushrooms such as *Ganoderma lingzhi* and *Trametes versicolor*, other non-medicinal mushrooms like *Phlebia tremellosa* and *Heterobasidion* sp. samples also showed high inhibition activity. According to Linderquist [51], the responsible bioactive compounds in mushrooms belong to several chemical groups; usually they are polysaccharides, triterpenoids and proteins. As mentioned above, several ACE-inhibitory peptides and proteins have been identified from mushroom water extracts. From ethanol or methanol extracts only some ganoderic acids [40] and nicotianamine [121] with ACE inhibitory capacity have been identified. Recent studies indicated that phenolic compounds can also play a role in the inhibition of ACE [123], [124]. In this study, the average inhibition against ACE of *I. andersonii* and *P. gilvus* ethanol extracts (the two highest-phenolic-content samples) could be explained by the action of phenolic compounds in the mushroom extracts.

Among the studied samples, it seems that *Phlebia tremellosa* contained potent compounds having high ACE-inhibition capacity. To confirm the potential of mushrooms for ACE inhibitory activity we performed the IC_{50} value determination for water extracts of this mushroom and compared with *Ganoderma lingzhi's* capacity. The IC_{50} of *Phlebia tremellosa* was 16 times higher than that of *Ganoderma lingzhi* sample, with values of 32 µg/mL and 2 µg/mL, respectively. These results confirmed the potential of this mushroom for ACE inhibition, and it should be pursued in future studies.

Besides the fact that the ACE-inhibitory capacity of most mushrooms (except for *Ganoderma lingzhi*) in this study has never been reported, our results also indicated a clear relationship between mushroom genus and certain activities, as mentioned above. Five of six *Ganoderma* and four of five *Trametes* mushroom samples showed high inhibition rates at the studied concentration. This correlation may result from the similarity of the chemical structures of metabolites provided by fungal species belonging to the same genus [122]. Further investigation and more samples are needed to confirm the speculation, but assuming such correlations can be established, this should be valuable information for directing future researches.

2.4. Conclusion

Twenty nine mushroom samples of 21 species in 14 genera collected in Nepal were checked for antioxidant and angiotensin-converting enzyme in vitro inhibition capacity. Beside *Phellinus gilvus* which was reported as a potent mushroom for isolating antioxidant compounds in some previous studies, this time we showed that *Inonotus andersonii* is also a promising candidate for antioxidant investigation with an antioxidant capacity equivalent to the well-known antioxidant spice, cloves. The H_2O_2-scavenging assay on HaCaT cells also revealed the potential of these mushrooms in the

prevention of oxidative-stress. From the fact that other samples of the *Phellinus* genus also showed high antioxidant activity, we deduced the potential of this genus as an important antioxidant source for future studies. ACE inhibition assays indicated that *Phlebia tremellosa* is a novel and potent candidate for antihypertensive studies. This mushroom exhibited even higher in vitro ACE inhibition activity than *Ganoderma lingzhi*, with the IC_{50} values of the two mushrooms at 32 μg/mL and 2 μg/mL, respectively. With half of the mushrooms samples herein being reported for antioxidant properties for the first time and most of the mushrooms having never been reported for ACE-inhibitory activity, information from this study should be a valuable reference for future studies on antioxidant and ACE-inhibitory activities of mushrooms.

2.5. Chapter's summary and limitation

In this chapter, 29 mushrooms collected in the mountainous areas of Nepal were analyzed for antioxidant activity by different methods, including Folin-Ciocalteu, ORAC, ABTS and DPPH assays. Intracellular H_2O_2-scavenging activity was also performed on HaCaT cells. The results showed that phenolic compounds are the main antioxidant of the mushrooms. Among studied samples, *Inonotus andersonii* and *Phellinus gilvus* exhibited very high antioxidant activity with the phenolic contents were up to 310.8 and 258.7 mg GAE/g extracts, respectively. The H_2O_2-scavenging assay on cells also revealed the potential of these mushrooms in the prevention of oxidative-stress. In term of ACE inhibition, results showed that *Phlebia tremellosa* would be a novel and promising candidate for antihypertensive studies. This mushroom exhibited even higher in vitro ACE inhibition activity than *Ganoderma lingzhi*, with the IC_{50} values of the two mushrooms being 32 μg/mL and 2 μg/mL, respectively. This is the first time biological activities of mushrooms collected in Nepal were reported.

Information from this study should be a valuable reference for future studies on antioxidant and ACE-inhibitory activities of mushrooms.

As a screening research, 29 samples in this study is still a limited number, especially for drawing a conclusion about the relationship between mushroom genus and biological activities as suggested in discussion part. More sample from same area is needed for a more firmly conclusion about genus-activity relationship. Besides, there are some studies reported antioxidant becoming a pro-oxidant at certain concentration; determination of optimal concentration of the mushroom extract to act as antioxidant instead of pro-oxidant should also be considered as an important part in future studies on antioxidant agent.

Table 2.1. Information related to mushrooms used in present study

No.	Scientific name	Locus*	Habitat	INSDC Acc. No.
N001	*Ganoderma carnosum*	Mt. Phulchoki/2765 m	Decayed wood	AB763348
N002	*Ganoderma lingzhi*	Mt. Phulchoki/2765 m	Decayed wood	AB811848
N003	*Ganoderma australe*	Mustang/3150 m	Decayed wood	AB811849
N004	*Ganoderma australe*	Mt. Phulchoki/2765 m	Decayed wood	AB811850
N005	*Ganoderma australe*	Mt. Phulchoki/2765 m	Decayed wood	Not determined
N006	*Ganoderma australe*	Dawachok/1500 m	Decayed wood	AB811852
N007	*Postia stiptica*	Dawachok/1500 m	Decayed wood	AB811853
N008	*Phlebia tremellosa*	Mt. Phulchoki/2765 m	Soil	AB811854
N009	*Trametes versicolor*	Mt. Phulchoki/2765 m	Decayed wood	AB811855
N010	*Inonotus andersonii*	Mt. Phulchoki/2765 m	Soil	AB811856
N011	*Trametes versicolor*	Mt. Phulchoki/2765 m	Decayed wood	AB811857
N012	*Inonotus* sp. 1	Mt. Phulchoki/2765 m	Living tree	Not determined
N013	*Heterobasidion linzhiense*	Surya Binayak/1400	Living tree	AB811859
N014	*Trametes versicolor*	Mt. Phulchoki/2765 m	Living tree	AB811860
N015	*Heterobasidion linzhiense*	Mt. Phulchoki/2765 m	Living tree	AB811861
N016	*Phellinus gilvus*	Mt. Phulchoki/2765 m	Decayed wood	AB811862
N017	*Inonotus* sp. 2	Mt. Phulchoki/2765 m	Decayed wood	Not determined
N018	*Phellinus conchatus*	Nagarkot/2500 m	Decayed wood	AB811863
N019	*Phellinus conchatus*	Nagarkot/2500 m	Decayed wood	AB811864
N020	*Inocybe* sp.	Mt. Phulchoki/2765 m	Soil	Not determined
N021	*Collybia peronata*	Nagarkot/2500 m	Fallen leaves	Not determined
N022	*Inonotus* sp. 3	Mt. Phulchoki/2765 m	Decayed wood	AB811865
N023	*Lactarius hatsudake*	Mustang/3150 m	Soil	Not determined
N024	*Lenzites betulina*	Mt. Phulchoki/2765 m	Soil	AB811866
N025	*Panellus* sp.	Mt. Phulchoki/2765 m	Decayed wood	Not determined
N026	*Rigidoporus* sp.	Surya Binayak/1400	Decayed wood	Not determined
N027	*Trametes versicolor*	Mt. Phulchoki/2765 m	Decayed branch**	AB811867
N028	*Trametes versicolor*	Mt. Phulchoki/2765 m	Decayed branch**	AB811868
N029	*Tricholoma caligatum*	Mt. Phulchoki/2765 m	Soil	Not determined

**Number in this average height of samples-collection area;* ** *Decay branch of living tree*

Table 2.2. ORAC values (μmol TE/g extract) of mushrooms extracts

No.	Scientific name	ORAC values	
		EtOH	H_2O
N001	*Ganoderma carnosum*	**1938.6** ± 64.5	764.2 ± 29.8
N002	*Ganoderma lingzhi*	**2136.2** ±100.8	**1046.1** ± 18.5
N003	*Ganoderma australe*	**1406.9** ±71.4	663.0 ± 19.4
N004	*Ganoderma australe*	**1602.9** ± 88.5	497.1 ± 69.7
N005	*Ganoderma australe*	**1781.4** ±123.3	813.5 ± 23.6
N006	*Ganoderma australe*	**2578.5** ± 99.0	**1196.9** ± 48.7
N007	*Postia stiptica.*	449.5 ± 27.7	660.5 ± 15.5
N008	*Phlebia tremellosa*	960.1± 38.1	629.2 ± 14.6
N009	*Trametes versicolor*	615.6 ± 34.0	650.0 ± 6.4
N010	*Inonotus andersonii*	**21015.4** ± 121.3	83.2 ± 31.9
N011	*Trametes versicolor*	**2168.0** ± 33.1	111.3 ± 36.7
N012	*Inonotus* sp. 1	**1848.0** ± 77.6	761.1 ± 15.4
N013	*Heterobasidion linzhiense*	616.1 ± 83.1	655.9 ± 26.8
N014	*Trametes versicolor*	410.6 ± 49.8	745.3 ± 3.7
N015	*Heterobasidion linzhiense*	691.0 ± 22.1	419.7 ± 19.8
N016	*Phellinus gilvus*	**9564.0** ± 281.5	280.7 ± 22.4
N017	*Inonotus* sp. 2	**1746.6** ± 100.9	358.2 ± 43.0
N018	*Phellinus conchatus*	**3856.4** ± 296.6	558.1 ± 18.9
N019	*Phellinus conchatus*	**4431.5** ± 211.6	570.3 ± 22.4
N020	*Inocybe* sp.	472.7 ± 38.8	447.6 ± 35.8
N021	*Collybia peronata*	802.4 ± 45.9	461.3 ± 15.9
N022	*Inonotus* sp. 3	342.8 ± 7.9	936.5 ± 69.5
N023	*Lactarius hatsudake*	425.5 ± 27.4	941.9 ± 54.9
N024	*Lenzites betulina*	548.9 ± 14.3	741.6 ± 13.8
N025	*Panellus* sp.	462.8 ± 12.0	**1070.1** ± 42.1
N026	*Rigidoporus* sp.	**1025.9** ± 48.7	534.1 ± 28.9
N027	*Trametes versicolor*	435.3 ± 14.2	**984.4** ± 14.1
N028	*Trametes versicolor*	522.2 ± 18.8	499.9 ± 26.5
N029	*Tricholoma caligatum*	620.5 ± 18.8	247.1 ± 10.8

Table 2.3. Phenolic content, ORAC values, ABTS and DPPH radical scavenging results of top 10 extracts

No.	Scientific name	Ethanol extract			
		Total phenolic (mg GAE/g)	ORAC	ABTS (%)	DPPH (%)
N010	*Inonotus andersonii*	310.8 ± 2.7	21015.4 ± 121.3	36.4±1.0 (63.5)	72.9±2.1 (102.0)
N016	*Phellinus gilvus*	258.7 ± 4.3	9564.0 ± 281.5	38.1±0.4 (56.0)	55.2±3.2 (109.0)
N017	*Inonotus* sp. 2	97.1 ± 0.9	1746.6 ± 100.9	24.1±0.3 (62.5)	22.4±1.6 (106.0)
N006	*Ganoderma australe*	88.3 ± 2.4	2578.5 ± 099.0	21.5±0.7 (63.5)	19.2±0.7 (108.0)
N005	*Ganoderma australe*	82.7 ± 8.2	1781.4 ± 123.3	16.1±2.3 (53.5)	14.7±0.9 (100.0)
N019	*Phellinus conchatus*	74.9 ± 1.3	4431.5 ± 211.6	19.3±0.2 (58.5)	14.5±1.5 (103.0)
N018	*Phellinus conchatus*	68.2 ± 0.2	3856.4 ± 296.6	12.6±0.3 (47.5)	17.0±0.2 (115.0)
N001	*Ganoderma carnosum*	70.2 ± 3.0	1938.6 ± 064.5	16.0±2.4 (61.0)	11.3±1.3 (103.0)
N002	*Ganoderma lingzhi*	66.8 ± 3.0	2136.2 ± 100.8	15.1±0.4 (63.0)	10.7±0.6 (126.0)
N011	*Trametes versicolor*	50.4 ± 0.5	2168.0 ± 033.1	10.3±0.3 (74.0)	NA**
Correlation coefficient*		**-**	**R = 0.923**	**R = 0.936**	**R = 0.986**

**Correlation coefficients in each column were between phenolic content and correspondent antioxidant activity;* ***not available; numbers in bracket of ABTS and DPPH columns were final concentrations (µg/mL)*

Table 2.4. Effect of mushrooms extract on UVB-induced intracellular H_2O_2 generation in HaCaT cells

No.	Scientific name	DFF*	No.	Scientific name	DFF*
N016	*Phellinus gilvus*	**7.3 ± 6.1**	N019	*Phellinus conchatus*	31.7 ± 3.1
N001	*Ganoderma carnosum*	**19.0 ± 9.6**	N018	*Phellinus conchatus*	32.7 ± 4.0
N010	*Inonotus andersonii*	**26.7 ± 4.0**	N002	*Ganoderma lingzhi*	35.3 ± 7.4
N005	*Ganoderma australe*	**26.7 ± 7.6**	N011	*Trametes versicolor*	35.7 ± 4.9
N017	*Inonotus* sp. 2	31.3 ± 10.7	N006	*Ganoderma*	53.3 ± 5.8
	Control	**26.3 ± 7.0**		Resveratrol	**53.3 ± 4.0**
	Negative control	**36.0 ± 9.8**			

** DFF = difluorofluorescein fluorescent intensity; proportionally related to H_2O_2 concentration*

Table 2.5. ACE inhibition rate of mushroom extracts at concentration of 100 µg/mL

No.	Scientific name	ACE inhibition (%)	
		EtOH	H_2O
N001	*Ganoderma carnosum*	22.99 ± 6.45	**71.30** ± 2.22
N002	*Ganoderma lingzhi*[*]	21.42 ± 3.77	**76.98** ± 1.22
N003	*Ganoderma australe*	22.11 ± 5.03	38.61 ± 4.65
N004	*Ganoderma australe*	15.37 ± 7.67	**50.72** ± 6.43
N005	*Ganoderma australe*	20.99 ± 3.44	**65.26** ± 6.76
N006	*Ganoderma australe*	33.10 ± 8.53	**61.59** ± 2.98
N007	*Postia stiptica*	18.23 ±1.16	26.81 ± 6.71
N008	*Phlebia tremellosa*[**]	6.14 ± 1.19	**92.57** ± 1.25
N009	*Trametes versicolor*	19.72 ± 2.11	38.18 ± 0.41
N010	*Inonotus andersonii*	**52.76** ± 1.80	39.38 ± 7.52
N011	*Trametes versicolor*	17.03 ± 0.82	**58.92** ± 7.82
N012	*Inonotus* sp. 1	18.25 ± 2.47	18.22 ± 1.40
N013	*Heterobasidion linzhiense*	1.47 ± 3.33	**54.97** ± 2.67
N014	*Trametes versicolor*	nd[***]	**69.09** ± 1.41
N015	*Heterobasidion linzhiense*	nd	**73.38** ± 3.08
N016	*Phellinus gilvus*	40.96 ± 2.60	13.80 ± 4.16
N017	*Inonotus* sp. 2	14.54 ± 4.45	nd
N018	*Phellinus conchatus*	18.21 ± 0.86	nd
N019	*Phellinus conchatus*	19.56 ± 4.59	48.39 ± 4.00
N020	*Inocybe* sp.	nd	**56.05** ± 7.40
N021	*Collybia peronata*	9.72 ± 1.23	38.99 ± 7.34
N022	*Inonotus* sp. 3	nd	15.71 ± 1.71
N023	*Lactarius hatsudake*	nd	nd
N024	*Lenzites betulina*	16.31 ± 1.03	**84.87** ± 2.04
N025	*Panellus* sp.	23.92 ± 1.49	35.36 ± 3.32
N026	*Rigidoporus* sp.	1.95 ± 2.32	10.17 ± 9.07
N027	*Trametes versicolor*	4.22 ± 5.10	**71.10** ± 1.28
N028	*Trametes versicolor*	0.17 ± 3.17	**75.40** ± 1.80
N029	*Tricholoma caligatum*	nd	**55.40** ± 3.89

[*] IC_{50} = 32 µg/mL; [**] IC_{50} = 2 µg/mL; [***] *nd = not detected;*

Chapter 3

ACE inhibitory peptides from auto-digested Reishi (*Ganoderma lingzhi*)

Ganoderma lingzhi
(source: improntaunika.it)

3.1. Introduction

The medicinal values of the reishi mushroom (*lingzhi* in Chinese) were documented more than 2,000 years ago [125]. Nowadays, reishi's pharmacological effects are the subject of renewed interest among researchers. A recent study reported by Cao *et al.* [126] indicated that the reishi commercially cultivated in East Asia, formally known as *Ganoderma lucidum*, is a different species from the true "*Ganoderma lucidum*" which was originally described as being from Europe. Cao *et al.* proposed the name "*Ganoderma lingzhi* Sheng H. Wu, Y. Cao & Y.C. Dai" for the reishi distributed in East Asia.

Containing approximately 400 different bioactive compounds, many of which have been found to be unique to this fungus, reishi has been reported to have effects on many

kinds of diseases [125]. Besides triterpenoids and polysaccharides, which have been isolated and thoroughly investigated [127], some proteins and lectins have also been identified in reishi [128], [129]. Previous studies have also provided valuable information on the existence of bioactive peptides in reishi [130], [131]. Unlike other mushrooms, which have been considered rich sources of proteins, reishi contains only around 7%–8% of these macromolecules [132]. However, studies on reishi have indicated that the mushroom could be a good source for bioactive proteins and peptides used in the treatment of a variety of diseases.

Angiotensin-Converting Enzyme (ACE) plays an important physiological role in regulating blood pressure. It converts angiotensin I to angiotensin II, which constricts the blood vessels and therefore increases blood pressure. Inhibition of the enzyme remains one of the first-line options for treatment of hypertension [133]. Since the first ACE-inhibitory peptide was discovered in viper's venom, there has been an ongoing search for natural ACE-inhibitory peptides, especially from food-derived proteins [134]–[136], and mushrooms [72], [120], [137], [138]. Although proteolytic peptides are less potent (IC_{50} values in μM range) than synthetic ACE inhibitors (IC_{50} values in nM range), they have a potential as active components in the diet by integration into functional food products.

Currently, studies of ACE-inhibitory peptides have focused mainly on the production and characterization of peptides isolated from microbial fermentation or digestions of proteins by enzymes supplied from outside [44]. Relatively little is known about auto-digested peptides formed by the "inside enzymes". In this study, we present for the first time the hypotensive effects of an auto-digested reishi extract (ADR) and the existence of ACE-inhibitory peptides in this extract. The results of this study will contribute to the development of functional foods or antihypertensive medication using reishi as a source

of the bioactive compounds, as well as to the understanding and use of auto-digested products.

3.2. Materials and Methods

3.2.1. Materials

Ganoderma lingzhi (BMC 4049 strain) powder was obtained from the Beauty Care Products Division of Nagase & Co. (Kobe, Japan). Synthetic peptides (>95%) were purchased from Medical & Biological Laboratories Co., Ltd. (Nagoya, Japan). Positive control, Isoleucine-Proline-Proline (IPP, >95%) was from Phoenix Pharmaceuticals (Funakoshi, Tokyo, Japan). Colorimetric assay kits for ACE activity were purchased from Dojindo Corp. (Dojindo Laboratories, Kumamoto, Japan) while protease assay kit was from Thermo Scientific (Pittsburgh, PA, USA). Spontaneous hypertensive rats (SHRs) were purchased from Japan SLC (Tokyo, Japan). The standard diet for rats was obtained from Nosan Corporation (Yokohama, Japan). Other reagents were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and were of the finest analytical grade.

3.2.2. Preparation of reishi's protease extract and its proteolytic activities

Reishi powder was air-dried and extracted by ion-exchange water for 4 h at 4 °C using an orbital shaker. The resulting mixture was then centrifuged for 10 min at 10,000 rpm and the supernatant was decanted and freeze-dried. Lyophilized samples were subjected to the Pierce™ Fluorescent Protease Assay Kit (Pierce, Rockford, IL, USA) which uses fluorescence detection following proteolytic digestion of fluorescein isothiocyanate-labeled casein (FTC-casein) to test the protease activities of mushroom extract. Briefly, the procedures were as follows: 100 µL of FTC-casein working solution was incubated with 100 µL of varied concentrations of reishi protease extract (5, 10 and 50 µg/mL) in a 96-well plate at room temperature. After 60 min of incubation time, the fluorescence intensity (excitation/emission was 485/530 nm) was measured using CytoFluor II

Microplate Reader (PerSeptive Biosystems, Framingham, MA, SA). TBS buffer (25 mM Tris, 150 mM NaCl, pH 7.2) and bovine pancreas TPCK trypsin (>14,000 BAEE units/mg, supplied with the kit) dissolved in TBS buffer were used as the negative control and positive control, respectively. The proteolytic activities were defined as the difference of fluorescent intensity between sample solutions and the negative control.

3.2.3. Preparation of reishi and auto-digested reishi's extracts

Reishi hot-water extract (HWR): Reishi powder (280 g) was boiled with ion-exchange water for 3 h; the sample mixture was then filtered, the resulting extract was lyophilized, and the final extract powder (15.3 grams) was stored in a capped plastic bottle sealed with Parafilm and stored at 4 °C until assayed.

Auto-digested extract (ADR): Totally, 12 liters of ion-exchange water was added to a tank containing 840 grams reishi powder, and the mixture was incubated at 37 °C for 24 h with shaking every 1 h. The whole mixture was then autoclaved at 121 °C for 15 min. After being filtered with filter paper, the resulting extract was lyophilized and the dried powder (58.9 gram) was stored in a capped plastic bottle sealed with parafilm and stored at 4 °C until assayed.

3.2.4. ACE inhibition assay

Dojindo ACE Kit – WST A502 was used for testing the ACE-inhibitory activity. Principles of the kit were described in details in a previous report [139]. Testing procedures were run strictly according to the manufacturer's instructions using a 96-well plate, and inhibition rate was calculated based on the comparison of optical absorbance of sample-treated wells (As), control wells (Ac) and blank wells (Ab) as in the equation below. Absorbance was measured at 450 nm using microplate reader-Biotek-ELX800 (BioTek, Winooski, VT, USA):

$$\text{Inhibition rate (\%)} = [(Ac - As)/(Ac - Ab)] \times 100$$

For HWR and ADR extracts, four concentrations (100, 167, 300 and 1670 µg/mL) were assayed against ACE to test for the ACE inhibitory potency of the extracts. For the peptides, six different concentrations of each peptide, ranging from 10 to 100 µg/mL, were assayed, and dose-response curves were plotted for the calculation of the IC_{50} values. IPP has been proven to be an ACE inhibitor [36] and thus was used as positive control (at concentrations ranging from 0.15 to 5 µg/mL).

3.2.5. Hypotensive effects of HWR and ADR extract on SHR rats

Twelve-week old spontaneously hypertensive male rats, SHR/Izm (Japan SLC, Shizuoka, Japan), weighing 269 to 290 grams, were divided randomly and housed in polycarbonate cages (three rats per cage) in a room kept at 23 ± 2 °C with a 12-h light/dark cycle. Experimental animals were fed a standard diet *ad libitum* and tap water. After a 1-week adaptation period rats were administered orally with HWR and ADR solutions at doses of 500 and 1,500 mg/kg body weight. A group in which ultrapure water (20 mL/kg body weight) was injected instead of reishi samples was used as control. Blood pressure was measured before as well as 4, 8 and 24 h after administration by the tail-cuff method using a BP-98A machine (Softron Corporation, Tokyo, Japan). At each time point, the rats' blood pressures were measured three times and mean values were retained for each rat; totally 6 mean values (for 6 rats in each group) were used for calculation of mean of the group. All of the animal experiments in this study were conducted in compliance with the guidelines of the Japanese Association for Laboratory Animal Science (2007) and approved by Animal Experiments of the Research and Development Division of Kyushu University (Approved date: 2010/12/28; Permitted Number: A22-234-0).

3.2.6. Ultrafiltration and RP-HPLC for fractionation

Auto-digested reishi extract was subjected to ultrafiltration using a 3-kDa cut-off membrane to obtain 02 fractions having molecular weights in the ranges >3 kDa and ≤3 kDa. The latter was applied to preparative HPLC (Series 600 HPLC, Waters, Milford, MA, USA) for 2 further fractionation steps. For the first preparative HPLC, an Inertsil ODS-3 (20 × 250 mm, 5μm) column was used while an Eclipse-XDB C_{18}, (9.4 × 250 mm, 5μm) column was selected for the second step. Water-A and acetonitrile-B (both were in mixture with TFA at the concentration of 0.1%) were used as the mobile phase, and the absorbance of the eluent was monitored at 215 nm in both steps. In the first step, elution was performed with a linear gradient of B in A from 10% to 60% for 60 min at a flow rate of 5 mL/min; in the second step the solvent program was started at 5 percent of B and the separation process was executed by a linear increase of B up to 13% over 60 min, and then from 60 to 70 min, B was increased to 25%; the process was finished by a washing step with 25% of B in 20 min without samples collection. The flow rate of this step was 2 mL/min. All of the collected fractions were freeze-dried, kept in capped glass vials and stored in a refrigerator until assayed.

3.2.7. Identification of peptides by LC-MS/MS

Identification of peptides was performed on a LCMS-IT-TOF system (Shimadzu, Kyoto, Japan) equipped with an electrospray ionization source. First, samples were separated by HPLC equipped with a ZIC-HILIC column (1.0 × 150 mm, Merck KGaA, Darmstadt, Germany). Then, the eluate from the HPLC was directly injected to an octopole ion-trap/time-of-flight system. For HPLC separation, all fractions (concentrations were 0.1 or 0.3 mg/mL) underwent a linear gradient elution, from 10% to 90% water (1% CH_3COOH) in a mixture with acetonitrile (1% CH_3COOH) for 45 min at a flow rate of 0.1 mL/min. Mass analysis was set up to run in both positive and negative modes in which mass spectra were acquired over the range of 57–1000 *m/z*.

Molecular mass was determined by the singly charged $(M + H)^{+1}$ state in the mass spectrum. The peptide molecular mass was automatically selected for fragmentation, and sequence information was obtained from tandem MS analysis.

3.2.8. Statistical analysis

All ACE-inhibitory assays were performed in triplicate whereas hypotensive test were repeated in 6 different rats for each group. Significant differences of *in vitro* assays were determined by Student's *t*-tests (unpaired; one-tailed) while two-way repeated measures ANOVA (Group × Time), with "H" as the within-subjects variable and "Group" as the between-subjects variable followed by a simple main effect test was applied for animal experiments. Values of $p < 0.05$ were considered to indicate statistical significance.

3.3. Results and Discussion

3.3.1. Reishi proteases' activities

In this study, reishi was extracted with ion-exchange water for 4 h at 4 °C. This reishi extract was then centrifuged, lyophilized and applied to a protease assay kit to test for proteolytic activities. The results are shown in Figure 3.1.

A study on proteolytic activities of 43 mushrooms by Sabotic *et al.* [140] revealed a large variety of proteases from basidiomycetes mushrooms as in other kingdoms. The fact that many proteases from mushrooms showed distinctive characteristics, and could be exclusive to basidiomycetes, makes mushrooms a vast and potent source of novel proteases [141]. However, information on proteases of reishi is very limited. We could not find any report for proteases in *G. lingzhi;* and only two published studies reporting the metalloproteases of the *G. lucidum* mushroom could be found [142], [143].

From the results presented in Figure 3.1, we can see that the proteolytic activity of reishi extract increased in a dose-dependent manner. This is the first time the total protease activity of reishi extract has been reported, and the results should provide

useful information for future studies on reishi's proteases. After reishi's proteolytic activity was confirmed, reishi powder was subjected to an auto-digestion process and the resulting extract (ADR) was used for further investigation of *in vitro* ACE inhibition and *in vivo* hypotensive effects. Reishi hot water extract (HWR) was also used for comparison.

3.3.2. ACE inhibition of auto-digested reishi extract

As shown in Figure 3.2, in all of the tested concentrations, both ADR and HWR showed ACE inhibition potential but ADR exhibited higher inhibitory activity than HWR. At a concentration as low as 100 μg/mL, ADR showed more than 50% inhibition. The inhibition rate increased to 75% at 167 μg/mL, and the enzyme was nearly completely inhibited at 1670 μg/mL. HWR also showed a dose-dependent inhibitory effect. The extract exhibited the same inhibition rate as that of ADR at1,670 μg/mL, even though it showed lower inhibition rates at other investigated concentrations. The difference of ACE inhibitory activity between ADR and HWR might be a consequence of the method of preparation. The auto-digestion process may have created active short peptides by proteolytic action of the mushroom's own proteases while hot-water-extraction process did not. These peptides might be the main factors causing the observed increase in the inhibitory effect of the ADR.

ACE inhibitory activity has been reported for many mushroom extracts. However, due to the differences in the assay systems used for evaluation of ACE-inhibitory activity, it is difficult to compare the results of different studies. Assuming that IC_{50} values could reflect the inhibitory strength, we can partly compare the inhibitory capacity of samples reported in different studies. In this context, the ACE-inhibitory activity of ADR reported in this study exceeded those reported for water extracts of other mushrooms. In a study carried out by Ukawa [144] on nine mushrooms, cold-water extract of

Lyophyllum decastes was reported to have the highest inhibition against ACE, but the IC_{50} value was as high as 250 μg/mL. In another survey on 23 mushrooms (screened from among 500 mushrooms) done by Izawa and Aoyagi [121], the hot-water extracts of 22 mushrooms showed lower IC_{50} values, ranging from 110–2,250 μg/mL, and only one sample, *Pholiota adiposa*'s extract, gave a comparable result to that of ADR, with an IC_{50} value of 66 μg/mL. ADR also showed better inhibitory activities than most mushrooms' water extracts of a recent study on antihypertensive proteins of nine edible mushrooms reported by Lau *et al*. [145]. At a concentration of 1,670 μg/mL, ADR inhibited up to 93% ACE activity; however, most of the mushrooms' extract in Lau's experiment required concentrations as high as 10,000 μg/mL to reach this rate. Based on these comparisons, the ADR of this study certainly merits further investigation of its *in vivo* antihypertensive effect.

3.3.3. Hypotensive effects of reishi and auto-digested reishi extract on rats

Reishi has a long history of use for promoting health and has been believed to have many therapeutic properties including strengthening of the cardiovascular function. Reishi powder was reported to have hypotensive effects on spontaneously hypertensive rats [146] and hot-water extract of reishi showed ameliorating effects on essential hypertensive patients after six months' treatment [147]. In this study ADR and HWR were administered to spontaneous hypertensive rats (SHRs) at doses of 500 and 1,500 mg/kg bodyweight, respectively. The initial average blood pressures of the SHRs in the test group were in the range of 171–175 mmHg just before the experiments. Systolic blood pressure (SBP) was measured at 0 and 4, 8, and 24 h after administration (time points denoted as T_0, T_4, T_8 and T_{24}, respectively) and the results are shown in Figure 3.3.

As shown in Figure 3.3, at T_0, there was no difference among groups in both systolic and diastolic blood pressure (for diastolic blood pressure, DBP, see Appendices, Figure A3.1). However, after 4 h, both reishi-treated groups showed low systolic blood pressure (SBP); after 8 h, three groups including ADR-500, ADR-1500 and HWR-1500 were still at a low level of SBP. After 24 h, all groups' SBPs had returned to baseline levels. There was also a tendency of lowering of DBP from T_0 to T_4, but no difference between T_4 and T_8 was observed, and all groups' DBP had returned to starting levels at 24 h. To confirm the variations, SBP was subjected to repeated analysis of variance (five groups) × time (four points) followed by a simple main effect test. There was no significant Group effect ($F(4,25) = 1.93$, $p = 0.136$), but significant Time ($F(3,75) = 32.43$, $p < 0.001$) and Group × Time interaction effects ($F(12,75) = 2.08$, $p = 0.028$) were observed. A simple main effect test also revealed a significant difference at T_8 ($F(4,25) = 3.16$, $p = 0.031$) but not at other time points. There was no observable difference for the control group ($F(3,23) = 2.26$, $p = 0.116$), but the simple main effect test for each group at all time-points showed significant differences in all sample-administered groups (HWR-500 group's $F(3, 23) = 4.15$, $p = 0.017$; HWR-1500 group's $F(3, 23) = 8.29$, $p = 0.001$; ADR-500 group's $F(3, 23) = 14.95$, $p < 0.001$; ADR-1500 group's ($F(3, 23) = 27.66$, $p < 0.001$).

Four hours after administration of the extracts, SBP decreased in all test groups; the sharpest decrease was observed in the ADR-1500 group with the change of SBP reaching −34.3 mmHg (or 19.5% of change). Interestingly, 8 h after administration of the extract, while other groups showed a tendency to lose their hypotensive effect, the ADR-500 group maintained its effect with an activity even higher than that at 4 h and similar to that of ADR-1500 (15% of reduction). It is also worth noting that the water extracts of other mushrooms and their active compounds reported in previous studies

[72], [137], [138] tend to lose their hypotensive effect only 4 h after administration. The fact that the hypotensive activity of ADR is still present after 8 h of administration may be due to the existence of intestinal digestion-resistant hypotensive agents in the extract. Base on the data presented here, ADR could be a promising candidate for use in antihypertension medication or for incorporation into antihypertensive functional foods.

3.3.4. Fractionation and identification of ACE-inhibitory peptides

Presently, ACE inhibitors are the second most-commonly prescribed treatment for hypertension [148] and the popular assumption for hypotensive effect of peptides is due to this enzyme blockade [149]. Studies on ACE-inhibitory peptides have mainly focused on the ≤3-kDa fraction due to the limitation of the dimensions of the enzyme's active site. In a study carried out by Kumakura [150], the author also proposed that the ≤3-kDa fraction of reishi extract may contain ACE-inhibitory peptides. The ADR extract of this study was proven to have *in vitro* ACE inhibition and *in vivo* hypotensive effect. Therefore, in tandem with previous studies' results, we decided to make further investigation on the components of ADR to clarify the potent components causing the hypotensive effect on SHRs as reported above. ADR was subjected to ultrafiltration using a 3-kDa cut-off membrane and after confirming the existence of peptides and/or amino acid in the ≤3-kDa fraction by staining with ninhydrin (Appendices, Figure A3.2), the permeate was then subjected to RP-HPLC for further fractionation. A typical chromatogram of a semi-preparative RP-HPLC fractionation and the corresponding fractions' ACE inhibition rates are shown in Figure 3.4.

After the first fractionation by HPLC, 11 fractions were obtained (see Appendices, Figure A3.3, Table A3.1), but only fraction ADR5, which had the highest inhibition rate and a sufficient quantity for further investigation, was chosen for further separation by a semi-preparative HPLC to obtain 7 other sub-fractions. From the results shown in

Figure 4 we can see that at 100 µg/mL, most sub-fractions had inhibition rates of more than 80% and there were no great differences in ACE inhibition between fractions. These results suggested that the active peptides were distributed evenly in all fractions and there was no single dominant compound for the inhibition of ACE. From these results we decided to apply all fractions onto an LCMS system to identify the structures contributing to the inhibitory activities against ACE.

In order to identify the potent ACE inhibitory peptides, all fractions (ADR5-1–ADR5-7) were analyzed by LC-MS/MS. Firstly, the peptides candidates were identified by *de novo* sequencing. All the candidates were then chemically synthesized and applied to the LC-MS/MS system for checking the fragmentation models. Peptide sequences were only accepted if the candidate's mass spectrum and synthesized peptide's mass spectrum matched with each other (see Appendices, Figure A3.4). In total 11 peptides were recognized and all of them were checked for the ACE-inhibitory effect. The results are shown in Table 3.1.

This time, only di- and tripeptides could be found in all sub-fractions. Although several parts were unidentifiable, there was no evidence of oligopeptides with more than three residues (> 500 Da) in these fractions. It can be seen that four (boldface) of the 11 peptides have rather high inhibition activities. The IC_{50} values of these peptides range from 73.1 µM to 162.7 µM (or 25.4 to 40.8 µg/mL). Surprisingly, even ADR5-2 showed the lowest inhibition rate among seven sub-fractions of ADR5; its peptides were amongst the four highest activity peptides identified. This phenomenon might be attributed to the effect of unidentifiable part of the fraction. It can be predicted that the unidentifiable part contains some inactive components mixing with the active components of ADR5-2 reducing the inhibition rate of the fraction. Assuming that IC_{50} values partly reflect the inhibitory strength, the ACE-inhibitory activity of peptides

identified in this study exceeded those recognized from water extracts of *Grifola frondosa* [120], *Tricholoma giganteum* [72], *Pholiota adiposa* [137] and *Pleurotus cornucopiae* [138], but were surpassed by some peptides isolated from *Pleurotus cystidiosus* [151] and *Agaricus bisporus* [152].

Blood pressure is regulated by various factors involved in the renin-angiotensin-aldosterone system, the sympathetic nervous system, and the kidney and fluid balance mechanisms. One limitation of this study is that only ACE was targeted for the potential of hypotensive effect of ADR and the extract's peptides. However, ACE is reported to play a crucial role in blood pressure regulation and fluid and electrolyte balance [153] and data of ACE-inhibitory activity should give valuable information for further antihypertensive investigation. Besides, this limitation did not change the fact that the auto-digested extract containing ACE-inhibitory peptides showed hypotensive effect *in vivo*.

3. 4. Conclusions

In this study, we investigated the *in vitro* ACE-inhibitory activity and *in vivo* hypotensive effect of auto-digested reishi in comparison with hot-water extract of the mushroom. Components showing potent activity against angiotensin-converting enzyme were also studied. It was shown that auto-digested reishi extract had higher inhibitory effect against ACE than that of the hot-water extract. The *in vitro* ACE assay suggested that no single ACE inhibitor dominated in the extract, but rather an aggregate of active peptides was present. Eleven peptides were identified and their IC_{50} values against ACE confirmed the hypothesis that a series of strong ACE inhibitors exists in auto-digested reishi extract. The *in vivo* hypotensive effect of auto-digested reishi extract suggested it to be a good source of ACE-inhibitory peptides that could be used in functional food or for antihypertension medication. This study not only reveals the remarkable properties

of auto-digested reishi but also suggests a potentially more potent alternative to the water extracts of mushrooms, *i.e.*, auto-digested extracts.

3.5. Chapter's summary and limitation

In this chapter, we used reishi's own proteases to hydrolyze its protein and obtained auto-digested reishi (ADR) extract. The extract was subjected to *in vitro* assays and administered to spontaneous hypertensive rats (SHRs) to determine its potential for use in hypotensive medication. Bioassay-guided fractionation and *de novo* sequencing were used for identifying the active compounds. After 4-hour administration of ADR, the systolic pressure of SHRs significantly decreased up to 34.3 mmHg (19.5% of change) and the effect was maintained up to 8 hours of administration, with the decrease as low as 26.8 mmHg (15% of reduction). Eleven peptides were identified and 4 of them showed potent inhibition against ACE with IC_{50} values ranging from 73.1 μM to 162.7 μM. The results showed that ADR could be a good source of hypotensive peptides that could be used for antihypertensive medication or incorporation into functional foods.

Even though ACE inhibitory peptides were identified, their content in ADR extract was not determined. This lack of information make it be difficult to calculate how potent the extract when it is used for functional food or for antihypertensive medication. It was also not clear whether identified peptides existed in the HWR or it is really unique from the ADR. Using peptide standards for identification and quantification of peptide will help to get over these limitations.

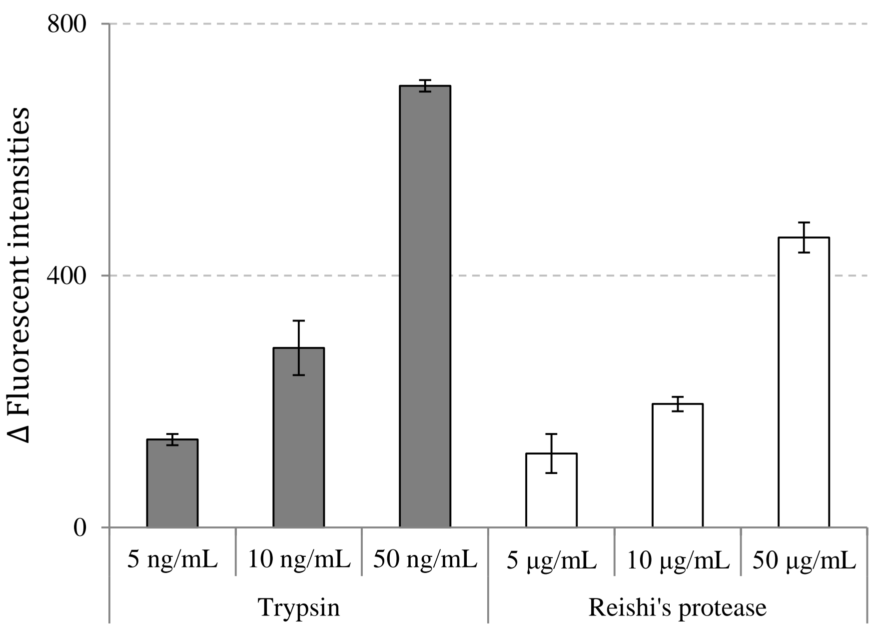


Figure 3.1. Proteolytic activities of reishi's proteases extract at different concentrations; trypsin and TBS buffer were used as positive and negative controls, respectively; the activities were defined as the difference of fluorescent intensity between sample solutions and the negative control (value = mean ± SD, n = 3).

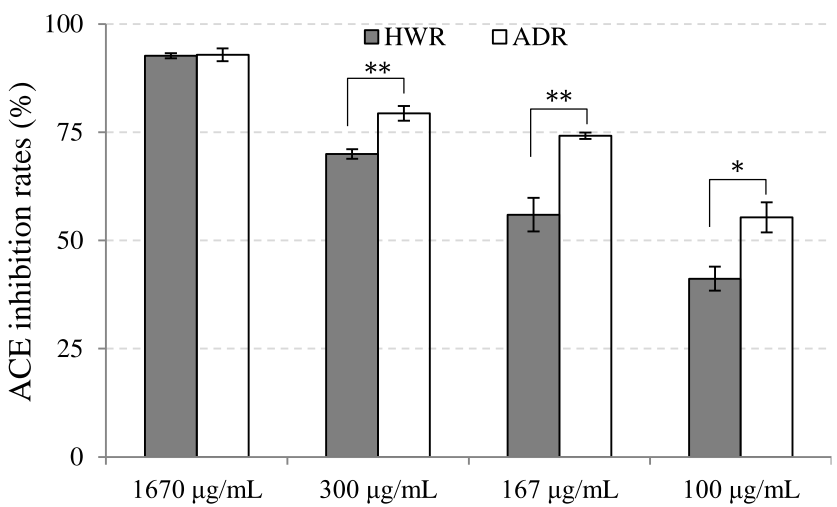


Figure 3.2. ACE inhibition rate of hot-water and auto-digested extracts of reishi at different concentrations (value = mean ± SD, n = 3). Significant differences were determined by *t*-test (unpaired; one-tailed): $p < 0.005$ (∗), $p < 0.001$ (∗∗).

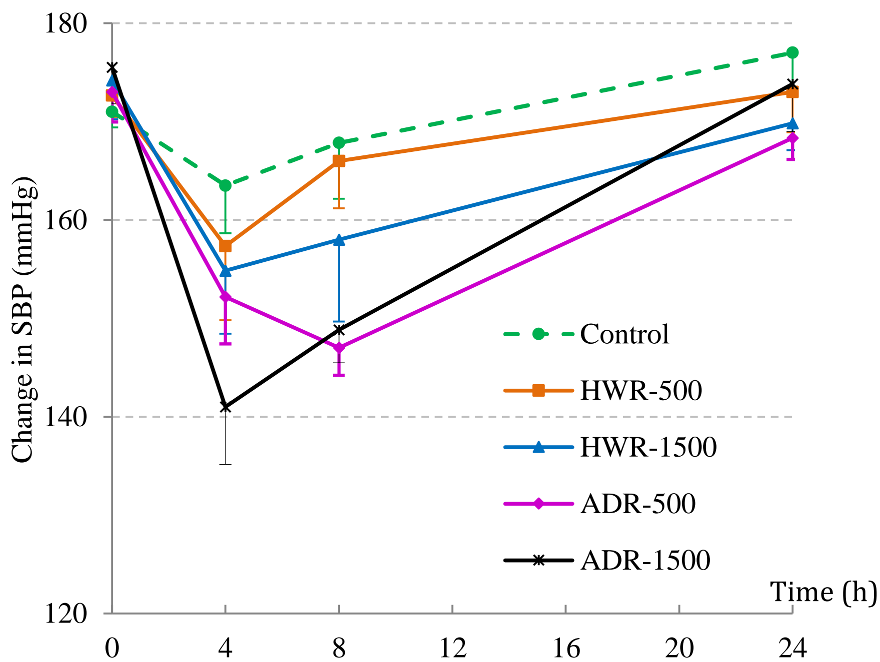


Figure 3.3. Time-course of changes in SBP of SHRs after administering HWR and ADR extracts (average ± SE, n = 6). Single oral administration was dosed at 500 and 1500 mg/kg body weight. Ultrapure water was used as control. Two-way repeated measures ANOVA (Group × Time), with "H" as the within-subjects variable and "Group" as a between-subjects variable followed by a simple main effect test was applied. There was no significant Group effects ($F(4,25) = 1.93$, $p = 0.136$), but significant Time ($F(3,75) = 32.43$, $p < 0.001$) and Group × Time interaction effect ($F(12,75) = 2.08$, $p = 0.028$) were observed.

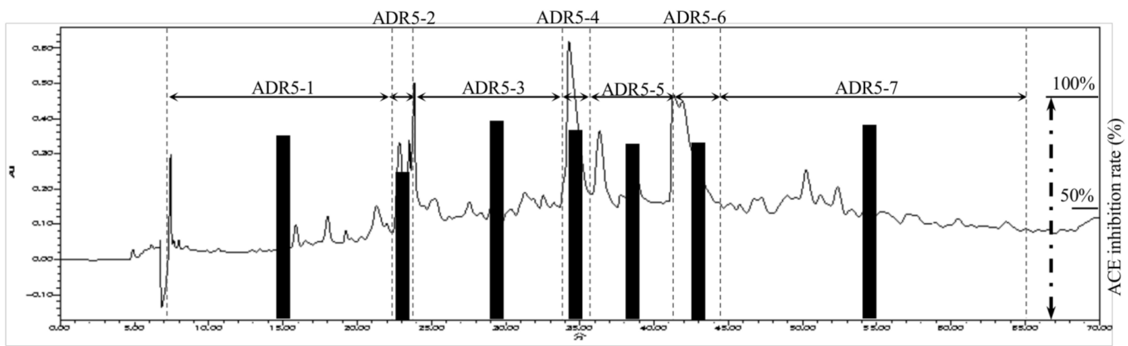


Figure 3.4. Typical chromatogram of RP-HPLC fractionation of the ADR-5 (solvent program was a linear gradient from 5%–13% of 0.1% TFA/AcCN in 0.1% TFA/H_2O for 60 min; then increased to 25% in other 10 min before finished) and the sub-fractions' ACE-inhibitory effects (100 µg/mL, n = 3; mean values and standard deviations were in Supplementary Materials, Table S2).

Table 3.1. Peptides existing in ADR extract and their ACE inhibition capacity

Fractions	**IC_{50} values (μM)**			
	Dipeptides		**Tripeptides**	
ADR5-1	undetectable			
ADR5-2	Ser-Ile	>200	undetectable	
	Ala-Tyr	**162.7**		
	Ser-Tyr	**94.7**		
ADR5-3	undetectable			
ADR5-4	Ser-Leu	>200	Asn-Ser-Ile	342.1
			Lys-Val-Pro	>500
ADR5-5	Ala-Leu	>200	undetectable	
	Thr-Leu	>200		
ADR5-6	**Ile-Arg**	**73.7**	**Ile-Pro-Thr**	**73.1**
ADR5-7	undetectable		Gly-Pro-Leu	>500
Positive control			Ile-Pro-Pro	< 0.5

Chapter 4

ACE inhibition activity of triterpenoids: the structure-activity relationship

Ganoderic acid A
(Source: pubchem.ncbi.nlm.nih.gov)

4.1. Introduction

Currently, angiotensin-converting enzyme (ACE) inhibition is one of the most popular therapies used for treatment of hypertension.[154] Various synthetic peptidomimetic drugs are presently dominant in the anti-hypertension drug market. However, there is still a need for new active compounds derived from natural sources due to the various drawbacks of using of currently available drugs [155].

Reishi, recently redefined as *Ganoderma lingzhi* Sheng H. Wu, Y. Cao & Y.C. Dai,[126] has been treasured for its medicinal value in traditional medicine in Japan and China.[125] Of all *Ganoderma* mushrooms, which account for more than 150 known triterpenoids, *G. lingzhi* has been the most well-studied species[156]. Our group has isolated several new triterpenoids from reishi [157], [158] and reported their

bioactivities, including 5α-reductase inhibition [159], anti-androgenic osteoclastogenesis [160] and α-glucosidase inhibitory activity [161]. Triterpenoids from reishi have been reported to have *in vitro* ACE-inhibitory activity [40] and *in vivo* anti-hypertensive effects in rats [162]. We conducted this study with the aim of identifying structural features involved in the ACE-inhibitory activity, an important factor contributing to the hypotensive effect [163], and the inhibition pattern of lanostane-type triterpenoids against the enzyme. By using a molecular modeling tool we could also draw a hypothesis about the inhibition patterns of the lanostanoids against the ACE. This hypothesis was then verified by *in vitro* experiments using Dixon plots.

4.2. Materials and Methods

4.2.1. Materials

Triterpenoids (>98%) were obtained from Chemfaces (Wuhan, Hubei, China) and Quality Phytochemicals (LLC, New Jersey, USA). All were used without further purification. ACE test kit was purchased from Dojindo (Dojindo Laboratories, Kumamoto, Japan). Docking software CLC Drug Discovery Workbench was afforded from CLC Bio (CLC Bio, Aarhus, Denmark).

4.2.2. Angiotensin-converting enzyme inhibition assay

The ACE inhibition test was performed in accordance with the manufacturer's instructions with some modification, using a 96-well plate. Briefly, 17 μL of substrate were mixed with 20 μL of sample solution (except for control and blank) and incubated for 5-10 minutes at room temperature; 3 μL of water/ethanol mixture of different ratio were added so that the final concentration of ethanol in all well was 5%. Enzyme reaction was initiated by adding 20 μL of ACE solution (except for blank) to each well. Reaction mixture was incubated at 37 ^{0}C in 60 minutes and absorbance of the solution was measured after indicator was added. We calculated inhibition rates based on

comparison of the absorbance of sample-treated wells (As), control wells (Ac) and blank wells (Ab) as shown in Equation (1). Absorbance was measured at 450 nm using a microplate reader ELX800 (BioTek, Vermont, USA).

$$\text{Inhibition rate (\%)} = [(Ac - As)/(Ac - Ab)] \times 100 \qquad (1)$$

To determine the IC_{50} values, we assayed five concentrations of each compounds in triplicate against ACE, and plotted dose-response curves for calculation of the values.

4.2.3. Inhibition pattern

Four substrate volumes (10 µL, 15 µL, 20 µL and 25 µL) were co-incubated with varied concentrations of triterpenoids (62.5 □g/mL, 125 □g/mL and 250 □g/mL) and ACE solution. Each reaction mixture was assayed as described above and reactions kinetics was determined using Dixon plots.

4.2.4. Molecular modeling

All triterpenoid structures were subjected to Docking experiments to check for the potent inhibitory pattern against ACE. All components of the complex (include crystalized water) subjected to the docking experiment were imported to the workspace of the Drug Discovery Workbench program without any structural modification. The compound were docked inside a 13-Å radius sphere covering the active site of 3 ACE structures extracted from the complexes of ACE with captopril, lisinopril and enalaprilat, respectively.

4.3. Results and Discussion

Thirty-two triterpenoids (>98% purity) from reishi (*G. lingzhi*) were subject to an assay of ACE inhibition using test kits - WST A502 (Dojindo, Kumamot, Japan); more details about materials and methods used in this study are present in the Supplementary data. There were 2 aldehydes, 5 alcohols and 25 acidic triterpenoid derivatives; however, to facilitate the investigation into the structure–activity relationship, the compounds were

divided into three groups based on a fused tetracyclic ring and a branch attached to this ring. The first group included compounds whose tetracyclic ring contains one double bond ($\Delta C^{8,9}$) and the branch ends with a carboxyl group; the second group was made up of compounds whose ring contains two double bonds ($\Delta C^{7,8}$, $\Delta C^{9,11}$) with a branch also terminated by a carboxyl group; and the third one was similar to the second except the branch of the compounds in this group have no carboxyl terminal. Details of this division and the triterpenoids' structures are shown in Table 4.1.

Among the investigated compounds, 15 showed measurable IC_{50} values. These values ranged from 100.1 to 499.5 μg/mL; the lowest recorded for ganoderic acid A and the highest for ganoderic acid N. The IC_{50}-measurable compounds mostly fell into the first group and only 1 compound (ganoderic acid TR) of the second group showed significant inhibition, with an IC_{50} value of 168.1 μg/mL. Other compounds in the second group and all compounds in the third group showed no significant inhibitory activity, with IC_{50} >500 μg/mL, 500 μg/mL being the solubility limit of the triterpenoids used in this study. There are some differences in IC_{50} values of compounds used in this study and those of a previous report [40]; however, the inhibitory-strength order was not that different. We think that the differences in IC_{50} values might have resulted from differences in the amount of organic solvent used in the inhibition assay as well as the enzyme source. In this study, we used ACE isolated from a rabbit lung and kept the ethanol constant at 5% of the assay volume; whereas the previous study's results were reported for ACE from a hog kidney with an ethanol percentage of 10%.

From the results shown in Table 1, it can be seen that the –COOH group at the branch is one of the key factors affecting the ACE-inhibitory activity of the triterpenoids. Structures without this carboxyl group showed no significant inhibition activity and gave unmeasurable IC_{50} values. However, the presence of this group in the structure

was not the "magic bullet" guaranteeing inhibitory activity; other factors including double bonds, and a –C=O or –OH group at some position also played roles. A double bond at $–C^{20,22}$ and $–C^{24,25}$ decreased the inhibition of the triterpenoids but the latter had a stronger effect. For example, ganoderic acid A and ganoderenic acid A differ from each other by the double bond at the $–C^{20,22}$ position, and these compounds showed a small decrease of inhibitory activity from 100.1 µg/mL (in ganoderic acid A) to 125.9 µg/mL (in ganoderenic acid A); however, ganoderic acid C1 and ganoderic acid LM2 also differ by a double bond at the $–C^{24,25}$ position, but they showed a sharp decrease from 383.6 µg/mL (in ganoderic acid C1) to >500 µg/mL (in ganoderic acid LM2). A double bond inside the tetracyclic ring also contributed to inhibitory activity. Except for ganoderic acid TR, other structures with two double bonds at $-C^{7,8}$ and $-C^{9,11}$ showed no significant activity, and IC_{50} values could not be obtained for these compounds. On the opposite side, however, most compounds with double bond at position of $-C^{8,9}$ showed an inhibition effect against the enzyme with measurable IC_{50} values. All compounds with measurable IC_{50} values have a –C=O or –OH group at C^{15}, whereas compounds without these group at this position, like ganoderic acids S, Sz, Y, showed unmeasurable IC_{50} values. The structural features of triterpenoids from reishi, *G. lingzhi*, affecting ACE-inhibition activity are briefly described and shown in Figure 4.1.

The fact that many triterpenoids with minor differences in structure showed quite different ACE-inhibition activity led us to use molecular modelling tools for investigation of compound–enzyme interactions. The first aim of this use was to visualize the major interactions between the triterpenoids and the enzyme as well as key binding sites of this protein. To that end, we conducted docking experiments using CLC Drug Discovery Workbench (CLC Bio, Aarhus, Denmark). The triterpenoids (ligand) whose structures were created by MarvinSketch (ChemAxon, Budapest, Hungary), were

docked inside a 13-Å-radius sphere covering the active site of the angiotensin-converting enzyme (receptor). Three protein structures extracted from complexes with captopril (PDB 1UZF), lisinopril (PDB 1O86) and enalaprilat (PDB 1UZE) were used as targets.

As with most docking software, the docking score obtained from the docking experiment was expected to be negative since the score mimics the potential energy change, when the ligand binds to the protein and makes it complex. However, with much surprise, the docking scores were positive for most structures docked to the targets used in this study; only a few cases gave negative scores, and their absolute values were quite small (details in Table S1, Supplementary data). These results indicated a certain violation of spatial distribution inside the active site of the proteins. We assumed that the docking ligand must have clashed with the receptors and/or could not form a strong enough bond at this docking site. To clarify the reason why most of the docking scores were positive, we investigated poses of the triterpenoids docked to the binding pocket of ACE in comparison with the poses of drugs inside the binding pocket. A typical result of a compound with highest inhibitory activity against ACE (ganoderic acid A) is shown in Figure 4.2.

As shown in the figure, the triterpenoid seemed too big to fit in with the pocket while the drugs fit well within this site. Some parts of the triterpenoid (shown by yellow arrows) dropped out of the binding pocket (dotted green space) and clashed with the protein. This clashing is likely what resulted in the positive docking scores.

Although the docking experiment produced unforeseen results, it otherwise can be used to make an interesting speculation about the inhibition pattern of the enzyme reaction. Based on the analysis of triterpenoids' poses inside the binding pocket and a previous report on the structural stability of the ACE,[164] we speculated that the inhibition

mode of the triterpenoids against the enzyme is not competitive but rather non- or uncompetitive or somewhat between these types. To verify this speculation, we ran an *in vitro* ACE-inhibitory assay as described by Lam et al. [165] with some modification (details shown in Supplementary data) using the above-mentioned ACE test kit. Two compounds with high water solubility were chosen for this experiment and results are shown in Figure 4.3.

As shown in the figure, the inhibition pattern was found to be mixed-type for ganoderic acid A and non-competitive for ganoderic acid C2. These results suggest that the triterpenoids could bind to the enzyme and perform an inhibition effect regardless of whether the substrate molecule was bound or not. The result also confirmed our speculation about the inhibition mode using the docking tool. This time, only 2 compounds were subjected to kinetics investigation; however, since the triterpenoids used in this study share common structural features and similar molecular weight and size, we suspect that they also show inhibition activity against the angiotensin-converting enzyme with the same pattern as ganoderic acid A and/or ganoderic acid C2.

4.4. Conclusion

In summary, in this study we demonstrated that triterpenoids from reishi (*G. lingzhi*) possessed inhibitory activity against angiotensin-converting enzyme at moderate inhibition rates. Our structure–activity investigation revealed the structurally important parts involved in this activity. The *in silico* and *in vitro* experimental data complementarily showed that the inhibition activity of the triterpenoids is not competitive but non-competitive and/or uncompetitive instead. This study also proposed a novel approach of using modelling software to predict enzyme inhibition patterns. As far as we know, this is the first time docking software has been used for investigation of ACE-inhibition modes.

4.5. Chapter's summary and limitation

Triterpenoids have been proven to inhibit angiotensin-converting enzyme effectively; however, there has been no report about the structure–activity relationship and inhibition patterns of these compounds. In this chapter, 32 lanostane-type triterpenoids derived from *Ganoderma lingzhi* were assayed to determine the structural features involved in the observed inhibition effects. *In silico* and *in vitro* experiments were also used to determine the kinetics of the reaction. Fifteen compounds showed measurable *in vitro* IC_{50} values ranging from 100.1 to 499.5 µg/mL. It was shown that carboxyl groups play an important role in inhibiting the enzyme; further, a hydroxyl group or carbonyl group at either C^7 or C^{15} increases the inhibition rate, and a double bond at $C^{24,25}$ decreases the activity. Based on the docking data we speculated that triterpenoids could not fit into the active site of the enzyme; therefore, the inhibition mode could not be competitive. Dixon plotting showed that the inhibition patterns should be uncompetitive and non-competitive instead.

Low *in vitro* inhibitory activity (compare to current-used drugs) and low water-solubility are two major limitations of triterpeneoids. Therefore, *in vivo* behavior and/or structural modification is needed for any intention of practical use.

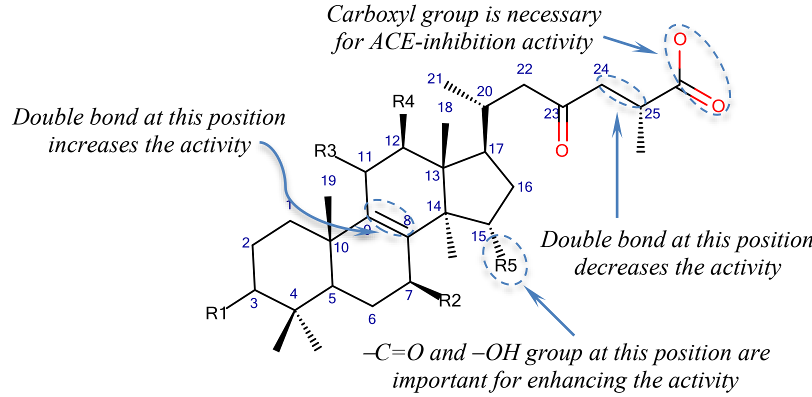


Figure 4.1. Structural features affecting to ACE-inhibitory effect

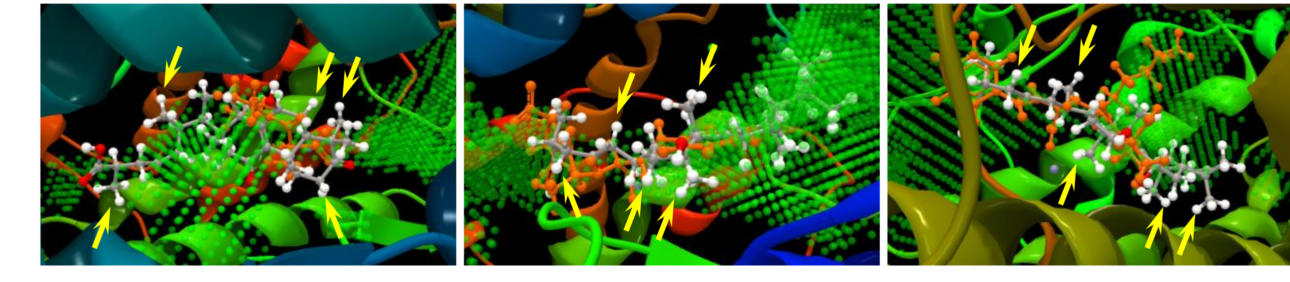


Figure 4.2. From left to right: Docking poses of ganoderic acid A (gray and white ball-stick) inside the active sites of proteins extracted from ACE-drug complexes 1UZF (ACE-captopril), 1UZE (ACE-enalaprilat) and 1O86 (ACE-lisinopril), respectively. Orange ball-stick skeletons were structures of captopril, enalaprilat and lisinopril in their own complexes.

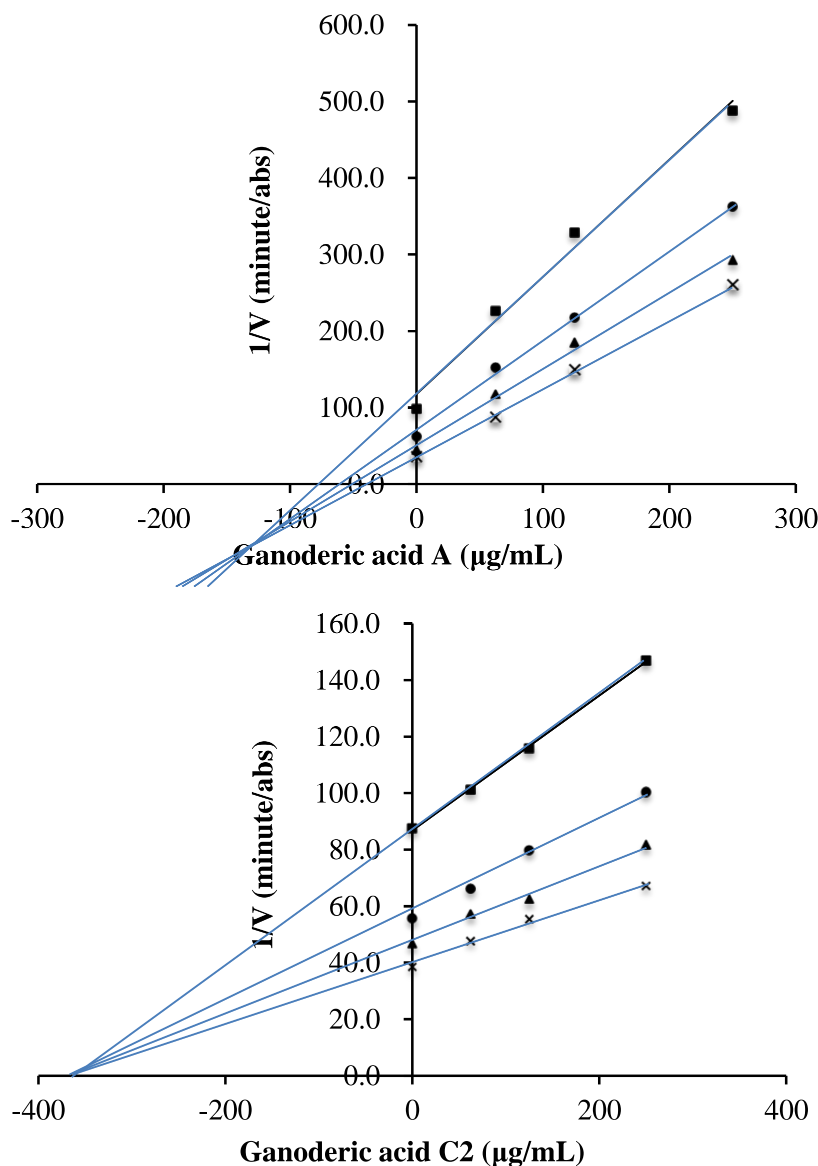


Figure 4.3. Dixon plots of ACE inhibition by ganoderic acid A and ganoderic acid C2. Substrate volumes were 10 µL (■), 15 µL (●), 20 µL (▲) and 25 µL (✕), respectively.

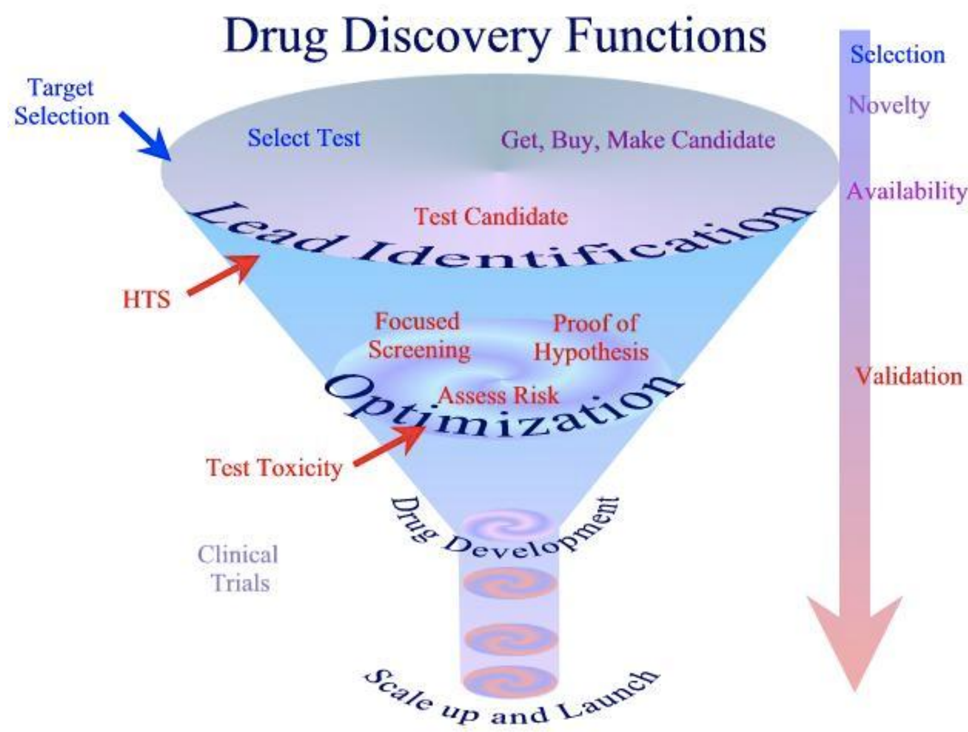
Table 4.1. Triterpenoids and their ACE-inhibitory activity

Compound	R1	R2	R3	R4	R5	Bond/Abs. confg.[a]	IC_{50} (μg/mL)[b]
Ganoderic acid A	=O	–OH	=O	–H	–OH	as drawing	100.1±8.9
Ganoderenic acid A	=O	–OH	=O	–H	–OH	$\Delta^{20,22}(E)$[c]	125.9±8.7
Ganoderic acid C2	–OH	–OH	=O	–H	–OH	as drawing	138.2±2.5
Ganoderic acid AM1	–OH	=O	=O	–H	=O	as drawing	184.0±9.9
Ganoderic acid K	–OH	–OH	=O	$–OCOCH_3$	=O	C^{25} not defined	202.9±4.0
Ganoderenic acid H	–OH	=O	=O	–H	=O	$\Delta^{20,22}(E)$, C^{25} not defined	217.5±18.2
Ganoderic acid H	–OH	=O	=O	$–OCOCH_3$	=O	$C^{20}(S)$	240.1±7.6
Ganoderic acid B	–OH	–OH	=O	–H	=O	as drawing	285.7±33.5
Ganoderic acid C6	–OH	=O	=O	–OH	=O	as drawing	381.7±6.0
Ganoderic acid C1	=O	–OH	=O	–H	=O	as drawing	383.6±8.4
Ganoderenic acid F	=O	=O	=O	–H	=O	$\Delta^{20,22}(E)$, C^{25} not defined	351.0±21.0
Ganoderenic acid C	–OH	–OH	=O	–H	–OH	$\Delta^{20,22}(E)$, C^{25} not defined	358.2±20.5
Ganoderenic acid D	=O	–OH	=O	–H	=O	$\Delta^{20,22}(E)$, C^{25} not defined	376.5±62.4
Ganoderic acid N	=O	–OH	=O	–H	=O	$-C^{20}(OH)$, $C^{20,25}$ not defined	499.5±19.4
Ganoderic acid DM	=O	=O	–H	–H	–H	$\Delta^{24,25}(E)$	>500
Ganolucidic acid A	=O	–H	=O	–H	–OH	as drawing	>500
Ganoderic acid ζ	–OH	=O	=O	–H	=O	$-C^{23}(OH)$, $\Delta^{24,25}(E)$	>500
Ganoderic acid LM2	=O	–OH	=O	–H	=O	$-C^{23}(OH)$, $\Delta^{24,25}(E)$	>500
Ganoderic acid E	=O	=O	=O	–H	=O	as drawing	>500
Ganoderic acid TR	=O	-[d]	-	-	–OH	$\Delta^{24,25}(E)$	168.1±20.2
Ganoderic acid T-N	–OH	-	-	-	$–OCOCH_3$	$\Delta^{24,25}(E)$	>500
Ganoderic acid T-Q	=O	-	-	-	$–OCOCH_3$	$\Delta^{24,25}(E)$	>500
Ganoderic acid Sz	=O	-	-	-	–H	$\Delta^{24,25}(Z)$	>500
Ganoderic acid S	=O	-	-	-	–H	$\Delta^{24,25}(E)$	>500
Ganoderic acid Y	–OH	-	-	-	–H	$\Delta^{24,25}(E)$	>500
Ganoderol A	=O	–H	-	$–CH_3$	$–CH_2OH$	$\Delta^{24,25}(E)$	>500
Ganoderol B	–OH	–H	-	$–CH_3$	$–CH_2OH$	$\Delta^{24,25}(E)$	>500
Ganoderiol F	=O	–H	-	$–CH_2OH$	$–CH_2OH$	$\Delta^{24,25}$	>500
Ganodermanondiol	=O	–OH	–OH	$–CH_3$	$–CH_3$	$C^{24}(S)$	>500
Ganodermanontriol	=O	–OH	–OH	$–CH_3$	$–CH_2OH$	$C^{24}(S)$, $C^{25}(R)$	>500
Lucialdehyde A	–OH	–H	-	$–CH_3$	–CHO	$\Delta^{24,25}(E)$	>500
Lucialdehyde B[e]	=O	–H	-	$–CH_3$	–CHO	$\Delta^{24,25}(E)$	>500

[a] absolute configuration; [b] IC_{50} is shown as mean±SD (n=3); [c] Δ-double bond; [d] do not exist; [e] double bonds $\Delta^{7,8}$ and $\Delta^{9,11}$ is replaced by $\Delta^{8,9}$ and C^7 is changed to –C=O; Unless otherwise specified, chiral centers are as drawn

Chapter 5

Rational design of ACE inhibitory peptides



A typical Drug Discovery process
(Source: newdrugapprovals.org)

5.1. Introduction

One of the most commonly used antihypertensive therapies is the inhibition of angiotensin-converting enzyme (ACE), the dipeptidyl carboxypeptidase that causes blood vessels to constrict and blood pressure to increase [166]. Since the discovery of the first ACE peptide inhibitor (from a viper's venom), significant efforts have been made to develop ACE inhibitors by both synthetic and nature-derived approaches. The identification of ACE inhibitor candidates has involved a costly and time-consuming process of screening and activity testing. In this context, high-throughput screening (HTS), in which large libraries of synthetic and natural compounds are subjected to

bioassays [167], seems to be a solution. However, the two drawbacks of the HTS approach are the low hit rates of active compounds and incomplete information about the biological target features [168].

Recently, the use of computers for evaluating the bioactivities of large compound libraries against a target structure, known as "virtual screening" [169], has been facilitated by the elucidation of protein structures by X-ray crystallography and the development of simulation software. In virtual screening, docking is a helpful tool for predicting the optimal pose and binding affinity of small molecules (ligands) when they bind to a target protein (receptor) to form a stable complex. Docking tools can also be used to identify potential candidates at a specified activity from a library of thousands of (or even more) compounds or to determine a lead structure by optimizing the potential candidates in the library [170].

In the field of ACE inhibition, peptides and peptidomimetics are the dominant structures, as most of the drugs currently used for ACE inhibition therapy are peptide-like compounds. Peptides have been reported with low oral bioavailability and intestinal absorption [171], but many studies (both *in vivo* and placebo-controlled trials) have shown the hypotensive effect of different peptides on rats and humans [38], [172]–[177]. For hypotensive peptides, proline at the C-terminus of peptides was proven to be more potent than other naturally occurring amino acids [178]. The presence of proline and proline-like moieties in the structures of antihypertensive drugs and many ACE inhibitor peptides demonstrates the vital role of this moiety in ACE-inhibitory agents.

There is virtually no absorption for peptide sequences with more than four amino acids, whereas di- and tripeptides have been demonstrated to be actively transported by many oligopeptide transporters such as PepT1 and PepT2 [179]. The objective of the present study was to identify potent ACE inhibitor tripeptides with proline at the C-terminal

position, using docking-guided experiments. Unlike previous virtual screening studies, which focused mostly on docking without considering the tautomers' form at the pH used in the assay (an important factor influencing screening results) or the bioavailability of the obtained candidates, we used a three-step screening process to identify potent peptides. The process includes compound tautomerization, virtual docking and ADME (absorption, distribution, metabolism, excretion) prediction. With this method, we sought to identify candidates with characteristics of a true drug with high potential for practical application.

5.2. Material and Methods

5.2.1. Materials

The protein structures were obtained from the Protein Data Bank (http://www.pdb.org), whereas the structures of peptides were created with the use of MarvinSketch (ChemAxon, Budapest, Hungary). ACE test kits were purchased from Dojindo (Dojindo Laboratories, Kumamoto, Japan), and synthetic peptides (> 95% of purity) were obtained from CS Bio (Shanghai, China).

5.2.2. Comparison of the drugs' structures

We used four commonly prescribed anti-hypertensive drugs (enalaprilat, lisinopril, trandolaprilat and perindoprilat) for the comparison, and we used them to identify the most common parts of an ACE inhibitor and to test whether the proline moiety is an important part of ACE inhibitors.

5.2.3. Peptide-structures design and tautomer selection

We created 529 C-terminal proline-containing tripeptides (from the 20 standard amino acids and three phosphorylated amino acids, pTyr, pSer and pThr), and we optimized their 3D-structures and calculated the tautomer forms at pH 8.0 (the pH of the ACE

inhibitory assay) using MarvinSketch. Only the two most dominant tautomers were selected and saved in a .mol2 format for further investigation.

5.2.4. Virtual Screening

5.2.4.1. Template Docking (ligand-based or pharmacophore)

To create the docking template, we used the lisinopril structure extracted from the ACE-lisinopril complex obtained from the Protein Data Bank (PDB code 1O86) [8]. We created the pharmacophore of the structure using the template-docking function of Molegro Virtual Docker 6.0 software - MVD (Molegro ApS, Aarhus, Denmark) in which the effective radius of each contributing factor was set at 1.8 Å and the charge threshold was set at 0.0; the default setting of other parameters were used. The binding site coordinates were the same as those of lisinopril (x=41.31; y=33.82; z=46.21), and the docking was run inside a sphere with a 15-Å radius centered at the active site. Peptide structures were stored in separated folder and docking software was directed to take the structures from the folder for docking.

We set the overall strength of the template scoring at −500. For each docking, five runs were done with each peptide structure and a maximum of five poses were retuned for each run. Only one pose, which had a SimilarityScore lower (lower is better) than that of IPP (isoleucine-proline-proline, a well-known ACE inhibitory peptide from sour milk), was used for further investigation. SimilarityScore reflects a reward or penalty from similarity of structural and chemical features (steric, hydrogen donor, hydrogen acceptor, ring….) of ligands and the template's. Therefore, the score can, in part, reflect the drug-like properties of studied tripeptides.

5.2.4.2. Docking (structure-based)

To prepare for docking, we saved the protein structure downloaded from the Protein Data Bank in .pdb format. We chose ACE-lisinopril complex (PDB code 1O86) for the

extraction of the protein structure used in the docking experiments, and we imported the extracted protein file into the docking program MVD 6.0 with the default setting of the software. Except for cofactor GLY_2000, all components of the complex (include crystalized water) subjected to the docking experiment were imported to the workspace of the MVD program without any structural modification. The docking space was restricted within a 15-Å radius sphere centered at the observed binding site of lisinopril (as mentioned above).

The docking was run with the MolDock SE docking algorithm [180] incorporated in the MVD program. We set the maximum iteration at 1500, the population size as 50, and the energy threshold at 100. For every structure, 50 runs were conducted, and five docking poses with the lowest MolDock score were retained. We then filtered the poses by RerankScore and used only the one pose with the lowest RerankScore lower than that of IPP for further investigation. The more computationally sophisticated RerankScore combines additional energy terms (such as steric self-interaction energy of the pose, protein steric interaction energy…), which are not used by the MolDock Score, generally producing better estimation of pose and interaction strength.

5.2.5. ADME properties evaluation

For our evaluation of some ADME properties, we imported SMILES strings of potential candidates to ACD/Percepta (Advanced Chemistry Development, Inc. Ontario, Canada). Lipinski's rule-of-five violations were also evaluated. Only structures with "moderate" or "good" agreement with Lipinski's rules and no significant first-pass metabolism (evaluated by Percepta) were selected and subjected to ACE inhibitor assays.

5.2.6. Angiotensin-converting enzyme inhibition assay

We performed inhibition activity assays using ACE kits in strict accordance with the manufacturer's instructions, using a 96-well plate. Principles of the method were

described elsewhere by Lam et al. [181]. We calculated the inhibition rates based on the comparison of the optical absorbance of sample-treated wells (A_s), control wells (A_c) and blank wells (A_b) as shown in Equation (1) below. Absorbance was measured at 450 nm using a microplate reader ELX800 (BioTek, Winooski, VT, USA).

$$\text{Inhibition rate (\%)} = [(A_c - A_s)/(A_c - A_b)] \times 100 \qquad (5\text{-}1)$$

To determine the IC_{50} values (the half maximal (50%) inhibitory concentration), we assayed five concentrations of each peptide in triplicate against ACE and plotted dose-response curves for calculation of the values.

5.2.7. Stereochemical effect evaluation

To evaluate the stereochemical effect of peptides on the inhibitory activity, we selected the four most active peptides *in vitro* with IC_{50} values <20 μM (i.e., WQP, WTP, IYP and WMP) and subjected them to the structural change. Only the middle residue of the tripeptides was kept original; the N-terminus and C-terminus were replaced by the D-form of the corresponding amino acids. We then evaluated the modified peptides for ACE inhibition activity and subjected them to docking studies to clarify the effects of the configurational changes.

5.2.8. Statistical analysis

The *in vitro* experiments were performed in triplicate, and the results are expressed as the mean±SD. The correlation coefficient between docking score and IC_{50} values was determined by a least-squares linear regression analysis using Microsoft Excel 2010.

5.3. Results and Discussion

5.3.1. Structural comparison

Dozens of ACE inhibitors are currently used for hypertension medication, but we chose only four compounds for a comparison of their structural similarity and to identify the most common parts that play a role in ACE inhibitor activity. We did this for three

reasons. First, the X-ray data of these compounds (in complex with ACE or an ACE homologue; 1UZE - enalaprilat, 1O86 - lisinopril, 2X93 - trandolaprilat and 2X94 - perindoprilat) have been deposited in the Brookhaven Protein Data Bank and could be accessed freely when needed. Second, these compounds have molecular weights similar to those of tripeptides, and their structures can be divided into parts corresponding to the three parts of tripeptides, the main target of this study. Third, these four compounds contain three structural changes that can be used to demonstrate the role of each part of tripeptides.

In Figure 1, the common parts of all compounds are covered by a dashed line; all four compounds contain the proline moiety connecting to an alanine-like chain by a peptide bond. Changing these compounds' structures without touching the common parts retains the inhibitory activity, although small changes in IC_{50} values were reported [182]. The similarity in molecular weight and structure between proline-C-terminal tripeptides and these antihypertensive substances suggest the existence of peptides with high ACE inhibitor potency

5.3.2. Virtual Screening

Virtual screening is being used more and more in drug design as a complement to industrial HTS. Virtual screening also provides academic institutions lacking HTS capacities applicable methods of hit identification [183]. Normally, either ligand-based or structure-based screening is chosen for a certain target bioactivity. Tan et al. [184] proposed that the integration of both techniques could give complementary and improved results for screening processes. We used this integrated approach in the present study. After docking by ligand-based and structure-based techniques (all 529 peptide structures were screened), we kept the structures with both a SimilarityScore

and a RerankScore lower than those of IPP for further investigation. A total of 387 structures (see Appendices Table A5.1) had scores lower than those of IPP.

The results are shown in Table 1; there are five groups in which all members (23 members in each group) have both a SimilarityScore and a RerankScore lower than those of IPP and another six groups that contain more than 20 members with these lower scores (see Appendices Table A5.1). Among these 11 groups, there are five groups whose members contain an amino acid with an electrically charged side chain at the penultimate position (i.e., Xaa-Arg-Pro, Xaa-His-Pro, Xaa-Lys-Pro, Xaa-Glu-Pro and Xaa-Asp-Pro), three groups that have phosphorylated amino acids (pTyr, pSer and pThr) at this position, and three groups in which an aromatic ring-containing amino acid (Xaa-Phe-Pro, Xaa-Trp-Pro and Xaa-Tyr-Pro) occupies this position.

In their study of 308 food-derived small peptides, Wu et al. [185] proposed that for ACE inhibitor tripeptides, the most frequent residues for the C-terminus were ring-containing amino acids, whereas amino acids with a positively charged side chain were common in the middle position, and hydrophobic amino acids were common for the N-terminus. In the present study, groups of tripeptides with electrically charged (both positive and negative) amino acids showed low docking scores for all members, which seems to be in agreement with the proposal by Wu et al. Our finding that phosphorylated amino acids at the penultimate position of peptides gave a low docking score (see Appendices Table A5.1) is also consistent with a previous report about docking of the peptides [44].

5.3.3. ADME properties evaluation and ACE inhibition

To be more focused on the most potent candidates, we then ranked the structures obtained from the virtual screening step again by RerankScore, and 100 structures with lowest RerankScore were extracted (see Appendices Table A5.2) and subjected to

Percepta for an ADME properties evaluation. Only structures evaluated as in "moderate" and "good" agreement with Lipinski's rule-of-five [186] and no significant first-pass metabolism were retained and subjected to ACE inhibiton assays. Of the 100 selected peptides, only 16 structures satisfied these criteria and were used for *in vitro* experiments. These peptides were synthesized and tested for ACE inhibitor activity. The results are shown in Table 5.2.

Due to the differences in assay conditions used for evaluation of inhibitor activity, it is difficult to compare results between studies; however, assuming that IC_{50} values reflect the inhibitory strength, we can somewhat use the values for comparing the inhibitory capacity of samples across different studies. In this context, compared with many previous reports on ACE inhibitor peptides, these 16 peptides showed quite high inhibitory activity with IC_{50} values ranging from 5.6 to 274.4 μM. The highest inhibition was recorded for Trp-Gln-Pro and the lowest was for Tyr-Leu-Pro. We could not find any published work concerning ACE inhibitor activity on 14 of the peptides, with WHP and IYP being reported elsewhere [187], [188]. Surprisingly, tripeptides with phosphorylated amino acids at the penultimate position were reported with low docking scores in previous step, but none were contained in the final 16 candidates. This may be because peptides containing phosphorylated amino acids have too many oxygen atoms in their structures and consequently, they violated Lipinski's rule-of-five and did not meet the criteria used for oral drugs. In addition to their high inhibition activity, all 16 peptides were predicted to be stable compounds with oral bioavailability ranging from 1.66% to 72% (or 0.83–36.0 mg after a virtual dose of 50 mg). These are quite high values compared to those of a report describing that nearly all orally active peptides have oral bioavailability lower than 5% [189]. Of course, these are the predicted values

and the experimental results may be lower than predicted data, but the values still show the peptides to be potential candidates as viable ACE inhibitors.

From the results presented in Table 5.2, it can also be inferred that the correlation between RerankScores (average scores from two tautomers of each peptide) and IC_{50} values was approximately 35% (correlation coefficient R value = 0.35). This is an acceptable value in the drug discovery field, where HTS is still the main “player” but the hit rate is only 0.01% to 0.1% [190].

5.3.4. Stereochemical effects

As is the case for other protein enzymes composed of chiral amino acids residues, it is logical that ACE inhibition depends on the steric configuration of its inhibitor [191]. In the present study we investigated the effect of the form of amino acids on the inhibition activity of inhibitory peptides by changing the amino- and carboxyl-termini residues from L-form to D-form. The modified peptides were also subjected to an *in vitro* ACE inhibition assay and docking experiments.

As shown in Table 3, the change to D-form amino acids at both the amino and carboxyl termini resulted in a decrease of inhibition activities. The change at the N-terminus led to less of a decrease in activity compared to the change at the C-terminus. Changing the N-terminal residues to the D-form resulted in a decrease in inhibition activities by approx. 20–50 fold, whereas changing the proline residue of the C-terminus to the D-form resulted in a decrease of inhibition activities by approx. 70–200 fold. These results not only confirmed the critical role of proline at the C-terminus in ACE inhibitory peptides but also emphasized the significance of the L-form amino acid at this position.

Our docking studies of tripeptides with the D-form at the C-terminus also showed an increase in the RerankScore or a decrease in the binding strength (see Appendices Table A5.3). Analysis of the docking poses using HBPLUS [192] revealed that a

configurational change at this position resulted in a change of the peptide-ACE interaction, especially in the interactions of the carboxyl group. In the L-form, both of the oxygen atoms of proline's carboxyl group interact with residues of the protein, usually Lys511, Tyr520, His513 and His353; however, in the D-form, only one of these atoms had an interaction with the residues, and the number of interactions at this position was also decreased. Peptides with L-proline at the C-terminus also showed the same spatial distribution as that of lisinopril's L-proline moiety (see Appendices Figure 5.1; the image was rebuilt from PDB 1O86). Figure 5.2 provides an example of tripeptides (with an L-form and D-form proline at the C-terminus) positioning inside the active site of ACE. Images of other peptides inside the active site are provided in Appendices Figure A5.2.

Surprisingly, the total number of interactions in the tripeptides with a D-form amino acid at the C-terminus was more than that of the tripeptides with an L-form (see Appendices Tables A5.4 and A5.5), but the inhibition activity was much lower. This result lets us assume that the number of interactions is less important than the position of the interaction and also helps us to reconfirm the important role of configuration in the inhibitory activity of ACE inhibitor peptides.

5.4. Conclusion

In this study, we used three-step virtual screening as a guiding tool for wet chemistry in designing angiotensin-converting enzyme inhibitors. Sixteen peptides with "acceptable" Lipinski's rule-of-five violations were obtained, 14 of which have not been reported elsewhere, and all of them showed quite high inhibition activity against ACE compared with previously reported inhibitory peptides. Predicted to have no significant first-pass metabolism and high bioavailability, the peptides should be recognized as potent ACE inhibitors and investigated further *in vivo*. Our investigation of configurational changes

also showed that the L-form of proline is essential for the inhibitory activity of tripeptides that ended with this amino acid. One question remains to be answered: why does only one small difference at the position next to P_1 (–CO–NH– in tripeptides and –(COOH)C–NH– in dicarboxylate-containing drugs) result in a major difference (approx. thousands of times) of inhibition strength? The detailed reason for this difference should be identified to determine the structure-activity relationship of potent ACE inhibitors.

5.5. Chapter's summary and limitation

Currently, peptides and peptidomimetics are the main focus in attempts to identify inhibitors of angiotensin-converting enzyme (ACE), the dipeptidyl carboxypeptidase that causes blood vessels to constrict and blood pressure to increase. This study was conducted to identify the most potent ACE-inhibitory tripeptides with a proline C-terminus, using a novel three-step (tautomerization-docking-ADME simulation) virtual screening process and *in vitro* assays. Sixteen candidates were identified, and their IC_{50} values ranged from 5.6 to 274.4 μM. ACE inhibition activity for 14 of the 16 tripeptides was reported for the first time. We also found that changing from the L-form to the D-form of the amino acid at the amino and carboxyl termini resulted in a decrease of inhibition, but a greater decrease was observed for C-terminal changes. With low IC_{50} values and high predicted bioavailability, the peptides identified by our protocol are comparable in terms of ACE-inhibition to those derived from costly and time-consuming wet screening. Our *in vitro* and docking results showed that the configuration of the C-terminus is a critical parameter contributing to the inhibitory activity of tripeptides with proline at this position. These findings will contribute to the use of simulation tools for rational drug design, especially for ACE inhibitors.

In silico docking is more and more popular in drug discovery. However, lack of "perfect algorithm", which can calculate all parameters affecting to ligand-receptor interaction,

decrease the reliability of this method. In this study, Tyr-Lys-Pro used for ligand-based docking showed high similarity to the structure of lisinopril but inhibition activity is nearly 40 thousand times lower than that of the drug. Beside that, all sixteen peptides passed the test of drug-like properties had better RerankScore and SimilarityScore than those of IPP but still showed weaker activity than the peptide's. Low correlation with *in vitro* experimental result is major barrier of computer-based methods. Considering factors which may affect ligand-receptor interaction and improving experimental procedures to obtain better *in silico – in vitro* correlation should be targeted for future study of computer-based drug design.

Enalaprilat

Trandolaprilat

Lisinopril

Perindoprilat

Figure 5.1. Structure of selected antihypertensive drug and their common part.

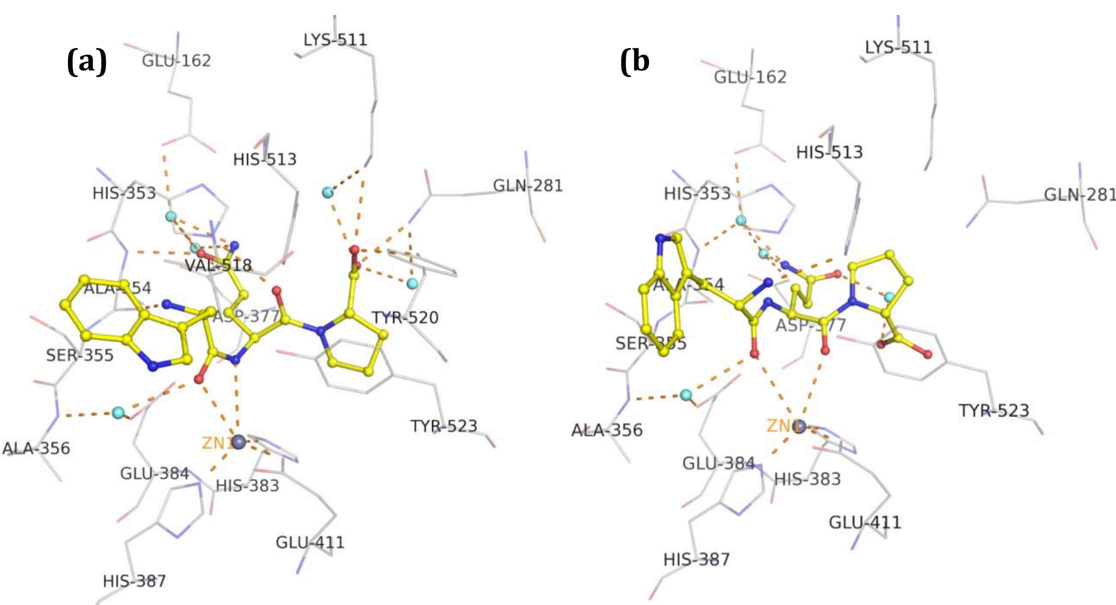


Figure 5.2. Close view of interaction between tripeptides WQP with L-proline **(a)** and D-proline **(b)** at C-terminus and ACE; yellow skeletons are tripeptides, cyan spheres are water molecules, grey spheres are zinc ion; PyMOL 1.3 (The PyMOL Molecular Graphics System, Schrödinger, LLC) was used for creating the image while HBPLUS [192] was used to calculate the interaction.

Table 5.1. Number of structures with both SimilarityScore and RerankScore lower than those of IPP.

No.	Tripeptide groups[a]	Common Structures[b]	No.	Tripeptide groups	Common Structures*
1	Xaa-Ala-Pro	5	13	Xaa-Met-Pro	17
2	Xaa-Arg-Pro	21	14	Xaa-Phe-Pro	21
3	Xaa-Asn-Pro	14	15	Xaa-Pro-Pro	12
4	Xaa-Asp-Pro	20	16	Xaa-pSer-Pro	22
5	Xaa-Cys-Pro	6	17	Xaa-pThr-Pro	21
6	Xaa-Gln-Pro	17	18	Xaa-pTyr-Pro	23
7	Xaa-Glu-Pro	23	19	Xaa-Ser-Pro	9
8	Xaa-Gly-Pro	9	20	Xaa-Thr-Pro	15
9	Xaa-His-Pro	23	21	Xaa-Trp-Pro	22
10	Xaa-Ile-Pro	9	22	Xaa-Tyr-Pro	23
11	Xaa-Leu-Pro	18	23	Xaa-Val-Pro	14
12	Xaa-Lys-Pro	23		**Total**	**387**

[a] *Xaa is any amino acid among 23 investigated ones*
[b] *Structures with both SimilarityScore and RerankScore lower than those of IPP.*

Table 5.2. ADME properties (calculated by Percepta) and ACE inhibition of selected peptides

No.	Peptides	RerankScore	IC_{50} (μM)	LogP	Lipinski's[a]	OralBio[b]
1	**Trp-Met-Pro**	**-166.92**	**16.6±0.1**	**Optimal**	**Good**	**17.5551**
2	Trp-Tyr-Pro	-160.04	39.4±0.2	Optimal	Moderate	8.1285
3	Trp-Val-Pro	-157.15	30.3±1.9	Optimal	Good	15.6372
4	**Trp-Gln-Pro**	**-155.88**	**5.6±0.1**	**Hydrophilic**	**Moderate**	**0.8309**
5	Tyr-Lys-Pro	-154.97	178.3±3.8	Hydrophilic	Moderate	2.2081
6	Trp-His-Pro	-154.86	71.1±0.6	Hydrophilic	Moderate	2.5754
7	Trp-Lys-Pro	-153.19	55.3±0.9	Optimal	Moderate	1.8899
8	Trp-Phe-Pro	-152.45	95.7±2.5	Optimal	Good	36.0245
9	**Trp-Thr-Pro**	**-151.90**	**12.8±0.5**	**Hydrophilic**	**Moderate**	**2.3846**
10	Tyr-Met-Pro	-151.63	142.0±5.0	Hydrophilic	Good	6.9838
11	Tyr-Leu-Pro	-151.53	274.4±8.2	Hydrophilic	Good	9.186
12	Tyr-Tyr-Pro	-149.95	231.7±13.9	Hydrophilic	Moderate	3.947
13	**Ile-Tyr-Pro**	**-148.98**	**15.8±0.4**	**Hydrophilic**	**Good**	**11.0819**
14	Tyr-Trp-Pro	-148.16	83.3±1.6	Optimal	Moderate	10.6072
15	Phe-Met-Pro	-147.97	176.0±2.6	Hydrophilic	Good	28.9321
16	Trp-Trp-Pro	-147.15	44.0±0.4	Optimal	Moderate	13.5831
17	**Ile-Pro-Pro**	-123.93	5.0[c]	Hydrophilic	Good	25.1891

[a] *In agreement with the Lipinski's rule-of-five.*
[b] *Oral bioavailability - remain amount after dosing 50 mg.*
[c] *from reference* [36].

Table 5.3. ACE inhibition activity of modified peptides

No.	**Tripeptides**	**IC_{50} values (μM)**
1	Trp-Gln-Pro	5.6±0.1
2	Trp-Thr-Pro	12.8±0.5
3	Ile-Tyr-Pro	15.8±0.4
4	Trp-Met-Pro	16.6±0.1
5	D-Trp-Gln-Pro	91.5±1.8
6	D-Trp-Thr-Pro	169.9 ±1.2
7	D-Ile-Tyr-Pro	723.0±37.9
8	D-Trp-Met-Pro	110.0±6.3
9	Trp-Gln-D-Pro	*>1165*
10	Trp-Thr-D-Pro	*>1243*
11	Ile-Tyr-D-Pro	*>1278*
12	Trp-Met-D-Pro	*>1157*

Chapter 6

Conclusion and Future Perspectives

6.1. Conclusion

Angiotensin-converting enzyme was discovered in mid-1950s but searching for this enzyme's inhibitor could not be progressed until late 1960s after two significant discoveries. The first event was clarification of carboxypeptidase A's structure, a homologue of ACE was clarified, and the second one was the identification of teprotide, a venom-derived ACE inhibitor. By structure–activity investigation using synthetic venom peptide analogs Cushman and Ondetti successfully developed proved "the first truly useful antihypertensive drug" [33] which then commercialized as Captopril. This discovery followed by creation of dozen of other ACE competitive inhibitors which many of them are peptidomimetic compounds. Like many other drug discovery process, invention of ACE inhibitors is a combination of synthetic chemistry and natural product sciences. In this study, a bilateral approach, which both use nature-derived products and synthetic compounds for discovering potential ACE inhibitors, was executed.

In chapter 2, by investigation methanol and water extracts of 29 wild-type mushrooms I found several potent ACE inhibitor-containing samples. Among them, *Phlebia tremellosa* showed highest inhibition against the enzyme with inhibition rate of crude water extract was as low as 2 μg/mL; the activity of this extract even higher than that of well-known medicinal mushroom *Ganoderma lingzhi* whose inhibition rate was 32 μg/mL. We also found that there was an appearance correlation between mushroom genus and biological activities that might cause by compounds with similar chemical structures provided by fungal species belonging to the same genus. With around 140,000 species are existing on earth, including both known and unknown [50], it is

likely that mushroom is a good source to seek for ACE inhibitors as well as other antihypertensive agents.

In chapter 3 and chapter 4, there were 11 peptides and 32 triterpenoids isolated from *Ganoderma lingzhi* were investigated for ACE inhibitory activity. Traditionally, *G. lingzhi* is used for antihypertensive therapies but action mechanism is unclear, my research partly explained the action behind this activity the activity. In this part, some structural features affect to inhibitory activity against the enzyme was also studied. Information on structure-activity relationship helps us to understand more about structural characteristics for potential inhibitors. It was found that treatment method affecting to inhibition activity of *G. lingzhi* extract and that peptides might be the main ACE-inhibitory compounds found in the extract. It was also proven that lanostane-type triterpenoids (mostly with molecular weight higher than 500 Da) inhibit ACE via non-competitive and/or uncompetitive modes.

In combination with previous studies, in chapter 5, we described a new approach for designing ACE inhibitory tripeptides with drug-like properties. By using this combined *in silico - in vitro* method 16 inhibitors were found. Among these peptides, 14 were reported for the first time. It was found that *L*-form amino, especially at position of *C*-terminus acid is essential for ACE-inhibitory tripeptides,. Functional group at P1 position play important role for enhancing the activity; a small change from –C=O group to –COOH group can results in thousands times difference in inhibition.

6.2. Future perspectives

Rational design for angiotensin-converting enzyme inhibitors has lasted for nearly 60 years and achievement in this field is enormous. However, the finding that ACE has two domains with somewhat difference in biological function surely re-directs future studies to domain-specific inhibitors. Such studies are now facilitated by availability of ACE

structure of both *N*- and *C*-domains and the development of computer programs. New types of inhibitors may help to improve present anti-ACE medication efficacy and reduce the drug's side effects that is the main barrier preventing patient's use.

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APPENDICES

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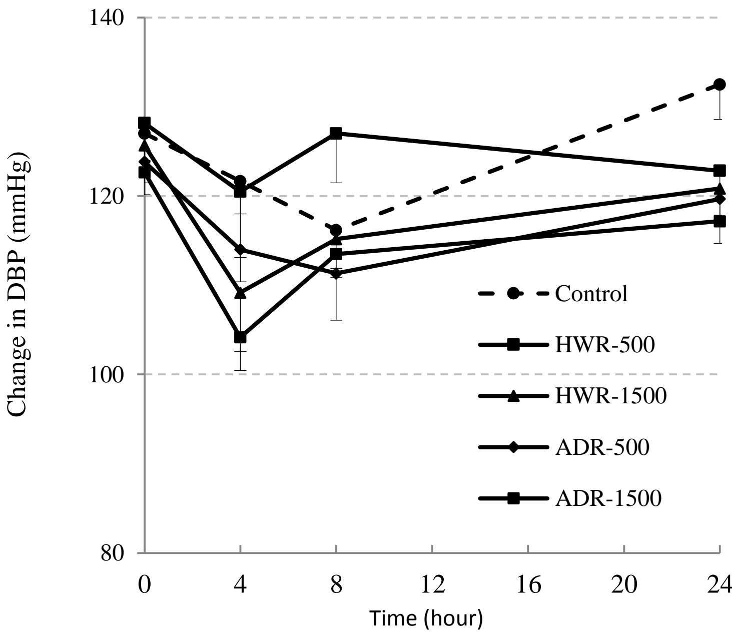

Figure A3.1. Time-course of changes (mean ± SE, n = 6) in DBP of SHRs after administering HWR and ADR extracts. Single oral administration was dosed at 500 and 1500 mg/kg body weight. Ultrapure water was used as the control (20 mL/kg body weight).

Figure A3.2. Ninhydrin staining of peptides and/or amino acid in the ≤3 kDa fraction of ADR extract (Reishi's auto-digestion extract was spotted onto silica-gel plate (TLC Plate Silica Gel 60 F254, Merck, Darmstadt, Germany); the plate was eluted with *n*-butanol:acetic acid:water 3:1:1 (by volume) mixture followed by ninhydrin stain (Wako Pure Chemical, Osaka, Japan)

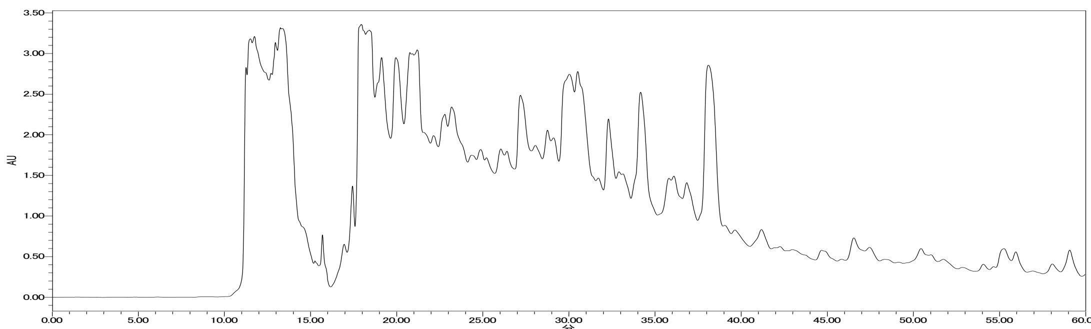

Figure A3.3. Preparative HPLC chromatogram of the ≤3 kDa fraction. Elution was performed with a linear gradient from 10% to 60% of B for 60 minutes at a flow rate of 5 mL/min.

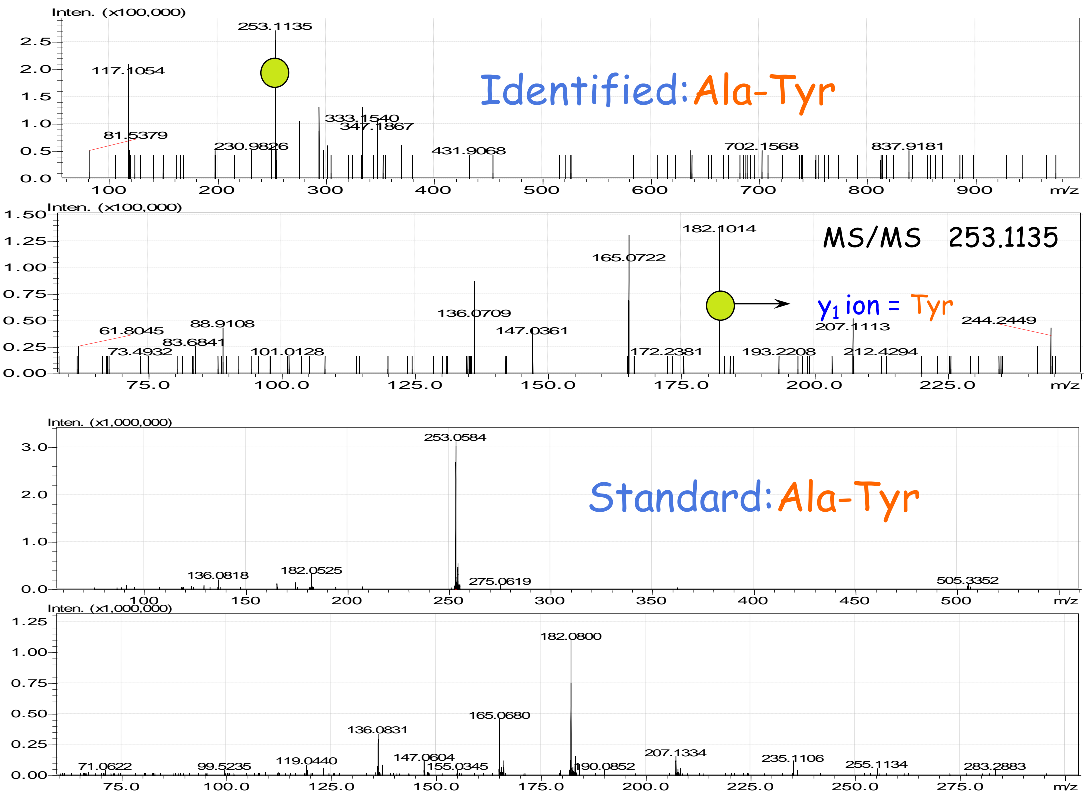

Figure A3.4.1: Mass spectra of sequenced Ala-Tyr and corresponding standards

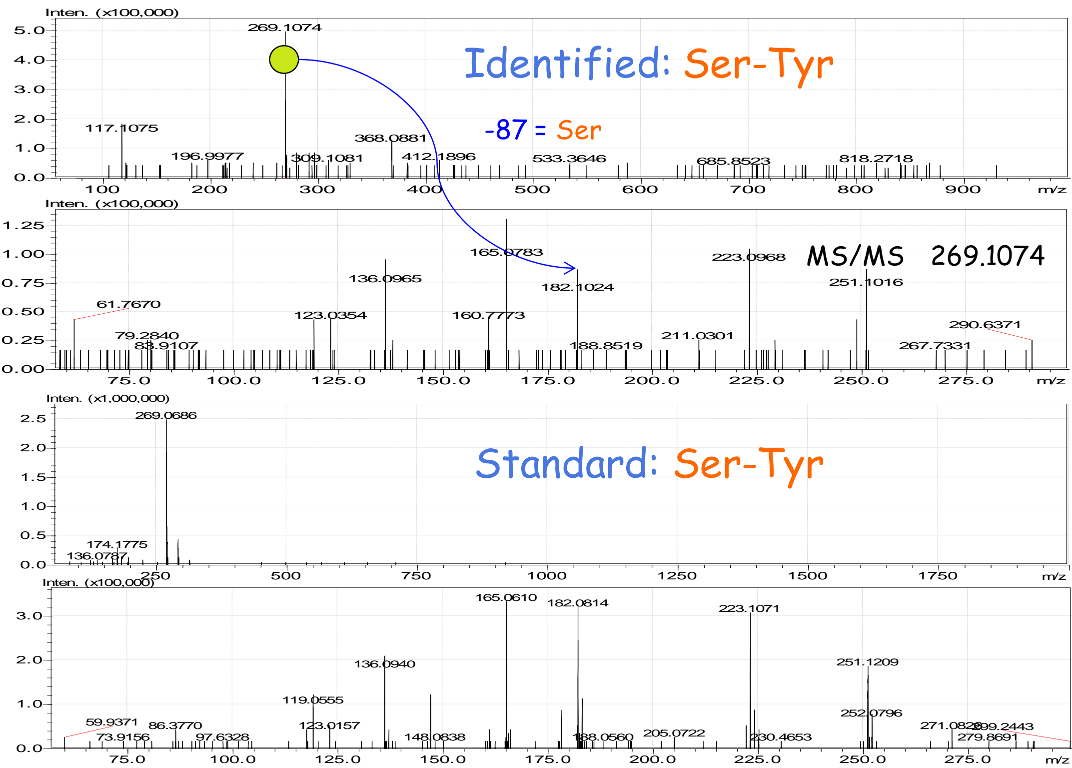

Figure A3.4.2: Mass spectra of sequenced Ser-Tyr and corresponding standards

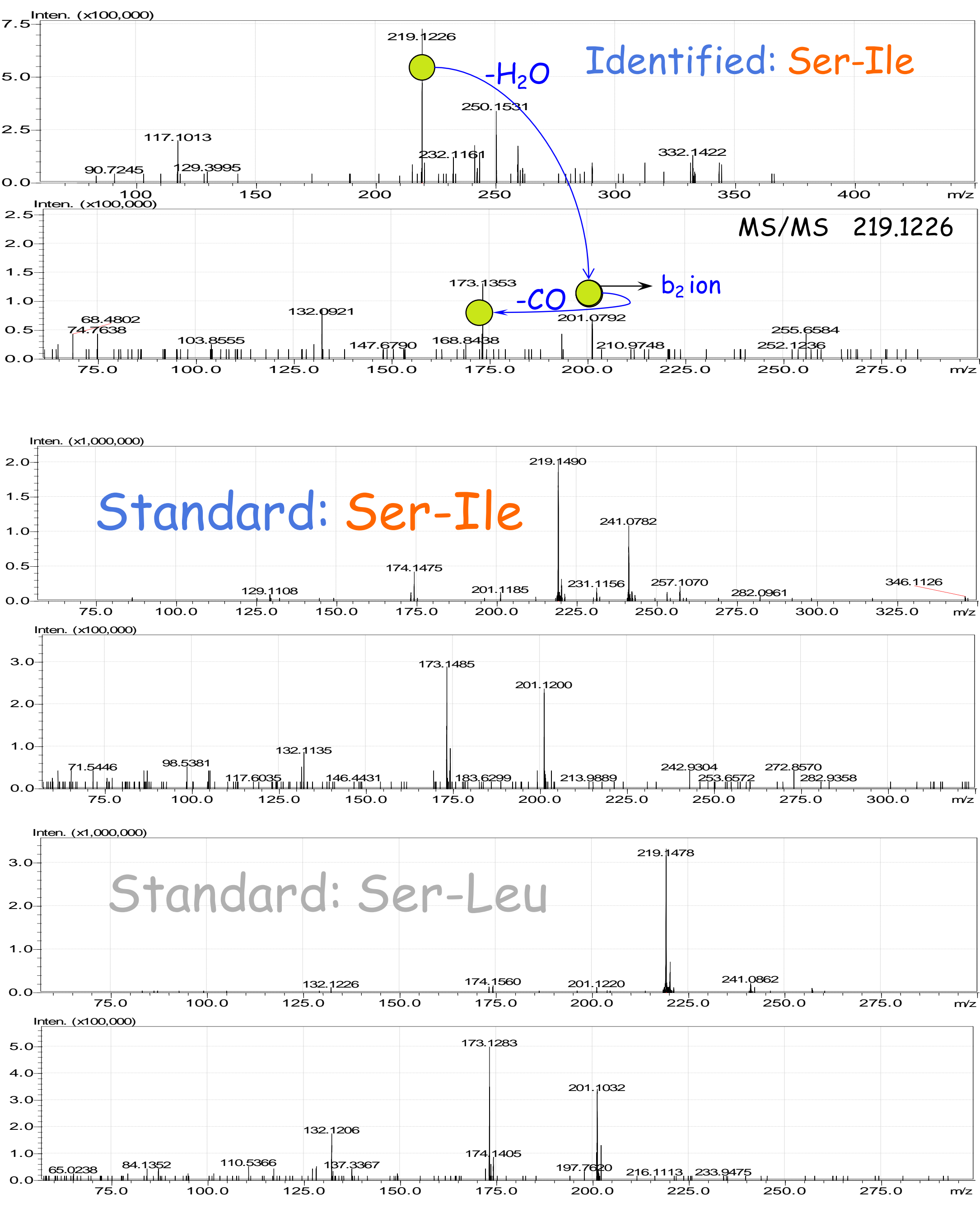

Figure A3.4.3: Mass spectra of identified Ser-Lxx and corresponding standards

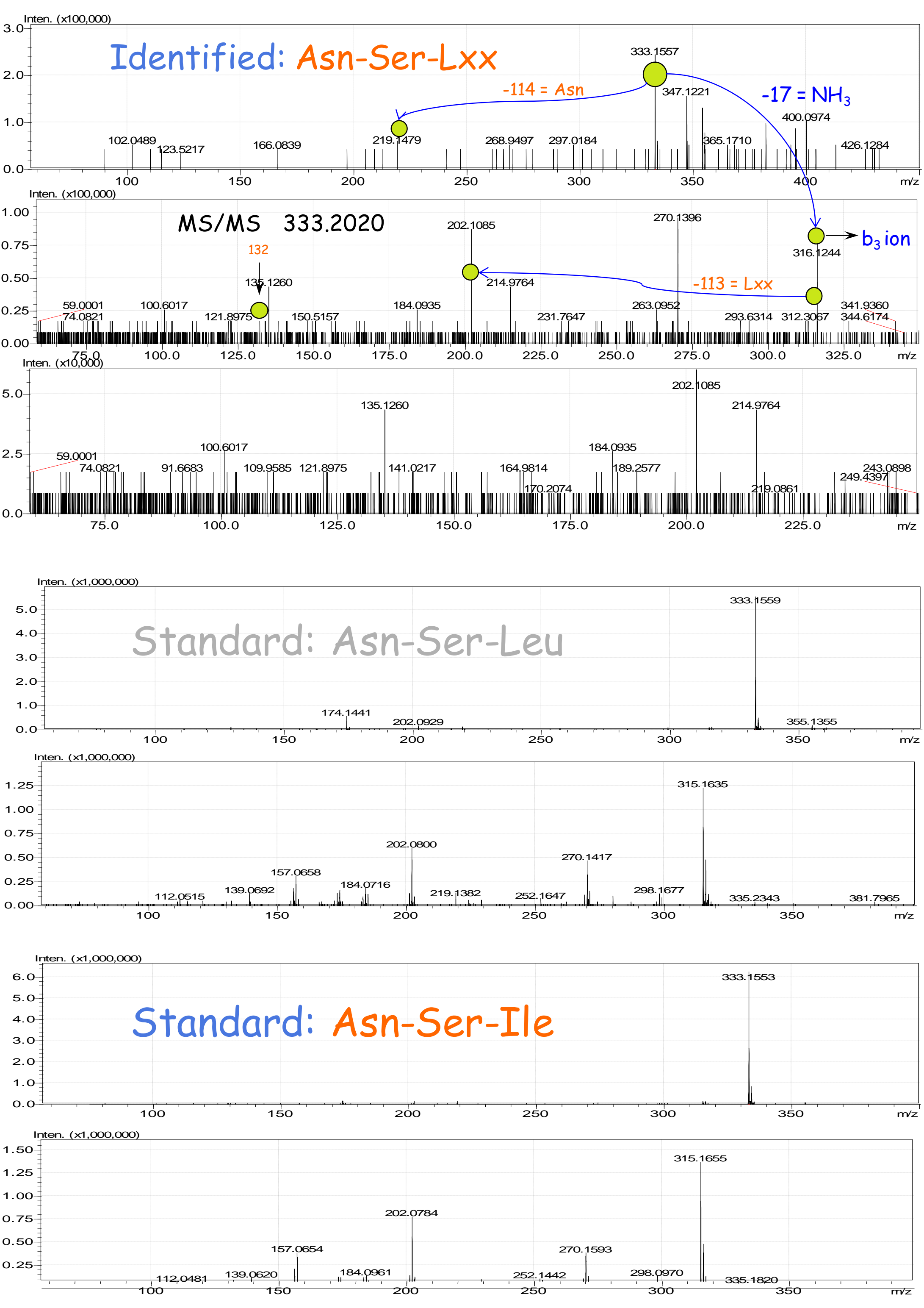

Figure A3.4.4: Mass spectra of identified Asn-Ser-Lxx and corresponding standards

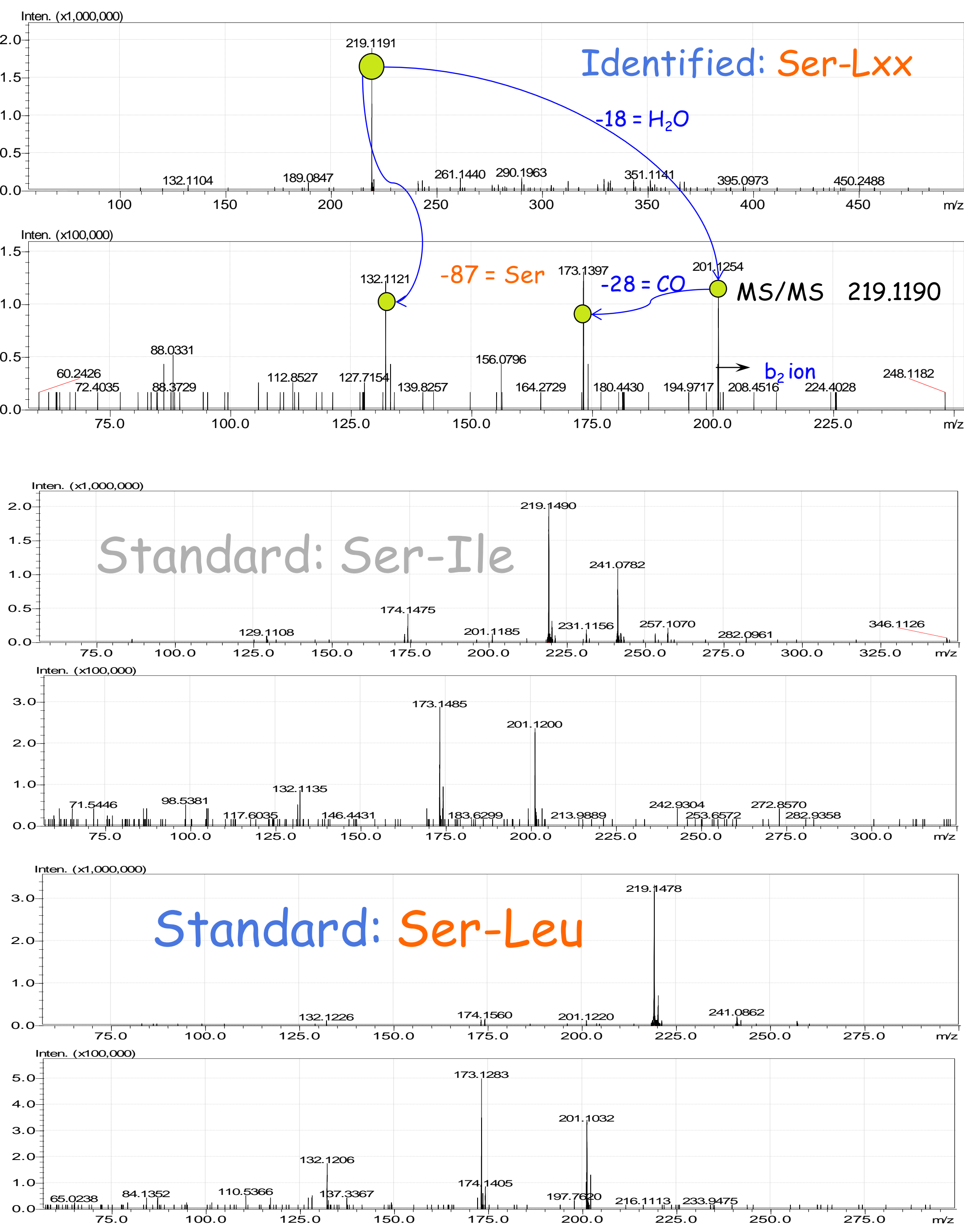

Figure A3.4.5: Mass spectra of identified Ser-Lxx and corresponding standards

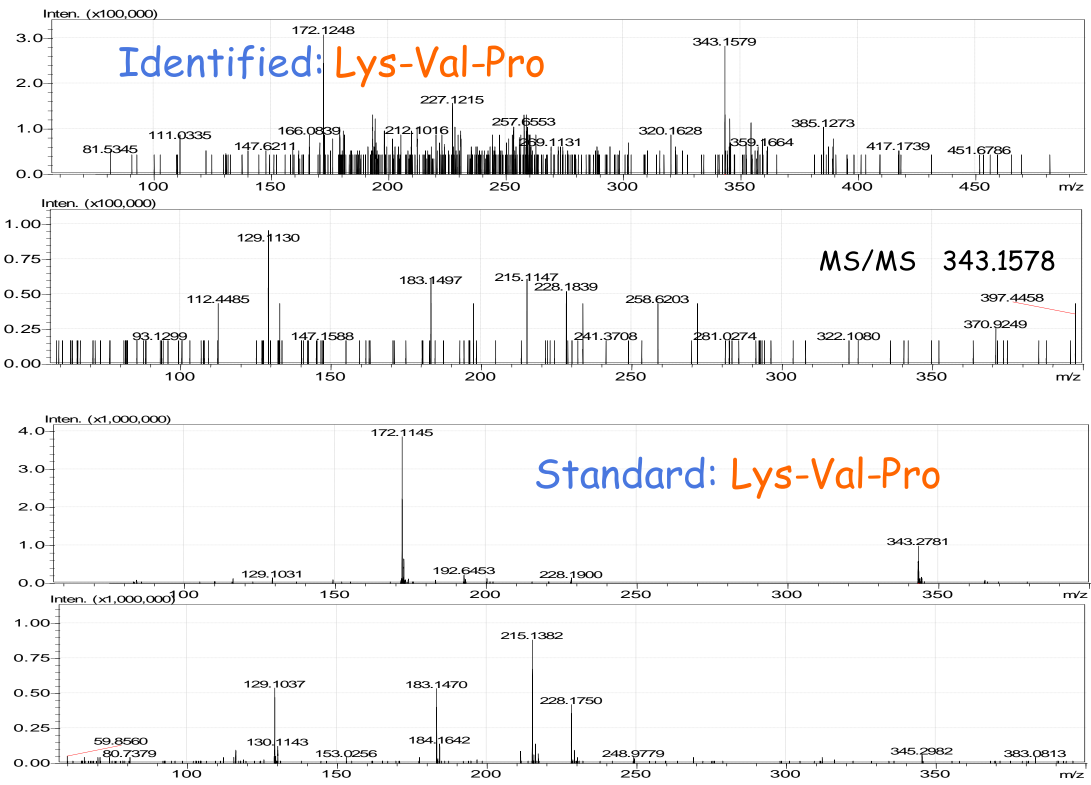

Figure A3.4.6: Mass spectra of identified Lys-Val-Pro and corresponding standards

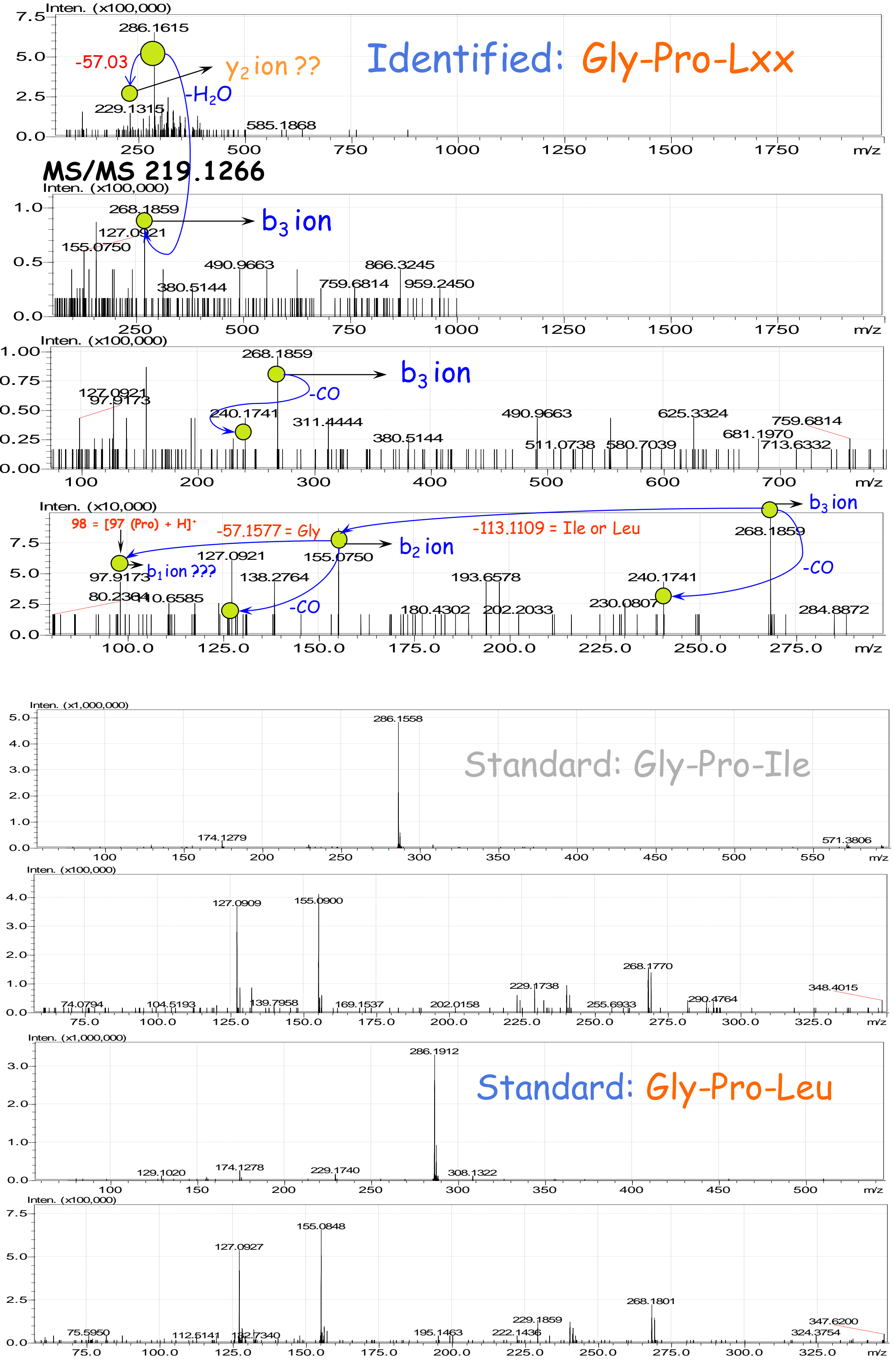

Figure A3.4.7: Mass spectra of identified Gly-Pro-Lxx and corresponding standards

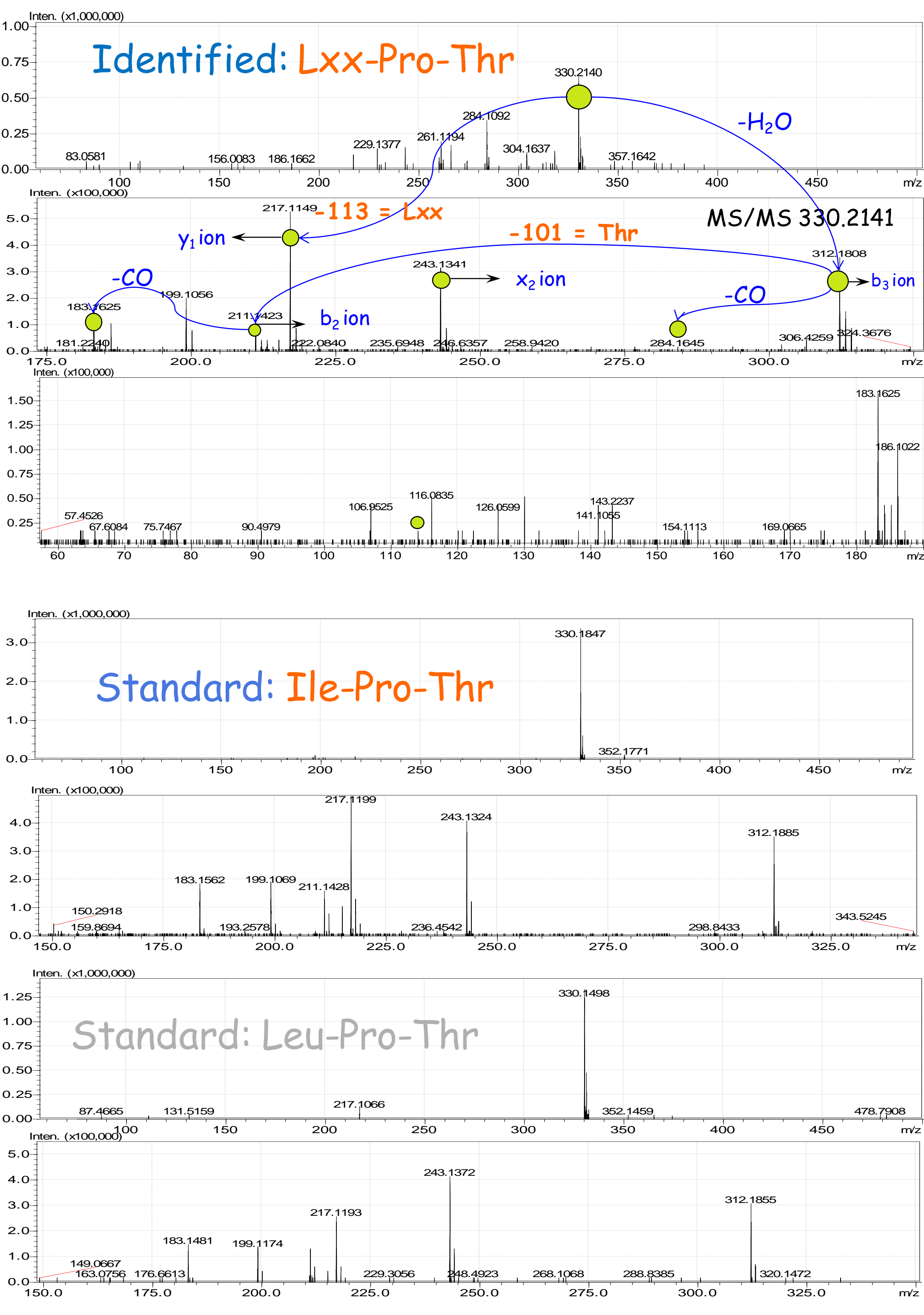

Figure A3.4.8: Mass spectra of identified Lxx-Pro-Thr and corresponding standards

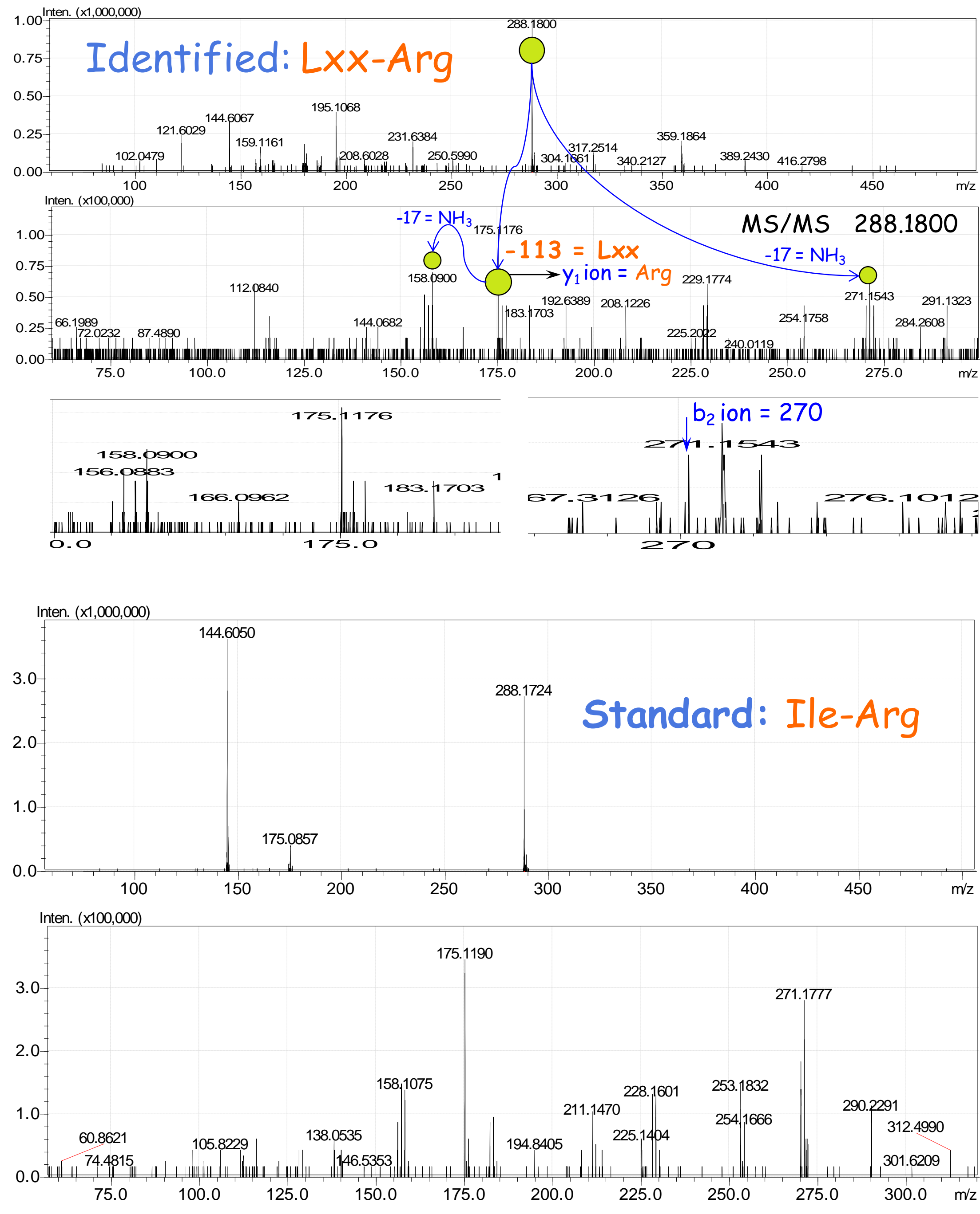

Figure A3.4.9: Mass spectra of identified Lxx-Arg and corresponding standards

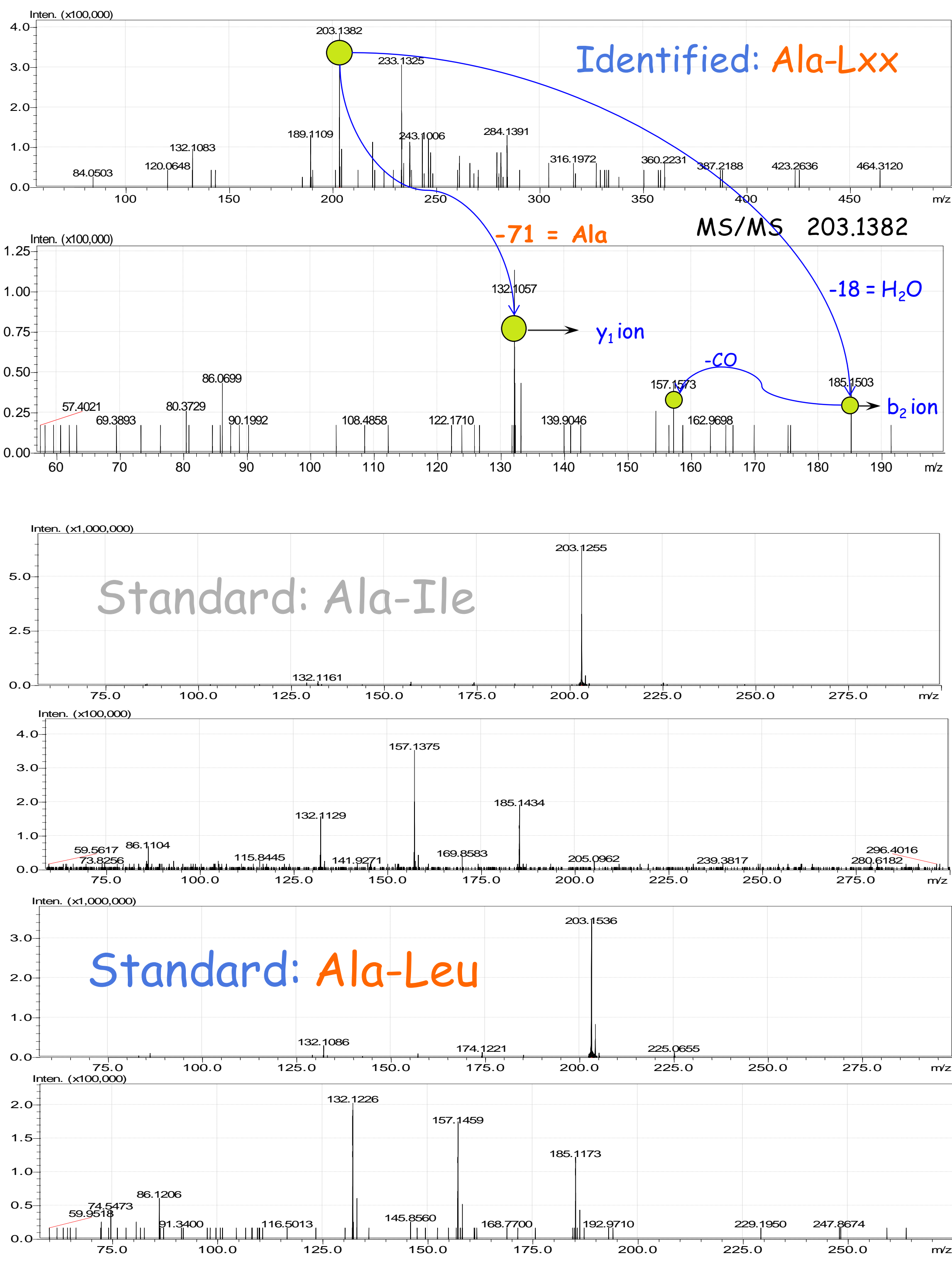

Figure A3.4.10: Mass spectra of identified Ala-Tyr and corresponding standards

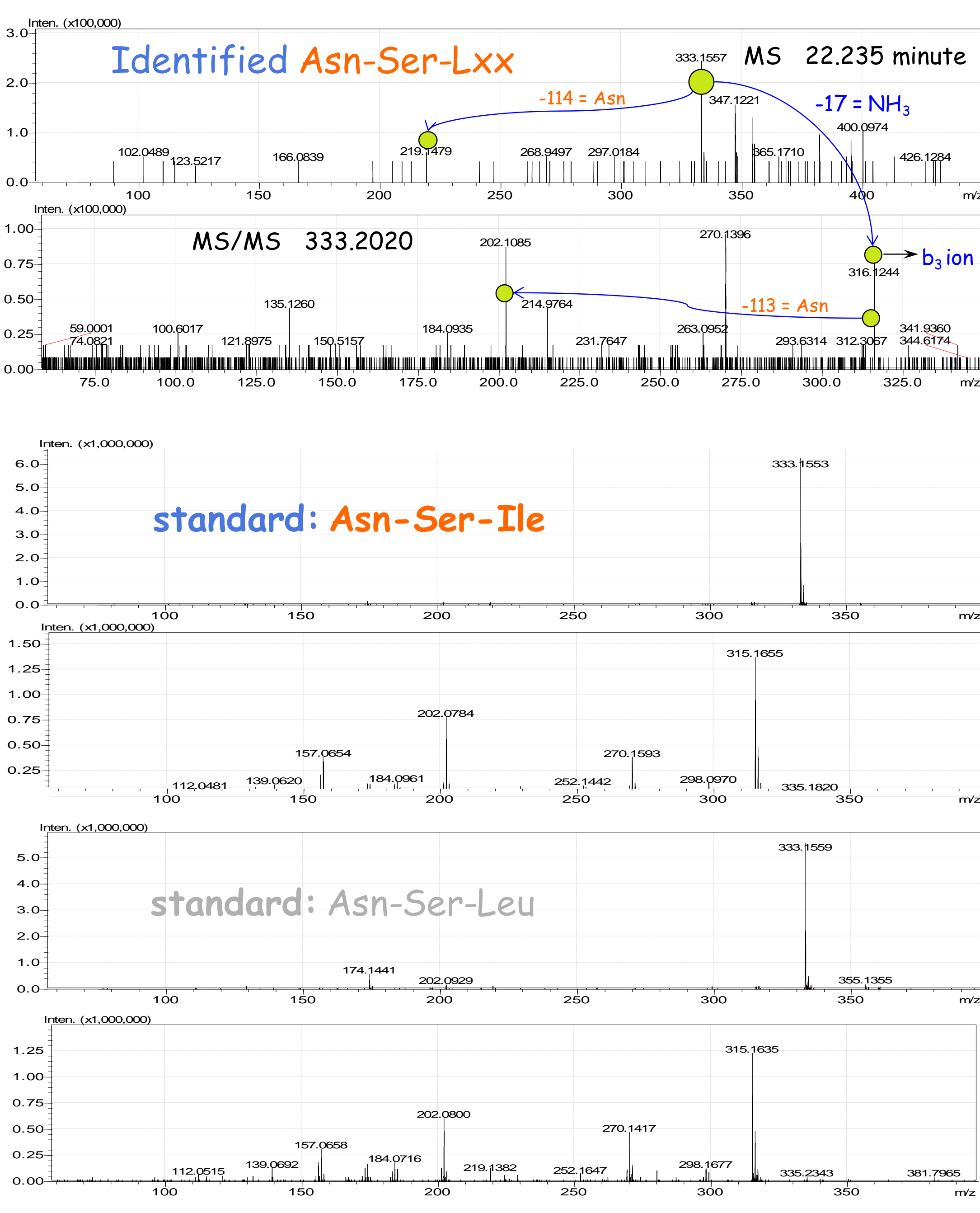

Figure A3.4.11: Mass spectra of identified Asn-Ser-Lxx and corresponding standards

Table A3.1. Eleven fractions and their ACE inhibition rates

No.	**Samples**	**Code**	**Sample final concentration (100 µg/mL)**				
			ACE Inhibition rate (%)			**Mean**	**Std.**
1	Hot water extract	HWR	43.33	42.12	38.04	41.16	0.87
2	Auto-digested Reishi	ADR	66.10	67.79	67.91	67.27	1.01
	time (min)**-amount (mg)**						
1	5–13 **(162.9)**	ADR1	21.04	22.50	22.94	22.16	1.00
2	13–17.5 **(48.7)**	ADR2	70.14	72.05	70.59	70.93	1.00
3	17.5–18.5 **(4.7)**	ADR3	74.29	74.51	75.41	74.74	0.59
4	18.5–22 **(14.4)**	ADR4	76.31	76.98	77.20	76.83	0.47
5	22–29.5 (**29.9**)	ADR5	88.42	89.87	90.66	**89.65**	1.14
6	29.5–31.5 **(6.45)**	ADR6	77.54	78.33	78.89	78.25	0.68
7	31.5–33.5 **(3.21)**	ADR7	83.48	83.93	84.16	83.86	0.34
8	33.5–35 **(1.8)**	ADR8	86.29	86.73	87.18	86.73	0.45
9	35–37.5 **(2.7)**	ADR9	86.51	88.30	87.07	87.29	0.92
10	37.5–39 **(1.2)**	ADR10	86.85	86.73	87.07	86.88	0.17
11	39–60 **(18.8)**	ADR11	80.23	80.90	79.67	80.27	0.62

Table A3.2. Sub-fractions and their ACE inhibition rates

No.	time (min)–amount (mg)	Code	Sample final concentration (100 μg/mL)				
			ACE Inhibition rate (%)			Mean	Std.
1	6–22.5 (3.48)	ADR5-1	85.41	83.11	83.77	84.10	1.18
2	22.5–24 (2.63)	ADR5-2	66.94	67.52	69.40	67.95	1.29
3	24–34.2 (2.48)	ADR5-3	91.16	91.16	92.80	91.71	0.95
4	34.2–35.6 (0.61)	ADR5-4	86.64	86.32	88.70	87.22	1.29
5	35.6–41.5 (4.39)	ADR5-5	80.49	81.64	81.39	81.17	0.61
6	41.5–44.5 (1.67)	ADR5-6	81.55	80.41	79.91	80.62	0.84
7	44.5–65 (5.68)	ADR5-7	88.78	88.45	89.60	88.94	0.59

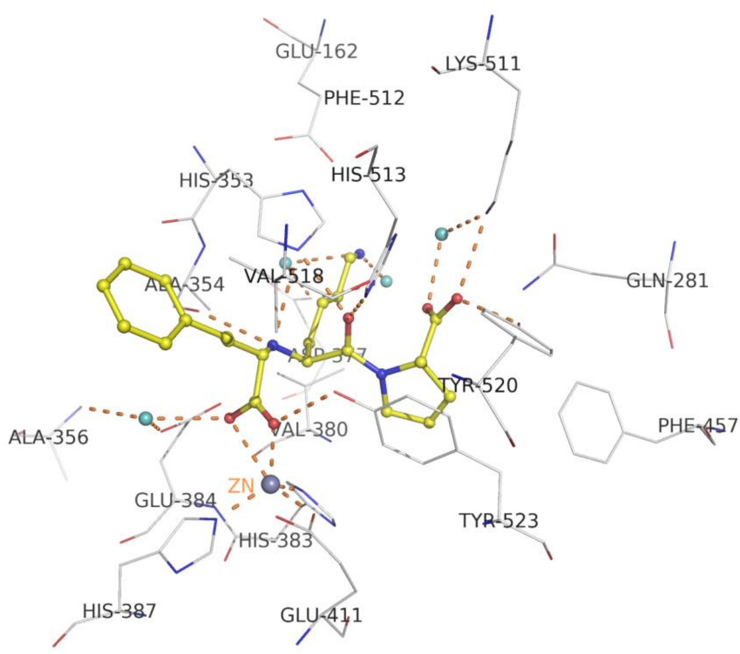

Figure A5.1. Image of lisinopril positioning inside the active of ACE (PDB 1O86)

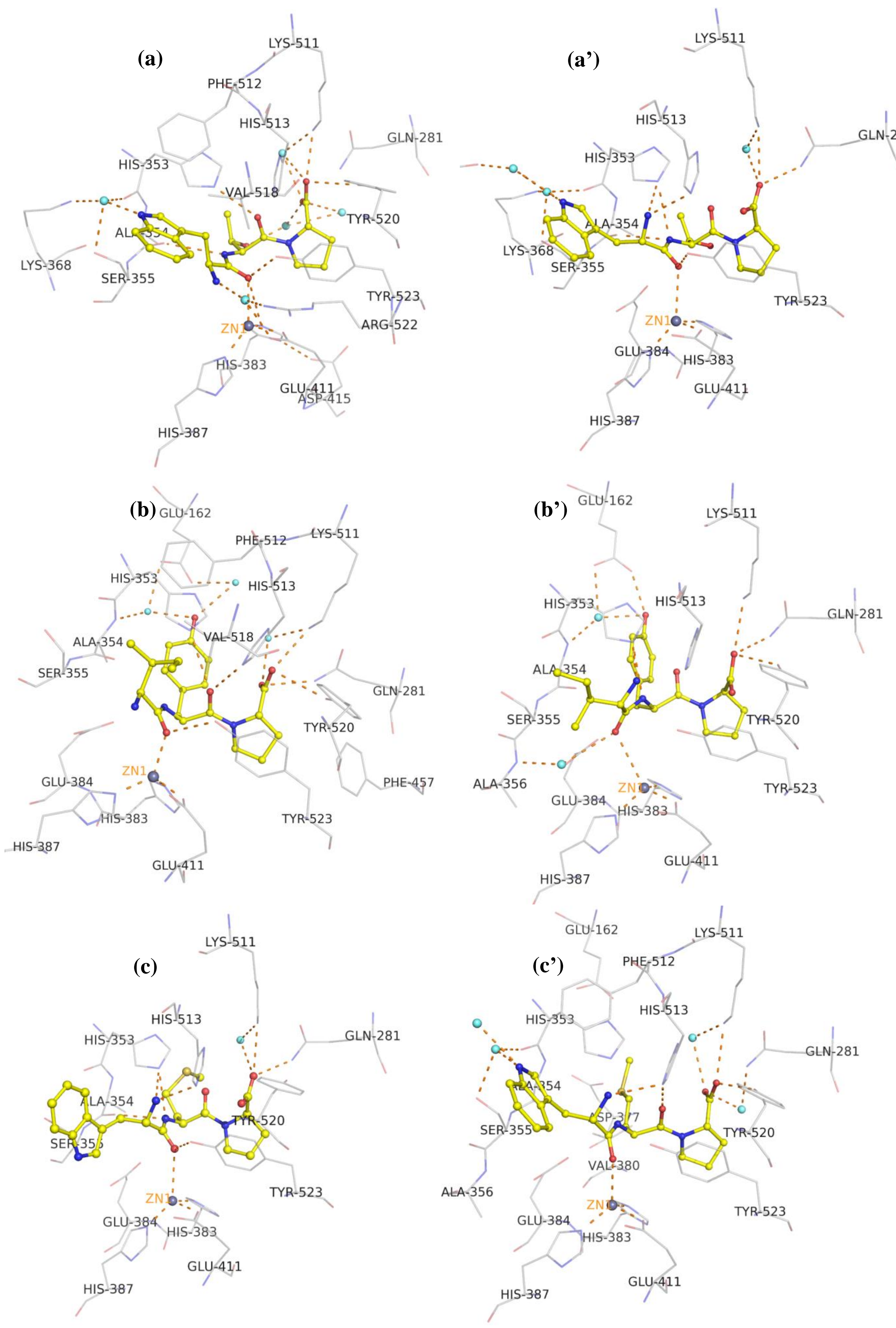

Figure A5.2. Images of tripeptides WTP, IYP and WMP positioning inside the active site of ACE; (a), (b), (c) are the peptides with C-terminus in L-form while (a'), (b'), (c') are the peptides with C-terminus in D-form.

Table A5.1. Number of structures with docking score lower than that of IPP

No.	Ligand	Number of Common Strutures	RerankScore	SimilarityScore	Property of side chain of amino acids*
1	X-Ala-Pro	5	(-142; -125)	(-437; -308)	**Ala** - nonpolar, aliphatic
2	X-Arg-Pro	21	(-168; -126)	(-484; -321)	**Arg** - positively charged
3	X-Asn-Pro	14	(-148; -126)	(-464; -312)	**Asn** - polar, uncharged
4	X-Asp-Pro	20	(-164; -125)	(-465; -310)	**Asp** - negatively charged
5	X-Cys-Pro	6	(-138; -124)	(-422; -327)	**Cys** - polar, uncharged
6	X-Gln-Pro	17	(-155; -124)	(-471; -310)	**Gln** - polar, uncharged
7	X-Glu-Pro	23	(-157; -128)	(-473; -316)	**Glu** - negatively charged
8	X-Gly-Pro	9	(-152; -126)	(-447; -306)	**Gly** - nonpolar, aliphatic
9	X-His-Pro	23	(-172; -129)	(-478; -319)	**His** - positively charged
10	X-Ile-Pro	9	(-150; -124)	(-463; -312)	**Ile** - nonpolar, aliphatic
11	X-Leu-Pro	18	(-165; -125)	(-464;-309)	**Leu** - nonpolar, aliphatic
12	X-Lys-Pro	23	(-169; -125)	(-474; -309)	**Lys** - positively charged
13	X-Met-Pro	17	(-170; -125)	(-475; -308)	**Met** - nonpolar, aliphatic
14	X-Phe-Pro	21	(-169; -125)	(-480; -339)	**Phe** - nonpolar, aromatic
15	X-Pro-Pro	12	(-166; -131)	(-445; -305)	**Pro** - polar, uncharged
16	X-pSer-Pro	22	(-169; -126)	(-482; -329)	**pSer** - not classified
17	X-pThr-Pro	21	(-164; -130)	(-456; -321)	**pThr** - not classified
18	X-pTyr-Pro	23	(-160; -131)	(-489; -358)	**pTyr** - not classified
19	X-Ser-Pro	9	(-152; -127)	(-456; -313)	**Ser** - polar, uncharged
20	X-Thr-Pro	15	(-164; -124)	(-467; -315)	**Thr** - polar, uncharged
21	X-Trp-Pro	22	(-167; -124)	(-491; -397)	**Trp** - nonpolar, aromatic
22	X-Tyr-Pro	23	(-166; -127)	(-489; -345)	**Tyr** - nonpolar, aromatic
23	X-Val-Pro	14	(-172; -125)	(-463; -315)	**Val** - nonpolar, aliphatic
Total		**387**	**(-172; -124)**	**(-491; -305)**	*OpenStax College, Biology. OpenStax College. 30 May 2013. <http://cnx.org/content/col11448/latest/>.
Ile-Pro-Pro		Lowest scores =>	(-123.93)	(-295.19)	

Table A5.4. Interaction of 4 most active peptides (amino acid in L-form) with residues of ACE analyzed by HBPLUS

IYP			WMP			WQP			WTP		
Ligand atoms	Interaction	Distance (Å)	Ligand atoms	Interaction	Distance (Å)	Ligand atoms	Interaction	Distance (Å)	Ligand atoms	Interaction	Distance (Å)
O2	K511NZ	3.06	O1	K511NZ	3.17	O1	K511NZ	3.13	O1	K511NZ	2.8
O2	Y520OH	3.14	O1	Y520OH	2.39	O1	Y520OH	2.6	O1	Y520OH	3.06
O4	H513NE2	3.27	O3	H513NE2	2.91						
O4	H353NE2	3.26	N3	H513NE2	2.93	O4	H353NE2	3.18	O4	H353NE2	2.91
						N4	A354O	2.05	N2	A354O	3.34
O5	Y523OH	2.88				O3	A354O	2.87	O5	Y523OH	2.74
O1	Q281NE2	3.01				O2	Q281NE2	2.64	O5	H383NE2	3.34
O5	Zn	2.27	O4	Zn	2.60	O5	Zn	2.73	O5	Zn	2.53
						N2	Zn	3.31			
O3	Wat2156	2.69	N4	Wat2029	2.97	O5	Wat2570	3.16	O2	Wat2311	3.14
O3	Wat2157	3.09	N4	Wat2365	2.68	N3	Wat2392	2.96	N4	Wat2365	2.67
			O2	Wat2311	3.04	O2	Wat2311	3.13	N3	Wat2409	2.79
O1	Wat2569	2.74	O2	Wat2569	3.04	O2	Wat2569	3.09	O2	Wat2568	2.39
						N3	Wat2156	2.80	O3	Wat2568	2.60
						O3	Wat2156	2.82	O1	Wat2569	3.11
									O2	Wat2569	2.96

The number come after oxygen and nitrogen atoms were automatically numbered by HBPLUS; number of residues and water molecules were as in original protein structure; interaction distances were calculated by HBPLUS

Table A5.5. Interaction of 4 most active peptides (C-terminus in D-form) with residues of ACE base on docking results

IY-d-P			WM-d-P			WQ-d-P			WT-d-P		
Ligand atoms	Interaction	Distance (Å)	Ligand atoms	Interaction	Distance (Å)	Ligand atoms	Interaction	Distance (Å)	Ligand atoms	Interaction	Distance (Å)
O1	K511NZ	2.97	O2	K511NZ	3.07		K511NZ		O2	K511NZ	3.14
O1	Y520OH	2.7		Y520OH			Y520OH			Y520OH	
			N3	H513NE2	2.67	N4	H513NE2	3.10	N4	H513NE2	2.95
N2	H353NE2	3.04					H353NE2		N2	H353NE2	3.25
N3	H353NE2	2.82	N2	H353NE2	2.82						
O3	E162NE2	2.67	N2	A354O	2.05		A354O		N2	A354O	3.27
O5	Y523OH		O4	Y523OH	2.94	O4	Y523OH	3.19	O5	Y523OH	2.79
O1	Q281NE2	3.12	O1	Q281NE2	2.78		Q281NE2		O2	Q281NE2	2.74
O5	Zn	3.3	O4	Zn	2.96	O4	Zn	3.18		Zn	
						O5	Zn	3.30			
O3	Wat2156	2.68		Wat2029		O5	Wat2570	3.08		Wat2311	
	Wat2157			Wat2365		N3	Wat2392	2.68	N4	Wat2365	2.95
				Wat2311			Wat2311		N3	Wat2029	2.82
	Wat2569		O2	Wat2569	3.14	O2	Wat2568	2.88		Wat2568	
O5	Wat2570	3.32				N3	Wat2156	2.66		Wat2568	
						O3	Wat2568	2.59		Wat2569	
									O2	Wat2569	3.17

The number come after oxygen and nitrogen atoms were automatically numbered by HBPLUS; number of residues and water molecules were as in original protein structure; interaction distances were calculated by HBPLUS

Table A5.2. 100 structures with lowest RerankScore

No.	Ligand	RerankScore	SimilarityScore
1	O-phosphotyrosylvalylproline	-172.415	-434.446
2	O-phosphotyrosylhistidylproline	-172.235	-445.572
3	O-phosphotyrosylmethionylproline	-170.296	-445.917
4	O-phosphotyrosyllysylproline	-169.715	-442.81
5	O-phosphotyrosylphenylalanylproline	-169.214	-447.829
6	O-phosphotyrosyl-O-phosphoserylproline	-169.114	-445.846
7	tyrosylarginylproline	-168.252	-453.068
8	O-phosphotyrosyltryptophylproline	-167.875	-465.703
9	O-phosphotyrosylprolylproline	-166.984	-410.837
10	tryptophylmethionylproline	-166.915	-475.398
11	O-phosphotyrosyltyrosylproline	-166.707	-461.495
12	O-phosphotyrosylleucylproline	-165.849	-429.485
13	tryptophylarginylproline	-164.983	-484.44
14	O-phosphotyrosylaspartylproline	-164.743	-433.776
15	O-phosphotyrosyl-O-phosphothreonylproline	-164.671	-426.242
16	O-phosphotyrosylthreonylproline	-164.662	-435.627
17	O-phosphothreonylarginylproline	-164.342	-368.269
18	phenylalanylarginylproline	-162.231	-456.953
19	prolyl-O-phosphotyrosylproline	-160.281	-397.865
20	tryptophyltyrosylproline	-160.039	-489.462
21	O-phosphothreonyl-O-phosphoserylproline	-159.114	-353.932
22	glutaminyl-O-phosphotyrosylproline	-159.076	-377.716
23	O-phosphoseryl-O-phosphoserylproline	-158.729	-343.364
24	Arginyl-O-phosphotyrosylproline	-158.596	-393.894
25	arginyl-O-phosphothreonylproline	-157.708	-350.091
26	arginylhistidylproline	-157.643	-362.442
27	O-phosphotyrosylglutamylproline	-157.538	-442.426
28	glutamyl-O-phosphotyrosylproline	-157.521	-377.631
29	tryptophylvalylproline	-157.151	-463.706
30	prolylarginylproline	-156.731	-360.965
31	tryptophylglutaminylproline	-155.876	-471.842
32	tryptophyl-O-phosphotyrosylproline	-155.858	-489.668
33	histidyl-O-phosphoserylproline	-155.675	-430.816
34	O-phosphothreonyltyrosylproline	-155.671	-393.517

Table A5.2. 100 structures with lowest RerankScore (cont.)

No.	Ligand	RerankScore	SimilarityScore
35	O–phosphotyrosylglutaminylproline	−155.456	−441.029
36	Arginyl–O–phosphoserylproline	−155.211	−352.793
37	glutaminyl–O–phosphoserylproline	−154.999	−337.969
38	arginylarginylproline	−154.977	−351.964
39	tyrosyllysylproline	−154.966	−449.17
40	glutamyl–O–phosphoserylproline	−154.93	−334.634
41	tryptophylhistidylproline	−154.862	−478.798
42	O–phosphoseryltyrosylproline	−154.651	−371.727
43	phenylalanyl–O–phosphoserylproline	−154.558	−446.208
44	aspartyl–O–phosphotyrosylproline	−154.535	−370.095
45	isoleucylarginylproline	−154.216	−358.258
46	O–phosphotyrosylglutaminylproline	−154.2519273	−345.114
47	Arginyl–O–phosphoserylproline	−154.1520818	−363.289
48	glutaminyl–O–phosphoserylproline	−154.0522364	−333.471
49	arginylarginylproline	−153.9523909	−443.123
50	tyrosyllysylproline	−153.8525455	−474.533
51	glutamyl–O–phosphoserylproline	−153.7527	−418.153
52	tryptophylhistidylproline	−153.6528545	−339.137
53	O–phosphoseryltyrosylproline	−153.5530091	−357.117
54	phenylalanyl–O–phosphoserylproline	−153.4531636	−327.528
55	aspartyl–O–phosphotyrosylproline	−153.3533182	−480.572
56	isoleucylarginylproline	−153.2534727	−427.023
57	O–phosphotyrosylglutaminylproline	−153.1536273	−329.74
58	Arginyl–O–phosphoserylproline	−153.0537818	−336.867
59	glutaminyl–O–phosphoserylproline	−152.9539364	−348.153
60	arginylarginylproline	−152.8540909	−467.547
61	tyrosyllysylproline	−152.7542455	−406.304
62	glutamyl–O–phosphoserylproline	−152.6544	−459.613
63	tryptophylhistidylproline	−152.5545545	−352.501
64	O–phosphoseryltyrosylproline	−152.4547091	−439.329
65	phenylalanyl–O–phosphoserylproline	−152.3548636	−371.395
66	aspartyl–O–phosphotyrosylproline	−152.2550182	−428.774
67	isoleucylarginylproline	−152.1551727	−482.63

Table A5.2. 100 structures with lowest RerankScore (cont.)

No.	Ligand	RerankScore	SimilarityScore
68	O−phosphoserylhistidylproline	−151.194	−351.568
69	threonylglutamylproline	−151.012	−321.514
70	tyrosylglutamylproline	−150.707	−441.561
71	O−phosphothreonylglutaminylproline	−150.597	−355.669
72	O−phosphotyrosylisoleucylproline	−150.25	−429.245
73	arginylaspartylproline	−150.165	−339.604
74	O−phosphoseryllysylproline	−150.124	−354.919
75	tyrosyltyrosylproline	−149.954	−456.36
76	asparaginyl−O−phosphotyrosylproline	−149.935	−373.757
77	Lysyl−O−phosphothreonylproline	−149.777	−335.546
78	prolyl−O−phosphoserylproline	−149.353	−366.456
79	alanyl−O−phosphotyrosylproline	−149.082	−364.697
80	lysyl−O−phosphoserylproline	−149.025	−334.852
81	threonyl−O−phosphotyrosylproline	−148.977	−365.437
82	isoleucyltyrosylproline	−148.976	−354.09
83	O−phosphoserylarginylproline	−148.884	−370.45
84	tryptophylasparaginylproline	−148.845	−464.664
85	aspartylhistidylproline	−148.837	−342.99
86	arginylleucylproline	−148.537	−342.835
87	lysylglutamylproline	−148.404	−332.209
88	tyrosyltryptophylproline	−148.159	−454.679
89	phenylalanylmethionylproline	−147.97	−438.984
90	O−phosphotyrosylarginylproline	−147.756	−468.308
91	cysteinyl−O−phosphoserylproline	−147.684	−333.259
92	methionylarginylproline	−147.576	−352.8
93	asparaginylglutamylproline	−147.224	−321.228
94	threonylglutaminylproline	−147.16	−324.244
95	arginyltyrosylproline	−147.151	−386.904
96	serylarginylproline	−147.149	−335.783
97	tryptophyltryptophylproline	−147.145	−491.622
98	tyrosylglutaminylproline	−146.62	−440.749
99	O−phosphothreonylglutamylproline	−146.458	−351.722
100	O−phosphothreonylhistidylproline	−146.455	−369.315

Table A5.3. RerankScore of 4 most active peptides with C-terminus in L- and D-form

Ligand	**RerankScore**	**IC_{50} (µM)**
Trp-Gln-L-Pro	-155.88	5.6
Trp-Thr-L-Pro	-151.90	12.7
Ile-Tyr-L-Pro	-148.98	16.4
Trp-Met-L-Pro	-166.92	16.7
Trp-Gln-D-Pro	-126.55	*>1165*
Trp-Thr-D-Pro	-138.38	*>1243*
Ile-Tyr-D-Pro	-114.16	*>1278*
Trp-Met-D-Pro	-136.31	*>1157*