OPTIMIZATION OF PROTEIN HYDROLYSATE PREPARATION AND THEIR ANGIOTENSIN I-CONVERTING ENZYME INHIBITORY ACTIVITY FROM SHIITAKE MUSHROOMS *Lentinula* sp.

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การหาค่าเหมาะที่สุดสำหรับการเตรียมโปรตีนไฮโดรไลเสตและฤทธิ์ยับยั้งแอนจิโอเทนซินวัน-คอนเวอร์ติงเอนไซม์จากเห็ดหอม *Lentinula* sp.

น.ส.สุปวีณ์ ไพศาลศักดิ์

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สุปวีณ์ ไพศาลศักดิ์ : การหาค่าเหมาะที่สุดสำหรับการเตรียมโปรตีนไฮโดรไลเสตและฤทธิ์ยับยั้งแอนจิโอเทนซินวัน-คอนเวอร์ติงเอนไซม์จากเห็ดหอม *Lentinula* sp.. (OPTIMIZATION OF PROTEIN HYDROLYSATE PREPARATION AND THEIR ANGIOTENSIN I-CONVERTING ENZYME INHIBITORY ACTIVITY FROM SHIITAKE MUSHROOMS *Lentinula* sp.) อ.ที่ปรึกษาหลัก : รศ. ดร.อภิชาติ กาญจนทัต

แอนจิโอเทนซินวัน-คอนเวอร์ติงเอนไซม์ (ACE) มีบทบาทสำคัญในการควบคุมความดันโลหิต โดยทำหน้าที่เปลี่ยนแอนจิโอเทนซินวัน (angiotensin I) ไปเป็นแอนจิโอเทนซินทู (angiotensin II) จะส่งผลให้ความดันโลหิตเพิ่มขึ้น ดังนั้นการยับยั้ง ACE มีผลทำให้ความเข้มข้นของแอนจิโอเทนซินทูต่ำลง และส่งผลให้ความดันโลหิตลดลง โปรตีนจากอาหารบางชนิดสามารถออกฤทธิ์ยับยั้งแอนจิโอเทนซินวัน-คอนเวอร์ติงเอนไซม์ ซึ่งไม่เกิดพิษต่อร่างกาย ปลอดภัย และมีประสิทธิภาพดีจึงอาจจะสามารถนำมาใช้ในการควบคุม และรักษาภาวะความดันโลหิตสูง เห็ดหอม (*Lentinula edodes*) เป็นเห็ดที่คนนิยมบริโภคมากในประเทศไทย ซึ่งจัดเป็นอาหารเพื่อสุขภาพมาอย่างยาวนาน และมีฤทธิ์ทางยาในด้านต่างๆ แต่ฤทธิ์ทางการลดระดับความดันโลหิตจากโปรตีนหรือเพปไทด์ของเห็ดหอม ยังมีการศึกษาไม่มากนัก ดังนั้น ในงานวิจัยนี้สนใจที่จะศึกษาโปรตีนไฮโดรไลเสตจากเห็ดหอมที่มีฤทธิ์ยับยั้งแอนจิโอเทนซินวัน-คอนเวอร์ติงเอนไซม์ ด้วยวิธีพื้นที่ผิวตอบสนอง (Response Surface Methodology; RSM) โดยใช้เอนไซม์แอลคาเลสในการย่อย จากการศึกษาพบว่า ภาวะที่เหมาะสม ให้ค่าการย่อยสลายสูงที่สุดร้อยละ 28.88 คือ อุณหภูมิ 50.2 องศาเซลเซียส ระยะเวลา 3 ชั่วโมง และอัตราส่วนเอนไซม์ต่อสารตั้งต้นร้อยละ 1.16 อย่างไรก็ตาม ภาวะที่เหมาะสมในการย่อยโปรตีนไฮโดรไลเสตที่มีฤทธิ์ยับยั้งแอนจิโอเทนซินวัน-คอนเวอร์ติงเอนไซม์ คือ 47 องศาเซลเซียส ระยะเวลา 3 ชั่วโมง 28 นาที และอัตราส่วนเอนไซม์ต่อสารตั้งต้นร้อยละ 0.59 โดยแสดงค่าความเข้มข้นต่ำสุดที่สามารถยับยั้งได้ร้อยละ 50 (IC_{50}) เท่ากับ 0.33 µg/mL จากนั้น คัดแยกเพปไทด์ตามขนาดโมเลกุลด้วยเทคนิคอัลตร้าฟิลเตรชัน 10, 5, 3, และ 0.65 กิโลดาลตันตามลำดับ พบว่าเพปไทด์ที่มีขนาดโมเลกุลต่ำกว่า 0.65 กิโลดาลตันมีฤทธิ์ยับยั้งแอนจิโอเทนซินวัน-คอนเวอร์ติงเอนไซม์ได้ดีที่สุดที่ระดับความเข้มข้น IC_{50} 0.23 µg/mL จากนั้นทำบริสุทธิ์โดยเทคนิคโครมาโตกราฟีของเหลวสมรรถนะสูง และนำเพปไทด์ที่มีฤทธิ์ยับยั้งสูงสุด (IC_{50} เท่ากับ 0.03287 µg/mL) ไปพิสูจน์เอกลักษณ์ด้วยเทคนิคแมสสเปคโตรเมตรี พบว่าลำดับกรดอะมิโนได้แก่ Lys-Ile-Gly-Ser-Arg-Ser-Arg-Phe-Asp-Val-Thr (KIGSRSRFDVT) น้ำหนักโมเลกุล 1265.43 ดาลตัน มีฤทธิ์ยับยั้งสูงสุด IC_{50} เท่ากับ 0.3714 µM การศึกษาจลน์ศาสตร์ของเอนไซม์ พบว่าเพปไทด์มีการยับยั้งแบบไม่แข่งขัน (non-competitive) ซึ่งเป็นไปในทิศทางเดียวกับการศึกษาโมเลคิวลาร์ด็อกกิง (Molecular Docking Study) พบว่า เพปไทด์เข้าจับกับเอนไซม์ ACE ที่ไม่ใช่บริเวณเร่ง จากผลการศึกษาดังกล่าวแสดงให้เห็นว่า เพปไทด์จากเห็ดหอมมีฤทธิ์ยับยั้งแอนจิโอเทนซินวัน-คอนเวอร์ติงเอนไซม์ และสามารถนำไปประยุกต์ใช้ในอุตสาหกรรมทางการแพทย์ และอุตสาหกรรมอาหารต่อไปได้

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Supawee Paisansak : OPTIMIZATION OF PROTEIN HYDROLYSATE PREPARATION AND THEIR ANGIOTENSIN I-CONVERTING ENZYME INHIBITORY ACTIVITY FROM SHIITAKE MUSHROOMS *Lentinula* sp.. Advisor: Assoc. Prof. APHICHART KARNCHANATAT, Ph.D.

Angiotensin-I converting enzyme (ACE) is a key enzyme involved in regulating blood pressure. ACE converts angiotensin-I into angiotensin-II, which results in raised blood pressure. Inhibition of ACE leads to a decreased concentration of angiotensin-II and lowering of blood pressure. Food protein-derived ACE inhibitory peptides are considered to be a safer alternative to antihypertensive drugs. Shiitake mushrooms, one of the most consumed mushrooms in Thailand, are recognized as a nutritive food with health-stimulating properties. The aim of this study was to optimize the preparation of a shiitake-protein hydrolysate and investigate the ACE inhibitory activity of the hydrosylate. Shiitake mushrooms were hydrolyzed by Alcalase using response surface methodology. The degree of hydrolysis (DH) and ACE inhibitory activity were monitored. The optimum conditions, under which the DH reached a maximum of 28.88%, were 50.2 °C, a hydrolysis time of 3 h, and an enzyme/substrate ratio of 1.16. However, the optimum conditions under which the hydrolysate exhibited the highest ACE inhibitory activity (IC_{50} = 0.33 μg/mL) were 47 °C, a hydrolysis time of 3 h 28 min, and an enzyme/substrate ratio of 0.59%. After fractionation into five ranges of molecular weight, the fraction < 0.65 KDa showed the highest activity with an IC_{50} value of 0.23 μg/mL. This fraction was purified by RP-HPLC and the peak with a retention time of 3.96 min had high ACE inhibitory activity (IC_{50} = 0.03287 μg/mL). The amino acid sequence of this peak, identified by mass spectrometry, was Lys-Ile-Gly-Ser-Arg-Ser-Arg-Phe-Asp-Val-Thr (KIGSRSRFDVT), with a molecular weight of 1,265.43 Da. The ACE inhibitory activity (IC_{50}) of the synthesized version of this peptide was 0.3714 μM. The peptide KIGSRSRFDVT was found to act as a non-competitive inhibitor from analysis of the Lineweaver-Berk plot. A docking study confirmed that the peptide binds at an ACE non-active site. These results indicate that peptides from shiitake mushrooms may be beneficial ingredients in pharmaceuticals, or functional foods, to treat hypertension.

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TABLE OF CONTENTS

Page

ABSTRACT (THAI) iii
ABSTRACT (ENGLISH) iv
ACKNOWLEDGEMENTS v
TABLE OF CONTENTS vi
LIST OF TABLES ix
LIST OF FIGURES x
LIST OF ABBREVIATIONS xi
CHAPTER 1 INTRODUCTION 1
CHAPTER 2 LITERATURE REVIEWS 3
2.1 Hypertension 3
2.2 Hypertension mechanism 3
2.3 Hypertension treatment 5
2.3.1 Antihypertensive drugs 5
2.3.2 Drug side effects 8
2.4 Bioactive peptide 8
2.5. Antihypertensive peptides 9
2.6 Protein hydrolysate 12
2.7 Edible mushroom 13
2.8 Shiitake mushroom 15
2.9 Response surface methodology (RSM) 16
CHAPTER 3 MATERIALS AND METHODS 19
3.1 Materials and chemicals 19
3.2 Preparation of shiitake mushroom protein hydrolysate by enzymatic hydrolysis 19
3.3 Experimental design for the optimization of the preparation of shiitake mushroom protein hydrolysate using Response Surface Methodology (RSM) 19

3.4 Determination of the DH 20
3.5 Determination of the soluble protein 20
3.6 ACE inhibitory activity assay 21
3.7 Purification of ACE inhibitory peptides 21
3.7.1 Ultrafiltration 21
3.7.2 RP-HPLC 22
3.8 Identification of the amino acid sequence of the purified peptide by MS 22
3.9 Peptide synthesis 23
3.10 Kinetics study 23
3.11 Molecular docking of KIGSRSRFDVT in the ACE binding site 23
3.12 Statistical analysis 24
CHAPTER 4 RESULTS AND DISCUSSION 25
4.1 Optimization of shiitake mushroom protein hydrolysis 25
4.2 Optimization of the ACE inhibitory activity of the peptides 29
4.3 Purification of ACE inhibitory peptides 32
4.4 Purifications of shiitake mushroom hydrolysates by RP-HPLC 33
4.5 Identification of peptide sequence 33
4.6 Determination of the kinetic properties of ACE inhibition by KIGSRSRFDVT 34
4.7 Molecular docking of KIGSRSRFDVT in the ACE binding site 36
CHAPTER 5 CONCLUSION 38
APPENDIX A 40
APPENDIX B 43
APPENDIX C 44
APPENDIX D 45
REFERENCES 46
VITA 53

LIST OF TABLES

Page

Table 2.1 The antihypertensive drugs and side effect (Bhagani et al., 2018; Rosas-Peralta & Jiménez-Genchi, 2018) 8

Table 2.2 Bioactive peptides derived from food protein: source and amino acid sequence. 9

Table 2.3 Antihypertensive peptides derived from food protein. 11

Table 2.4 Food protein hydrolysate and their bioactive activity 13

Table 2.5 Antihypertensive peptides derived from Mushroom 14

Table 4.1 Experimental design and results of the CCD 26

Table 4.2 Analysis of variance for the response of DH 27

Table 4.3 Analysis of variance for the hydrolysate response of ACE inhibitory activity 30

LIST OF FIGURES

Page

Figure 2.1 The renin-angiotensin system.....4

Figure 2.2 The structure of antihypertensive drugs 7

Figure 2.3 A tree-dimensional response surface 2 main effects 17

Figure 4.1 Response surface graphs for the %DH with the different factors. (a) The interactive effect of temperature and time. (b) The interactive effect of E/S and time. (c) The interactive effect of temperature and E/S. 28

Figure 4.2 Response surface graphs for the ACE inhibitory activity with the different factors. (a) The interactive effect of temperature and time. (b) The interactive effect of E/S and time. (c) The interactive effect of temperature and E/S. 31

Figure 4.3 RP-HPLC profile of the active fraction (< 0.65 kDa) from shiitake protein hydrolysate. Eluent A: 0.1% trifluoroacetic acid (TFA), eluent B: 70% acetonitrile containing 0.05% TFA.....33

Figure 4.4 Identification of amino acid sequence and molecular mass of the ACE inhibitor peptide purified from the shiitake mushroom hydrolysate (peak 1 from RP-HPLC). Mass fragmentation spectrum of the ACE inhibitory peptide KIGSRSRFDVT. 34

Figure 4.5 The Lineweaver-Burk plot of KIGSRSRFDVT ACE inhibition, No inhibitor (●), 0.4 mg/mL of peptide (▲), 0.6 mg/mL of peptide (■), and 0.8 mg/mL of peptide (♦). 35

Figure 4.6 (a) The 3D diagram of the predicted interactions between the purified peptide (KIGSRSRFDVT) and ACE complex. (b) The 2D diagram of the predicted interactions between the purified peptide (KIGSRSRFDVT) and ACE molecule. Images obtained with Discovery studio 2019 software. 37

LIST OF ABBREVIATIONS

α	Alpha
β	Beta
%	percentage
/	per
:	ratio
˚C	degree Celsius
μg	microgram
µl	microliter
µM	micromole
A	absorbance
BSA	Bovine serum albumin
CCD	Central composite design
DH	Degree of hydrolysis
et al.	and others
Da	Dalton
g	gram
hr	hour
IC_{50}	median inhibitory concentration, 50% maximum inhibition
kDa	Kilo Dalton
L	liter
LC/MS/MS	Liquid chromatography/Mass Spectrometry/Mass Spectrometry
M	molar
Min	minute
mL	milliliter
mg	milligram
mM	milimolar
MW	molecular weight
NaCl	Sodium Chloride
nm	nanometer
PBS	Phosphate buffer saline
Psi	Pound per square inch

RP-HPLC	Reversed Phase-High Performance Liquid Chromatography
rpm	revolution per minute (round per minute)
RSM	Response surface methodology
TFA	trifluoroacetic acid
U	unit activity
UV	ultraviolet
v/v	volume by volume
w/v	weight by volume

CHAPTER 1
INTRODUCTION

Hypertension is a chronic medical condition in which elevated blood pressure can be detrimental to health. There are many conditions associated with hypertension, including stroke, heart disease, chronic renal failure, and aneurysm disease. There are many predisposition factors for hypertension, such as a sedentary lifestyle, stress, and visceral obesity, which are not restricted to the elderly (Bhagani et al., 2018). The angiotensin I-converting enzyme (ACE, EC.3.4.15.1) plays a key physiological role in the control of blood pressure through the Renin-Angiotensin System (Regoli & Gobeil Jr, 2015; Sayer & Bhat, 2014), which mediates control of the extracellular volume (i.e. the volume of blood plasma, lymph, and interstitial fluids) and arterial vasoconstriction. ACE catalyzes the conversion of the decapeptide angiotensin I to the potent vasoconstrictor angiotensin II, and also degrades bradykinin, leading to the systemic dilation of the arteries and a decrease in arterial blood pressure (Li et al., 2004). Administration of some ACE inhibitor (ACEI) peptides results in the decreased formation of angiotensin II and thus, decreased blood pressure. However, these synthetic drugs are believed to have certain side effects—including cough, taste disturbances, skin rashes, and angioneurotic edema, all of which might be intrinsically linked to synthetic ACEIs (Kim & Wijesekara, 2010). Therefore, the development of non-toxic and economical ACEIs is desirable for the prevention and treatment of hypertension (Actis-Goretta et al., 2006).

Bioactive peptides usually contain 3–20 amino acid residues per molecule. A number of research reports have shown that bioactive peptides are capable of imparting antioxidative, antimicrobial, immunomodulatory, and antihypertensive effects (Erdmann et al., 2008; Moller et al., 2008). ACE inhibitory proteins and peptides are commonly produced by enzymatic hydrolysis (Kim & Wijesekara, 2010; Wang et al., 2010). Using this method, the protease, protein substrate, and hydrolysis conditions can greatly influence the release of ACE inhibitory peptides (Kim & Wijesekara, 2010). Commercial enzymes used to produce protein hydrolysates with ACE inhibitory activity, include Alcalase, pepsin, trypsin, papain, and Protamex (Gómez-Guillén et al., 2011; Hernández-Ledesma et al., 2011). Response surface methodology (RSM) is a statistical tool for evaluating process parameters, using the fewest number of experiments, when many factors and interactions affect the desired responses for a given process. RSM is widely used in the industrialization of active peptides obtained from proteolysis (Turan et al., 2015). Evaluating the effects of the enzyme substrate ratio (E/S),

proteolysis time, pH, temperature, and other factors affecting the yield, as well as the degree of hydrolysis (DH), activity, and other outcome values, the proteolysis method can be optimized to obtain active peptides with a high yield and/or potent activity. Currently, ACE inhibitory peptides are prepared from stone fish proteins (Auwal et al., 2017), whey proteins (Guo et al., 2009), lizardfish muscle proteins, and other similar materials using RSM (Wu et al., 2012).

Shiitake mushrooms (*Lentinula* sp.) are one of the most cultivated and consumed mushrooms in Thailand and are rich in proteins, carbohydrates, fiber, and several vitamins. Shiitake mushrooms have shown health-stimulating properties and medicinal effects, including antioxidant activity, antimicrobial activity, reduced plasma glucose levels with induced diabetes, lower cholesterol and triglyceride levels, anticancer properties, anti-inflammatory activity, and antiviral activity (Bisen et al., 2010; Rahman et al., 2018; Ren, 2018). To the best of our knowledge, previous research has primarily focused on the optimization of selected variables to obtain the DH in shiitake mushroom protein isolate production. Herein, RSM was employed to optimize the hydrolysis conditions, including the pH, hydrolysis temperature, and E/S. Then, the ACE inhibitory peptides prepared using Alcalase were evaluated. Ultrafiltration and reversed-phase high-performance liquid chromatography (RP-HPLC) were used to purify ACE inhibitory peptides. The sequence of the peptide was identified by LC-MS/MS. A possible binding interaction of the purified peptide within the active site of ACE was also proposed according to the results of molecular docking experiments.

CHAPTER 2

LITERATURE REVIEWS

2.1 Hypertension

Hypertension or high blood pressure is a condition which the pressure in the blood vessels has raised consistently. To provide nourishment and maintain homeostasis, the heart beats to pump the blood for circulates and transport nutrients, oxygen, carbon dioxide and body fluid. The forces of blood pushing against the walls of arteries create the pressure. The pressure when heart constricted call systolic blood pressure (SBP) and diastolic blood pressure (DBP) as heart relaxed. An average of systolic and diastolic blood pressures is above 140/90 mmHg in adults aged 18 years are defined as hypertension while normal blood pressure are less than 120/80 mmHg.

Hypertension is a one of major health problem leading risk factors for mortality rate and affecting nearly a billion people worldwide. World Health Organization (WHO) reported that hypertension is cause of death 9.4 million people globally in 2010. And by the year 2025 is expected 1.56 billion people suffer from this disease. (Bhagani, Kapil, & Lobo, 2018; Hanafi et al., 2018)

In Thailand, according to ministry of public health's report hypertension is a major cause of death. The statistics data from Bureau of non-communicable disease report expanding of hypertension patients' ratio to 100000 in Thai populations, there are 287.5 in 2001 and increase about 5 times to 1,433.61 in 2010. And the numbers of patients dramatically increase every year.

The high rate of mortality of hypertension by reasons of rarely symptoms in the early stages because many people go undiagnosed. It often called silent killer. And another important reason, hypertension is not a single disease. It generally contribute other health risk factors that increase the probabilities of heart attack, stroke, and kidney failure, myocardial infarction, cerebral infarction, renal disorders, ischemic heart disease, and cardiovascular disease. (Silva et al., 2011)

2.2 Hypertension mechanism

The blood pressure is controlled by various interacted biochemical pathway. One of the most normally conduct blood pressure is the renin-angiotensin system or RAS. RAS system adjusts sodium homeostasis, body fluid volume, and maintains arterial blood pressure.

The regulator which plays an important role in RAS system is an angiotensin I-converting enzyme also called ACE (dipeptidyl carboxy peptidase I, kinase II, E.C 3.4.15.1). ACE belongs to the class of zinc proteases that needs zinc and chloride for its activity (Zhao et al., 2009). ACE controls the blood pressure by balancing the fluid and salt in cardiovascular and homeostasis. In lowing blood pressure situation, enzyme renin (angiotensinogenase) is secreted to blood circulation from the kidneys. While angiotensinogen release from liver is hydrolyzed into angiotensin I (Ang I; Asp-Arg-Val-Tyr-Ile- His-Pro-Phe-His-Leu) by renin. After that, ACE which is located on the surface of epithelial cell on blood vessels walls especially in the lungs convert the inactive prohormone decapeptide Ang I into active hypertensive hormone octapeptide angiotensin II (Ang II; Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) by cleaving dipeptide histidyl-leucine (His-Leu) from the C-terminus resulting in potent vasoconstriction act to raise blood pressure (Giani et al., 2010). (Figure 2.1)

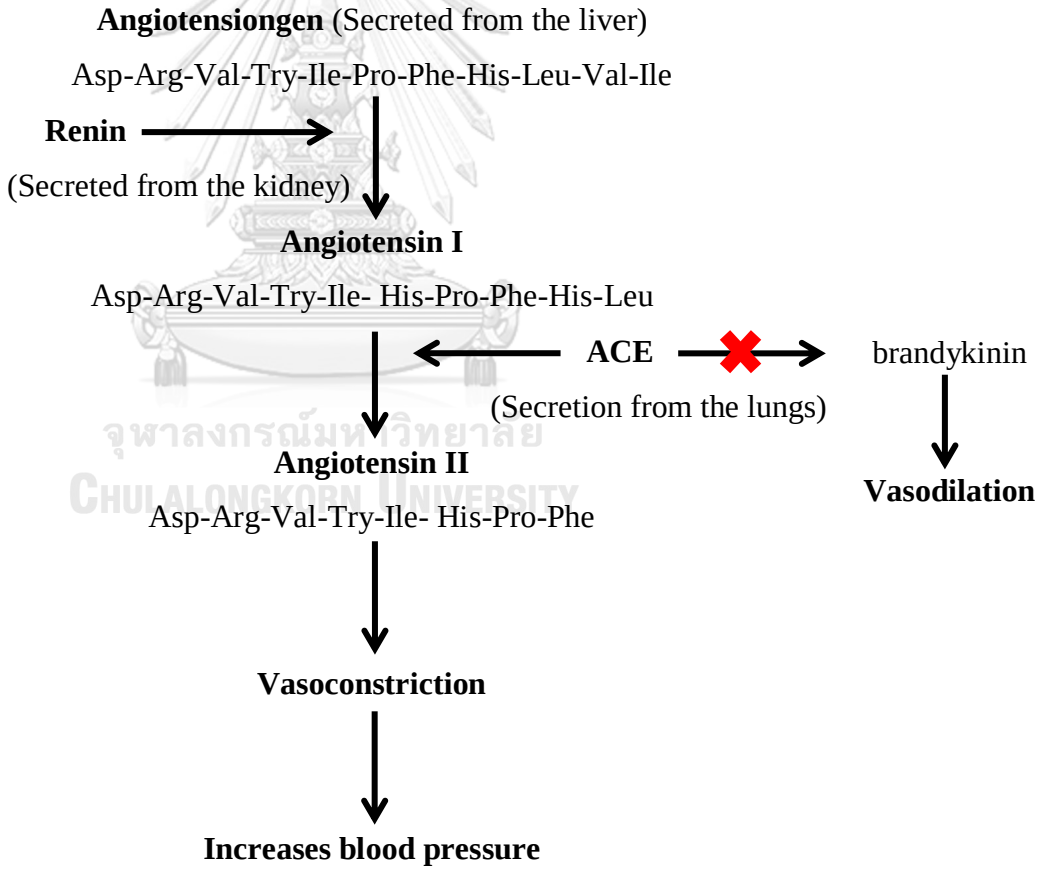


Figure 2.1 The renin-angiotensin system

ACE also degrades the C-terminal dipeptide from brandykinin which is a potent vasodilator and stimulates the release of aldosterone in the adrenal cortex. Bradykinigen, from

the liver is converted to active hypertensive bradykinin but it turns into inactive fragments by the actions of kinase II (ACE) (Asoodeh et al., 2012). Inhibition of ACE may exert an antihypertensive effect as a consequence of the decrease of angiotensin-II as well as increase of bradykinin. ACE inhibition has therefore been targeted for blood pressure reduction.

2.3 Hypertension treatment

Management of hypertension is crucial to prevent hypertension related diseases and death. A primary approach to non-pharmacological treatment by lifestyle modification includes a series of behavioral changes. The life style measures recommended are the following salt restriction, moderation of alcohol consumption, increased vegetables, fruit intake, avoidance of diets in high saturated fat and carbohydrate-rich products, weight loss, improved physical activity or exercise, and smoking cessation. Lifestyle modification management for hypertension also includes reducing chronic exposure to environmental and occupational stress. (Bazzano et al., 2017)

However, the dominant treatment for hypertension was taking antihypertensive drugs. The major antihypertensive drug classes that are currently available used include diuretics, β blockers, calcium channel blockers, angiotensin converting enzyme inhibitors, and angiotensin II blockers, etc. These antihypertensive drugs can be used alone (monotherapy) or in combination to lower blood pressure. Furthermore, most patients require at least two medicines to control BP and it has been suggested that pharmacotherapy should start with combination therapy. So the patients take a lot of drugs in hypertension treatment. Although antihypertensive drugs are effective in reducing blood pressure, long-term use of these chemical synthetic drugs may lead to liver and kidney failure of hypertension patients because of the negative side effects.

2.3.1 Antihypertensive drugs

There are several antihypertensive drugs with remarkable efficient in reducing blood pressure such as captopril, enalapril, lisinopril, ramipril show in figure 2.2. The drugs widely used as clinical are divined in to 4 classes following mechanism as follows Thiazide–type diuretics, Calcium channel blockers (CCBs), Angiotensin converting enzyme inhibitors (ACEIs), and Angiotensin receptor blockers (ARBs)

1) Thiazide–type diuretics

The diuretics are accounted first-line for the treatment of hypertension. The diuretics drug effect on vasodilator and salt/water excretion. The drugs exhibit in the nephron at the

apical membrane in the thick ascending limb of the loop of Henle. They inhibit Sodium (Na^+) and chloride (Cl^-) reabsorption. Other major effects of loop diuretics include a decrease in free water excretion during water loading and reabsorption during dehydration, because of reduced osmotic gradient in the medulla in order to lowers blood volume. Examples of diuretics include: Bumetanide, Chlorthalidone, and Chlorothiazide. (Laurent, Schlaich, & Esler, 2012)

2) Calcium channel blockers (CCBs)

Calcium channel blockers (CCBs) block the entry of calcium through L-type (long-lasting) channels. Because of calcium react to the heart and blood vessels by increases the strength and force of contraction mostly cause vasodilation. The Calcium channel blockers drugs such as verapamil, nifedipine, nimodipine, nicardipine, amlodipine.

3) Angiotensin converting enzyme inhibitors (ACEIs)

The renin-angiotensin system plays a major role in control blood pressure. The key enzyme of this system is ACE which catalyzes angiotensin I into a potent vasoconstrictor, angiotensin II. Thus, ACE inhibition mainly effect in an overall hypertension component. Several drugs are listed in this type e.g. ramipril, enalapril, lisinopril, ramipril.

4) Angiotensin II receptor blockers (ARBs)

Angiotensin II is the end product of the renin–angiotensin system (RAS) which acts via two receptors subtypes: AT1 and AT2. Angiotensin II receptor blockers (ARBs) specifically block the AT1 receptor subtype, leading to a lower incidence of cough and angioedema; hence, compliance is improved compared to ACEIs. Drug in this group are example losartan, irbesartan, candesartan, valsartan, and Azilsartan. (Giani et al., 2010)

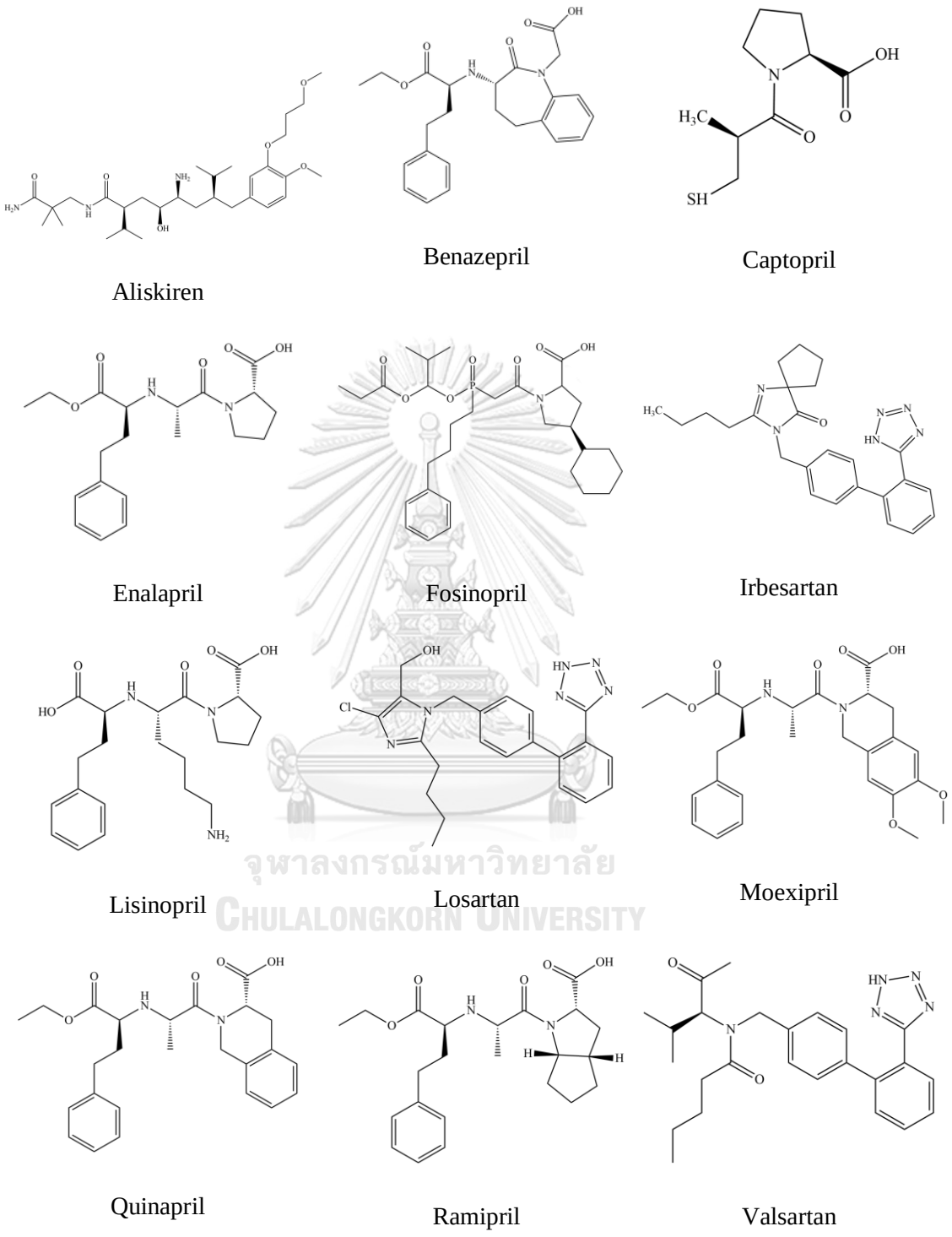


Figure 2.2 The structure of antihypertensive drugs

2.3.2 Drug side effects

The series of antihypertensive drug that clinical used have many undesirable side effects show in Table 2.1 According numerous studies, mostly synthesized drug although remarkably potent at regulating blood pressure, these drugs have often serious side effect such as headaches allergic reactions, skin rashes, chronic dry coughing, taste disturbances, insomnia, diarrhea, inflammatory response, angioedema, hyperkalemia, tachycardia, decrease in the white blood cells, and their long term administration may cause aldosterone escape phenomenon and reduce the efficacy of ACE inhibitors in addition synthetic drugs are easy digested by various protease in the body (Zhang et al., 2017). Therefore, the development of novel ACE inhibitors derived from natural sources is required to reduce side effects while maintaining a strong antihypertensive activity which could be used as a functional food or nutraceutical compounds.

Table 2.1 The antihypertensive drugs and side effect (Bhagani et al., 2018; Rosas-Peralta & Jiménez-Genchi, 2018)

Drugs	Class	Side effects
Doxazosin	α-adrenergic blocker	an increased risk of heart failure compared
Amlodipine	CCB	Peripheral oedema, tachycardia, gum hyperplasia
Indapamide	Diuretic (thiazide-like)	Hypokalaemia/hyponatraemia (increase/reduce in potassium loss), hyperuricaemia, Hyperglycaemia, increase risk for gout
Atenolol	β-adrenoceptor blockers	Affect the respiratory system; Bradycardia, bronchospasm, disturbed sleep, lethargy.
Doxazosin	α-adrenoceptor blockers	Postural hypotension, urge incontinence, peripheral oedema, increased risk of heart failure
Ramipril, Losartan	ACE-i, ARB	Reversible renal decline, dry cough, hypotension; headache.

2.4 Bioactive peptide

Bioactive peptides characterizes as specific protein fragments that show beneficially one or more functions in the human body and may ultimately influence health (Siow & Gan, 2013). Bioactive peptides are typically possessing specific, low molecular weight and small protein fragments 2-30 amino acids residues in size that are inactive within the sequence of

their parent protein (Ngo et al., 2011). And the lower the molecular weight, the higher their chance to cross the intestinal barrier and exert biological functions (Gu et al., 2011).

According to several researches, it has been recognized that dietary proteins provide a rich source of bioactive peptide for example green soybean, defatted peanut flour, whey protein, milk, fish, rice, and egg. Food-derived peptides are attributed health beneficial effect including antimicrobial properties, blood pressure lowering (ACE inhibitory) effects, cholesterol lowering ability, antithrombatic and antioxidative effects, enhancement of mineral absorption (binding properties), immunomodulatory effect, osteoprotective, and opioid activities (Hartmann & Meisel, 2007).

The peptides derived from food proteins are studied and shown strong effect on inhibit ACE (Table 2.2) and considered to be milder and safer than synthetic drugs; furthermore, these peptides usually have multifunctional properties and are easily absorbed in the gastrointestinal tract. Therefore, inhibitors derived from food protein are focused.

Table 2.2 Bioactive peptides derived from food protein: source and amino acid sequence.

source	method	sequence	Activity	Reference
silkworm larvae	gastrointestinal enzymes	-	Ferrous ions chelating capacity (IC_{50}=2.03 mg/ml).	Wu et al. (2011)
rice bran protein	trypsin	YSK	DPPH free radicals scavenging (IC_{50} = 76 mM)	Wang et al. (2017)
Pacific cod	papain	TCSP, TGGGNV	DPPH = 72% Hydroxyl = 56%	Ngo et al. (2011)
porcine hemoglobin	peptic digestion	LGFPTTKTY FPHF	ACE inhibitory (IC_{50} = 4.92 mM)	Yu et al. (2006)
bitter bean	Alcalase	VLNSNAAPL PN	DPPH free radical scavenging (IC_{50} = 2.9 mg GAE/g) reducing power (IC_{50} = 11.7 mM) ACE inhibitory = 80.2 %	Siow & Gan (2013)
Enoki mushroom	5.67 M sulfuric â 100˚C for 4.03 h	MPLLRPAA	ACE inhibitory (IC_{50} = 18.036 mg/mL)	Zhang et al. (2017)

2.5. Antihypertensive peptides

Due to ACE plays a key role in regulating blood pressure therefore ACE inhibition has been targeted for blood pressure reduction. Among concerning about negative side effects

of chemicals synthesis ACE inhibitors drugs like captopril, enalapril, and lisinopril, the safe and mild peptides with ACE inhibitory activity may be an interesting candidate to use as food additive for prevention and remedy of high blood pressure.

A number of peptides with antihypertensive ACE inhibitors have been isolated and characterized from various natural sources including animal products, marine organisms, and plants. (Table 2.3)

Stylotella aurantium, marine sponge was hydrolyzed by various enzymes namely protamex, kojizyme, neutrase, flavouzyme, Alcalase, α-chymotrypsin, trypsin, papain, and pepsin. Among all of the enzymatic hydrolystate, peptic hydrolysate evidenced the highest level of activity relative to the other hydrolysates. The amino acid sequences with highest ACE inhibitory activity were Tyr-Arg (337.2 Da), and Ile-Arg (287.2 Da) had an IC_{50} value of 237.2 μM and 306.4 μM, respectively. (Ko et al., 2017)

Salampessy et al. (2017) studied leatherjacket (*Meuchenia* sp.) which is a cheap fish species abundantly found in Australian waters, in the Pacific and Indian Oceans. Leatherjacket was hydrolysed by using papain, bromelain, and flavourzyme. They found that the peptide with ACE inhibitory activity which potential to be used in the preparation of functional foods are EPLYV, DPHI, AER, EQIDNLQ and WDDME, having IC_{50} values of 0.05, 0.02, 0.11, 0.24 and 0.01 g/L, respectively.

The ACE inhibitory activity of pistachio (*Pistacia vera* L.) was researched by Li et al. (2004). Pistachio kernel was hydrolyzed by pepsin and trypsin. The Pe–Tr–H is the amino acid sequence that presented in vitro ACE inhibitory activity as IC_{50} 0.87 mg/ml. Moreover, the Pe–Tr–H can *in vivo* decrease around 22 mmHg in systolic blood pressure (SBP) and 16 mmHg in the diastolic blood pressure (DBP) at 4 h after the oral administration. The Pe–Tr–H was then separated and purification. A novel ACE inhibitory peptide, ACKEP, was isolated and identified, that has same C-terminal construction as that of lisinopril and enalapril, which plays a key role in binding with ACE. It indicates that the pistachio kernel protein could be utilized for production of ACE inhibitory peptides.

Table 2.3 Antihypertensive peptides derived from food protein.

source	method	sequence	ACE inhibitory activity (IC_{50})	Reference
green soybean	Alcalase	PSLRSYLAE	532 μM	Hanafi et al. (2018)
whey protein	pancreatin	-	0.097 μM	Silvestre et al. (2012)
sea cucumber	bromelain, Alcalase	MEGAQEAQGD	4.5 mM, SHR* at 3 mM/kg dosage	Zhao et al. (2009)
broccoli	water-soluble extract	YPK	10.5 μg protein/ml.	Lee et al. (2006)
oyster	pepsin	VVYPWTQRF	66 μmol/L, SHR* at 20 mg/kg dosage	Wang et al. (2008)
hen egg white lysozyme	trypsin, papain	FESNFNTQATNR	0.03 mg/ml	Asoodeh et al. (2012)
sweet sorghum	Alcalase	TLS	102.1 μM.	Wu et al. (2016)
Cirrhinus mrigala	papain	-	1.15 mg/ml	Elavarasan et al. (2016)
atlantic salmon	Alcalase, papain	VR	0.332 mg/ml	Gu et al. (2011)
sipuncula	pepsin, trypsin	GNGSGYVSR	29 μM	Guo et al. (2017)
sea cucumber	Alcalase	EVSQGRP	0.05 mM	Forghani et al. (2016)
Ulva rigida protein	pepsin, bromelain	IP	0.020 mg/mL	Actis-Goretta et al. (2006)
cauliflower	trypsin, pepsin, pancreatin	FFAPYAPNFPFK	0.461 mol/L	Chiozzi et al. (2016)
oyster mushroom	5.67 M sulfuric â 100˚C for 4.03 h	RLDGSIKGELW	0.587 mg/mL	Zhang et al. (2017)

* Spontaneously hypertensive rat

Normally, physiologically active peptides can be release from several food proteins during hydrolysis by digestive enzymes, hydrolysis by proteolytic microorganisms, and the action of proteolytic enzymes derived from microorganisms or plants.

2.6 Protein hydrolysate

Protein hydrolysis defines as splits the peptide bonds to give smaller peptides and amino acids**.** Generally, there are 2 ways of hydrolysate method widely used including chemicals hydrolysate, enzymatic hydrolysate. Hydrolysate by chemicals is difficult to control condition, producing toxic substances and using extreme temperature and pH levels. Contrastingly, enzymatic hydrolysate is using moderate conditions, no toxic, have specificity amino acid sequences, and can control degree of hydrolysate. Until now, several commercial proteinases such as alcalase, pepsin, trypsin, thermolysin, and flavourzyme have been used to produce protein hydrolysates to recover and improve the nutritional and functional properties of proteins from food protein (Toopcham et al., 2015)**.**

Protein hydrolysates are also a source of bioactive peptides that are inactive in the intact protein but become active after hydrolysis. Moreover, enzymatic hydrolysis can hydrolyze proteins to peptides possessing hydrophobic amino acids at the end of their peptide chains. These hydrophobic amino acids increase the affinity of the enzymatic hydrolysis can hydrolyze proteins to peptides possessing hydrophobic amino acids at the end of their peptide chains. These hydrophobic amino acids increase the affinity of the peptides and finally increasing the antioxidant activities of the hydrolysates.

The hydrolysated peptides have many beneficial actions in the health, such as antihypertensive, antioxidant, anticancer properties (Hartmann & Meisel, 2007). The examples of bioactive peptides are shown in Table 2.4.

Table 2.4 Food protein hydrolysate and their bioactive activity

source	method	DH	sequence	Activity	Reference
Tilapia	pepsin, papain, bromelain.	23	FPHP	ACE-inhibitory activity (IC_{50}= 0.41 mg/mL), reduced SBP 33 mmHg, DBP 24 mmHg in SHRs	Lin et al. (2017)
peanut	Alcalase	40	-	95% ferrous ion chelating, DPPH radical-scavenging (IC_{50}= 2.0 mg/mL), 97% ACE inhibitory	Jamdar et al. (2010)
Tilapia	*Virgibacillus halodenitrificans* SK1-3-7 proteinases	48	MILLLFR	ACE inhibitory (IC_{50} = 0.12 mg/mL)	Toopcham et al. (2015)
grass carp	Alcalase	17.25	VAP	ACE inhibitory (IC_{50} = 0.00534 mg/mL)	Chen et al. (2012)
Enteromorpha clathrata	Alcalase	24.35	PAFG	ACE inhibitory (IC_{50} = 35.9 μM)	Pan et al. (2016)
red snow crab shell	Alcalase	12.5	-	ACE inhibitory (IC_{50} = 0.307 mg/mL) $ABTS^+$ radical scavenging (IC_{50} = 0.135 mg/mL) Reducing power (IC_{50} = 2.836 mg/mL)	Yoon et al. (2013)

2.7 Edible mushroom

Mushrooms are well-established that exhibit diverse nutritional with health-stimulating properties and medicinal effects due to the presence of proteins, carbohydrates, fiber, mineral elements and other bioactive compounds.

Extensive studies have revealed that different mushroom species are of value in the prevention and treatment of several human diseases. Therefore, edible mushrooms can be considered as a functional food.

There are number of studies report that substances in macro fungi, mushrooms have been identified as showing promising anti-tumor, immune potentiating, antiviral, antibacterial, and hypocholesterolemia effects.

Zhao et al. (2017) work aims to determine activity of mushroom foot protein bioactive peptide to activate alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH). The mushroom foot was enzyme hydrolysis by alkaline proteases and conducted degradation of alcohol by test activation of ADH and ALDH were 72.00% and 73.43%, respectively. From this study was further evidence of the mushroom foot peptides had a potential effect on the activation of alcohol metabolic enzymes in vitro, suggested that they can be used to treat alcohol intoxication.

Terfezia claveryi and *Agaricus bisporus* were hydrolyzed by gastrointestinal enzymes**.** Some produced peptides from both mushrooms had antioxidative and antimicrobial activity. The most effective samples were protein of *A. bisporus* with absorbance of 0.305 for reducing power and 77.77 % for DPPH scavenging activity. The hydrolysates from *T. claveryi* were effective for inhibition of linoleic acid oxidation with 85.85 % and chelating activity with 21.36 %. In addition, inhibition of growth of microorganisms, *Pseudomonas aeruginosa* and *Bacillus cereus* were conducted, *A. bisporus* inhibit 26.64 % and *T. claveryi* 27.44 %. (Farzaneh et al., 2018)

There are various studies investigate the mushroom peptides with antihypertension by inhibition of ACE activity are show in Table 2.5

Table 2.5 Antihypertensive peptides derived from Mushroom

source	method	sequence	ACE inhibitory activity (IC_{50})	Reference
Pholiota adiposa ASI 24012	extract with distilled water at 30°C for 12 h.	GEGGP	0.044 mg	Koo et al. (2006)
Tricholoma giganteum	extract with distilled water at 30 ◦C for 3 h.	GEP	0.04 mg	Lee et al. (2004)
Straw mushroom	5.67 M sulfuric acid at 100 ℃ for 4.03 h	EVNILAF	2.926 mg/mL	Zhang et al. (2017)
Shiitake mushroom	5.67 M sulfuric acid at 100 ℃ for 4.03 h	MTVVRR	3.092 mg/mL	Zhang et al. (2017)
Wood ear mushroom	5.67 M sulfuric acid at 100 ℃ for 4.03 h	TVLICVGPF	4.837 mg/mL	Zhang et al. (2017)
Lentinus polychrous	Water extract	-	60.93-79.94% (1.066 mg protein/ml)	Kokram et al. (2016)
Agaricus bisporus (J.E. Lange)	Distilled water extract	AHEPVK	63 μM	Lau et al. (2014)

Moreover, the amino acid sequence, GEGGP, extract with distilled water at 30°C for 12 hours of fruiting body of *Pholiota adiposa* ASI 24012 shows clear antihypertensive effect on spontaneously hypertensive rats (SHR) at a dosage of 1 mg/kg.

2.8 Shiitake mushroom

Recently, mushrooms have received attention because they are nutritious food with health-stimulating properties and medicinal effects. *Lentinula edodes*, commonly called "Shiitake mushroom," is classified as *Lentinus* genus of the Pleurotaceae family.

Scientific classification

Common name: Shiitake (Shi – i – ta - ke), Black mushroom, Chinese mushroom
Subdivision: Basidiomycotina
Class: Hymenomycetes
Subclass: Holobasidiomycetidae
Order: Agaricales (Agarics)
Family: Pleurotaceae
Genus: *Lentinus*
Specie: *L. edodes*

Shiitake mushroom is one of the most staple diets for human especially in Asian countries and a traditional food consumed for thousands of years. In the global mushroom market shiitake mushroom consumption was on second ranks based on consumer demand. (Bisen et al., 2010; Ren et al., 2018)

Nutritionally, shiitake mushroom are a low-fat and low-calorie food. The proximate composition of fruit body of fresh shiitake is following 92% moisture, 1.35% proteins, 0.2% fat, 0.47% ash, and 5.42% carbohydrate. Among 1.35% of protein content consist of alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, isoleucine,lysine, proline, phenylalanine, serine, threonine, tryptophan, tyrosine, and valine. (Tabata et al., 2006)

The shiitake mushroom is used as a flavorful addition to foods especially vegetarian diets. These mushrooms are woodsy rich taste and meaty texture, and nutritional value.

The shiitake mushroom is associated with numerous potential health benefits. Shiitake also have medicinal properties the ability to improve the regulation of blood sugar levels and insulin secretion, inhibit the growth of various types of tumors, preventing heart disease and building resistance against viruses.

A large number of studies have shown that the mushroom is rich in nutrients and bioactive compounds as well as their biological properties.

Rahman et al. (2018) evaluated anti-atherosclerotic effect of shiitake mushroom (*L. edodes*) extraction through direct anti-oxidative and inhibitory effects upon LDL oxidation and antioxidant effect through scavenging on 2, 2-diphenyl-1-picrylhydrazil (DPPH) radical. They found that shiitake mushroom extraction by hexane mostly scavenged (67.38%, IC_{50} 0.55 mg/mL) the DPPH free radical, and most potently inhibited lipid peroxidation (67.07%). GC–MS analysis in the hexane fraction of *L. edodes* identified ergosterol act as a membrane antioxidant upon ingestion and might have been involved in chelating transition metal ion, Fe^{2+} and thus mediating reduced oxidative stress and consequent lowered LDL oxidation.

Chien et al. (2016) studied the antimicrobial and antitumor activities of chitosan from shiitake stipes. The result show that shiitake exhibited excellent antimicrobial activities against 8 species of Gram positive and negative pathogenic bacteria with inhibition zones of 11.4–26.8 mm at 0.5 mg/ml. The general pathogens namely *Bacillus cereus*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Escherichia coli*, *Flavobacterium* sp., *Pseudomonas aeruginosa*, *Salmonella typhimurium*, and *Vibrio parahaemolyticus*. In addition, from cell viability results shiitake extract inhibit cell proliferation on IMR 32 and Hep G2 cells by retarding cell cycle at S phase. Therefore, the shiitake effect on anti-tumor and antimetastatic.

Nutritional components of Shiitake mushroom aid in its pharmacological potency against hyperlipidemia and cardiovascular complications, depressed immunity, hepatic disorders, cancer, oxidative, fungal and microbial aspects have been duly attributed to its bio-functional components (Ren et al., 2018). However, there are a few studies in antihypertensive activity; it is interesting to focus in this activity.

2.9 Response surface methodology (RSM)

Response Surface Methodology (RSM) is useful technique for design experiment. It consists of a group of mathematical and statistical analysis in order to indicating effect of experimental variables on the system. (Mäkelä, 2017) The RSM generate an empirical model which represent an interaction between variables. Moreover, a predicted equation of response is performed. The equations are used for calculating the response by substitute the conditions required. The RSM is mostly used in optimization conditions for maximum response or minimum response.

Response surface model mostly originated from the graphical perspective generated after fitness of the mathematical model which plot response with level of variable (x_1, x_2) for easy to interpret of interaction between factors.

The relationship between independent variables (x) and response (y) is show in equation 1.

$$y = f(x_1, x_2,\ldots,x_k) + \varepsilon \quad (1)$$

When the term ε denotes error in the system and the function f is first-order or second order polynomial. And y represents surface response. Usually in RSM work, it is convenient to transform the real value of variables to coded variable which normally defined to be dimensionless with mean zero and the same standard deviation. The plot of response and the levels of variable (x_1, x_2) show the interaction of variable in 3D response surface or 2D contour plot. (Figure 2.3)

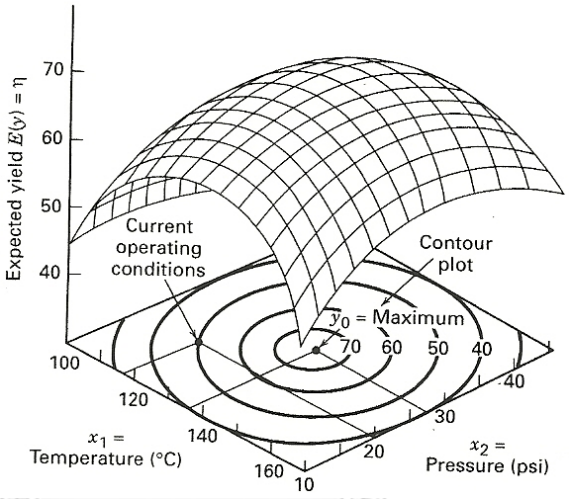


Figure 2.3 A tree-dimensional response surface 2 main effects

Two important models are regularly operated in RSM. The first-order model (Equation 2) is the approximating function when the response is modeled by a linear function of the independent variables. (Khuri & Mukhopadhyay, 2010)

$$y = \beta_0 x_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_k x_k + \varepsilon \quad (2)$$

If there is curvature in the response surface, the second-order model must be used. (Equation 3)

$$y = \beta_0 x_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum\sum_{i<j} \beta_{ij} x_i x_j + \varepsilon \quad (3)$$

The RSM is useful tool in predicted response of variable on system so it is developing in not only study or research but also industry. This process successfully determines optimum conditions and gives the information necessary to design a process. In comparison RSM beyond the factorial design by very advantages. The applications of RSM were developed the design in order to reduce the number of experiments. In case 3-levels of main effect, while number of full factorial optimization conditions are 3^n, RSM conditions are 2^n+n+6 when n is number of variables in the experiment. Moreover, the RSM is able to monitor the interaction

between several variable at the same time whereas the traditional methods are one-factor-at-a-time. So it is acceptable in effectiveness and be able to applied in optimization of product and food processing operations. (Shankar et al., 2013)

CHAPTER 3
MATERIALS AND METHODS

3.1 Materials and chemicals

Shiitake mushrooms, *Lentinula* sp., were purchased from *Tawan Produce Co., LTD.* (*Samutprakarn*, Thailand) and stored in a desiccator at room temperature. Alcalase® with an activity of 3.018 U/mL from *Bacillus licheniformis* was purchased from Novo (*Novo* Industries, Bagsvaerde, *Denmark*) and stored at 4 °C until use. ACE (E.C. 3.4.15.1) from rabbit lung, bovine serum albumin (BSA), hippuric acid, hippuryl-L-histidyl-L-leucine (HHL), acetonitrile, captopril, and 2, 4, 6-trinitrobenzenesulfonic acid (TNBS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other biochemicals and chemicals used were of analytical grade.

3.2 Preparation of shiitake mushroom protein hydrolysate by enzymatic hydrolysis

Whole shiitake mushroom fruiting bodies were dried using a hot air oven at 80 °C for 24 h, ground into fine powder by a grinder, then passed through an 80-mesh (177 μm opening) sieve and kept in a vacuum-sealed polypropylene bag in a desiccator at room temperature until use.

For single-factor analysis, the hydrolysis conditions were a temperature of 45–60 °C and a processing time of0.5–5 h. Briefly, 1 g of shiitake mushroom powder was suspended in 50 mL of phosphate buffer (PBS; 20 mM phosphate buffer, pH 7.2). The pH of the mixture was adjusted to 7.2 with 1 M sodium hydroxide (NaOH). Then, the hydrolysis was performed using Alcalase in a shaker incubator at 3,000 ×*g*. The hydrolysis was terminated by heating at 90 °C for 20 min, followed by centrifugation at 10,000 rpm for 30 min. The supernatant was collected and stored at –20 °C until use.

3.3 Experimental design for the optimization of the preparation of shiitake mushroom protein hydrolysate using Response Surface Methodology (RSM)

RSM was applied to find the optimum conditions for the desired responses (DH and ACE inhibitory activity). On the basis of preliminary single-factor experiments, the RSM was designed to evaluate the influence of independent variables (time, A; temperature, B; and E/S,

C) on the degree of hydrolysis (DH) and ACE inhibitory activity (Y). A five-level three-factor central composite design (CCD) was used to evaluate the effect of the hydrolysis time (30–360 min), temperature (30–70 °C), and enzyme/substrate (0.1%–2%). The complete design consisted of 17 combinations, including three replicates of the center as shown in Table 4.1. The response values for the hydrolysis were the DH and ACE inhibitory activity. The experimental design and statistical analysis were performed using Stat-Ease software (Design expert version 7.0.0 Trial). The hydrolysis was performed by adding Alcalase to a suspension of shiitake mushroom powder and PBS buffer and incubating in a shaker incubator at 120 rpm with different times and temperatures as shown in Table 4.1. After hydrolysis for a selected time, temperature, and enzyme/substrate ratio, the enzymatic hydrolysis reaction mixture was heated to inactivate the protease at 90 °C for 20 min in boiling water, followed by centrifugation at 10,000 rpm for 30 min. The resultant supernatant was collected and immediately stored at 4 °C for future use.

3.4 Determination of the DH

The DH of the shiitake mushroom hydrolysates was determined using the method described by Nielsen et al. (2001) with minor modifications. The free amino groups of the hydrolysates were measured using *o*-phthaldialdehyde (OPA solution). After hydrolysis, 400 μL of shiitake mushroom hydrolysate was added to 3 mL of OPA reagent and mixed for 5 s. The mixture was stood for exactly 2 min before being read at 340 nm in a spectrophotometer. Serine was used as a standard. All assays were performed in triplicate and the degree of hydrolysis was calculated from the formula:

$$\%DH = (h/h_{tot}) \times 100 \qquad \text{Eq. 1}$$

Where h_{tot} is the total number of peptide bonds per protein equivalent, and h is the number of hydrolyzed bonds. The h_{tot} factor is dependent on the amino acid composition of the raw material.

3.5 Determination of the soluble protein

The soluble protein content was determined using the Bradford assay (Bradford, 1976), which relies on the binding of the dye Coomassie Brilliant Blue G-250 to protein and results in a visible color change from brown to blue. Briefly, 20 μL of a sample was prepared in a 96-well plate and 200 μL of Bradford reagent working solution was added and the mixture was incubated at room temperature for at least 5 min but not more than 1 h. The absorbance was measured at 595 nm. A standard curve of absorbance versus micrograms of

protein was obtained and the amount of protein was determined from the curve. The concentration of protein in the original samples was determined from the amount of protein, the volume/sample ratio, and the dilution factor. Bovine serum albumin (BSA) was used as the standard protein for setting the standard curve.

3.6 ACE inhibitory activity assay

The assay for determining ACE inhibitory activity was conducted according to the protocol as described by Ibrahim et al. (2017) with slight modifications. The assay for ACE inhibitory activity is based on the specific binding of TNBS to the primary amine of the His-Leu dipeptide, produced by hydrolytic cleavage from Hippuryl-His-Leu (HHL) by ACE, forming TNP-His-Leu (TNP-HL) by desulfitation, followed by formation of a yellow complex with sulfite, which was detected at 420 nm. The inhibition assay was performed in a 96-well plate and started by adding 5 µL of 200 mU/mL ACE to an aliquot of the sample (10 µL), then 13 µL of the substrate (5 mM HHL) was added and the mixture was incubated for 1 h at 37 °C. Then, 50 µL of 0.68 mM TNBS was added and the mixture was incubated for 1 h at 37 °C, then the absorbance was measured at 420 nm to estimate the ACE inhibitory activity. The activity of each sample was tested in triplicate. Captopril was used as positive control. The percentage of ACE inhibitory activity was calculated according to the following equation:

$$\text{ACE inhibitory activity (\%)} = [(C\text{-}Bi) - (S\text{-}Bs)/ (C\text{-} Bi)] \times 100 \qquad \text{Eq.2}$$

Where C, S, Bi, and Bs represent the absorbances of the control (100% activity), sample (inhibitor peptide), blank inhibitor (only HHL), and blank sample (only peptide sample) respectively. The concentration of hydrolysate or peptide which was able to inhibit 50% of the ACE activity under the above assay conditions was defined as the IC_{50} value. Calculation of the IC_{50} values was performed by non-linear regression using GraphPad Prism Version 6 (GraphPad Software Inc., La Jolla, CA, USA).

3.7 Purification of ACE inhibitory peptides

3.7.1 Ultrafiltration

For fractionation of ACE inhibitory shiitake mushroom peptides, 500 mL of shiitake mushroom hydrolysate was prepared by filtration through a filter paper (Whatman No. 1) and fractionated using an ultrafiltration unit (Pellicon XL Filter; Merck Millipore, Billerica, MA, USA) through four ultrafiltration membranes with molecular weight (Mw) cut-off values of 10, 5, 3, and 0.65 kDa. The ultrafiltration was performed sequentially. Initially the material

was separated through a 10 kDa membrane to collect the retentate and permeate separately. The retentate was the >10 kDa fraction. The permeate obtained from the 10 kDa membrane was then filtered through a 5 kDa membrane and similarly progressed to 3 and 0.65 kDa membranes. Five fractions with MW values < 0.65, 0.65–3, 3–5, 5–10, and > 10 kDa were obtained. The fractions were stored at 4 °C until used. The soluble protein content and the ACE inhibitory activity of each fraction were evaluated. The fraction with the highest ACE inhibitory activity was concentrated and stored at –20 °C before further purification.

3.7.2 RP-HPLC

RP-HPLC used a Luna C18 (4.6 mm x 250 mm) column. A 1 mL aliquot of the concentrated fraction obtained from ultrafiltration was filtrated through a 0.45 μm NYL Whatman membrane. Two hundred microliters of this fraction was injected into the HPLC system. The separation was achieved by gradient elution using solvent A: 0.1% (v/v) trifluoroacetic acid (TFA) in distilled water and solvent B: 0.05% (v/v) TFA in acetonitrile (ACN). The elution was performed using a linear gradient of 88% elute A and 12% elute B for 55 min at a flow rate of 0.7 mL/min. The absorbance of the eluent was monitored at 280 nm by a UV detector. The high and clear peaks were collected, concentrated, and checked for ACE inhibitory activity, and the fraction showing the strongest activity was freeze-dried and the peptide sequence was identified by MS.

3.8 Identification of the amino acid sequence of the purified peptide by MS

Characterization of the purified ACE inhibitory peptide was performed using a LC-MS/MS quadrupole time-of-flight (Q-TOF) mass spectrometer (Bruker). LC-MS/MS Q-TOF was used to analyze peptide chains in the mass range of 25–20,000 m/Z. All collected LC-MS/MS Q-TOF data were analyzed by *de novo* peptide sequencing. The principle of *de novo* sequencing is to use the mass difference between two fragment ions to calculate the mass of an amino acid residue in the peptide chain. The mass obtained can usually be used to determine the residue. The definition of *de novo* peptide sequencing is peptide sequencing without using a database of the amino acid sequences. The samples were analyzed with Mascot database software (Matrix Science Ltd., London, UK). Protein identification was performed based on the SwissProt (Fungi) and NCBI (Fungi) databases.

3.9 Peptide synthesis

The identified potential ACE inhibitory peptide from the shiitake hydrolysate was chemically synthesized by standard Fmoc solid phase synthesis [at Bootech Bioscience & Technology Co., Ltd. (Shanghai, China)] using an Applied Biosystems Model 433A Synergy peptide synthesizer. The purification of the peptide was verified by an analytical MS system (Thermo Mod. FinniganTM LXQTM) coupled to a Surveyor HPLC. The ACE inhibitory activity of the peptide was determined by ACE assay. The synthetic peptide had the peptide sequence: KIGSRSRFDVT; molecular weight 1265.43 Da; and 98% purity.

3.10 Kinetics study

To estimate the ACE inhibition pattern, different concentrations of the synthesized peptide (0.8, 0.6, 0.4, and 0 mg/mL) were incubated with an ACE solution along with different concentrations of HHL (1, 2, 3, 4, and 6 mM) as illustrated in ACE inhibitory activity *assay* section. The inhibition kinetics in the presence and absence of peptide were investigated by Lineweaver–Burk plots. The Lineweaver-Burk plot was plotted with the reciprocal of the HHL concentration used as the independent variable (X-axis) and the absorbance at 420 nm (HL+TNBS complex) as the dependent variable (Y-axis).

3.11 Molecular docking of KIGSRSRFDVT in the ACE binding site

Molecular docking studies were performed using Hermes 1.10.1 software. The three-dimensional crystal structure of a human ACE-lisinopril complex (1O8A.pdb) was downloaded from the RCSB PDB Protein Data Bank (http://www.rcsb.org/pdb/home/home.do) and was used as the receptor in this study. The structure of the peptide was generated with Discovery Studio 20 software. Before the docking, all hetero atoms, including water molecules and the inhibitor lisinopril were removed from the ACE model. The polar hydrogens were then added to the ACE model. Evaluation of the molecular docking was performed according to the score of several scoring functions, including ChemScore, ChemPLP, ASP, and Goldscore. According to the scores and binding energy, the best pose for each residue was obtained. Discovery Studio 20 software was used to identified the hydrogen bonds, and the hydrophobic, hydrophilic, electrostatic, van der Waals, and coordination interactions between peptide residues and the ACE molecule, as well as the best pose for the peptide.

3.12 Statistical analysis

All the experiments were carried out in triplicate, and the results were expressed as mean ± standard deviation and analyzed by SPSS 11.5 statistical software. The hypothesis testing method included one-way analysis of variance (ANOVA) followed by least significant difference (LSD) test, $P < 0.05$ was considered to be significant.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Optimization of shiitake mushroom protein hydrolysis

According to preliminary tests (changing one factor at a time), the degree of hydrolysis of the proteins was measured and used for determining the center point, using time: 3 h; temperature: 50 °C; and enzyme/substrate ratio: 1%. In RSM experiments, based on the single-factor experimental results, the three factors in the hydrolysis were varied at five levels in a CCD and the response surface of the DH was analyzed with Design-Expert 8.0.5. The three independent factors varied were time, temperature, and enzyme/substrate. The design of the 17 experiments and the responses of each experiment are shown in Table 4.1. As seen in the table, the DH varied from 0% to 28.88%. The maximum DH (28.88%) was generated with the conditions: time 3 h; temperature 50 °C; and enzyme/substrate (E/S) ratio 1%. To examine the effects of the conditions on the DH, a second order polynomial model was obtained by multiple regression analyses as follows:

$$Y = 27.54-0.062A+0.45B+4.34C-3.61A^2-4.28B^2-6.76C^2+0.57AB-0.038AC-0.30BC$$

Where A, B, and C are the coded values of time (h), temperature (°C), and enzyme/substrate (E/S) ratio, respectively, while Y is the value of DH.

The optimum conditions for hydrolysis predicted by the model were: time 2.99 h; temperature 50.21 °C; and E/S 1.16%. The predicted DH was 28.244%. Under these optimum conditions, the achieved degree of hydrolysis was 28.992% with error 2.648%, which is close to the predicted value. Therefore, the response surface method predicted accurate, reliable, valid, and adequate values for shiitake mushroom protein hydrolysate preparation.

The linear (A, B, C), quadratic (A^2, B^2, C^2), and interaction terms (AB, AC, BC) of the effects of the variables on the DH of shiitake protein hydrolysate were evaluated in terms of their adequacy, fitness, and significance by analysis of variance (ANOVA) as shown in Table 4.2. The ANOVA for the DH results showed that the obtained second-degree polynomial model gave extremely significant results ($P < 0.0001$) with a satisfactory determination coefficient ($R^2 = 0.9866$), suggesting that this model could accurately predict the relationship between the independent variables and the response. The adjusted determination coefficient (adjusted $R^2 = 0.9694$) was also high, indicating the high accuracy of this model. The lack of fit ($P = 0.6177$, > 0.05) of this model was not significant. Thus, this model could accurately predict the DH values. The *P*-values were used to indicate whether a regression coefficient was significant. The coefficient becomes more significant in tandem

with a decrease in the *P*-value. Regression coefficient analysis indicated that the linear coefficients (C) were very significant ($P < 0.0001$) and A^2, B^2, and C^2 were also extremely significant ($P < 0.0001$) regarding the response DH. The response surfaces for the effects of variables on the DH values are shown in Figure 4.1. The results suggested that the effect of the enzyme/substrate ratio has the most influence on the DH, and the value of the E/S may affect the sufficiency of the reaction of Alcalase with the substrate. The 3D response surface plots shown in Figure 4.1 are graphical representations of the regression equation. These plots are used to understand the interactions of the factors and determine the optimal level of each factor for maximal response. Each response surface plotted for the DH value represents a different combination of two test variables at one time, while maintaining the other variables at the zero level.

Table 4.1 Experimental design and results of the CCD

Run	Coded values of variable			Experimental values			Response	
	A	B	C	Time (h)	Temp. (°C)	E/S (%)	DH (%)	ACE inhibitory activity (IC_{50}; μg/mL)
1	1	-1	-1	4	45	0.5	8.5	0.39
2	0	0	0	3	50	1	28.88	1.29
3	0	0	0	3	50	1	27.92	1.94
4	0	0	0	3	50	1	26.07	1.29
5	-1	-1	-1	2	45	0.5	8.31	7.68
6	-1	1	-1	2	55	0.5	9.83	11.90
7	0	0	1.68	3	50	1.84	15.52	4.59
8	0	1.68	0	3	58	1	15.28	8.23
9	1.68	0	0	4.68	50	1	16.04	4.53
10	1.68	0	0	1.32	50	1	17.25	10.06
11	-1	1	1	2	55	1.5	16.64	4.15
12	0	-1.68	0	3	41.6	1	14.20	6.88
13	1	-1	1	4	45	1.5	16.36	6.71
14	1	1	-1	4	55	0.5	10.37	5.52
15	-1	-1	1	2	45	1.5	18.23	9.70
16	1	1	1	4	55	1.5	18.95	4.32
17	0	0	-1.68	3	50	0.15	0	3.04

Table 4.2 Analysis of variance for the response of DH

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	856.80	9	95.20	52.14	< 0.0001
A-Time	0.053	1	0.053	0.029	0.8693
B-Temperature	2.82	1	2.82	1.54	0.2540
C-E/S	257.21	1	257.21	140.88	< 0.0001
AB	2.57	1	2.57	1.41	0.2745
AC	0.012	1	0.012	0.0065	0.9382
BC	0.71	1	0.71	0.39	0.5514
A^2	146.27	1	146.27	80.12	< 0.0001
B^2	206.17	1	206.17	112.93	< 0.0001
C^2	513.03	1	513.03	281.00	< 0.0001
Residual	12.78	7	1.83		
Lack of Fit	8.70	5	1.74	0.85	0.6177
Pure Error	4.08	2	2.04		
Cor Total	869.58	16			
$P < 0.05$			$R^2 = 0.9853$		

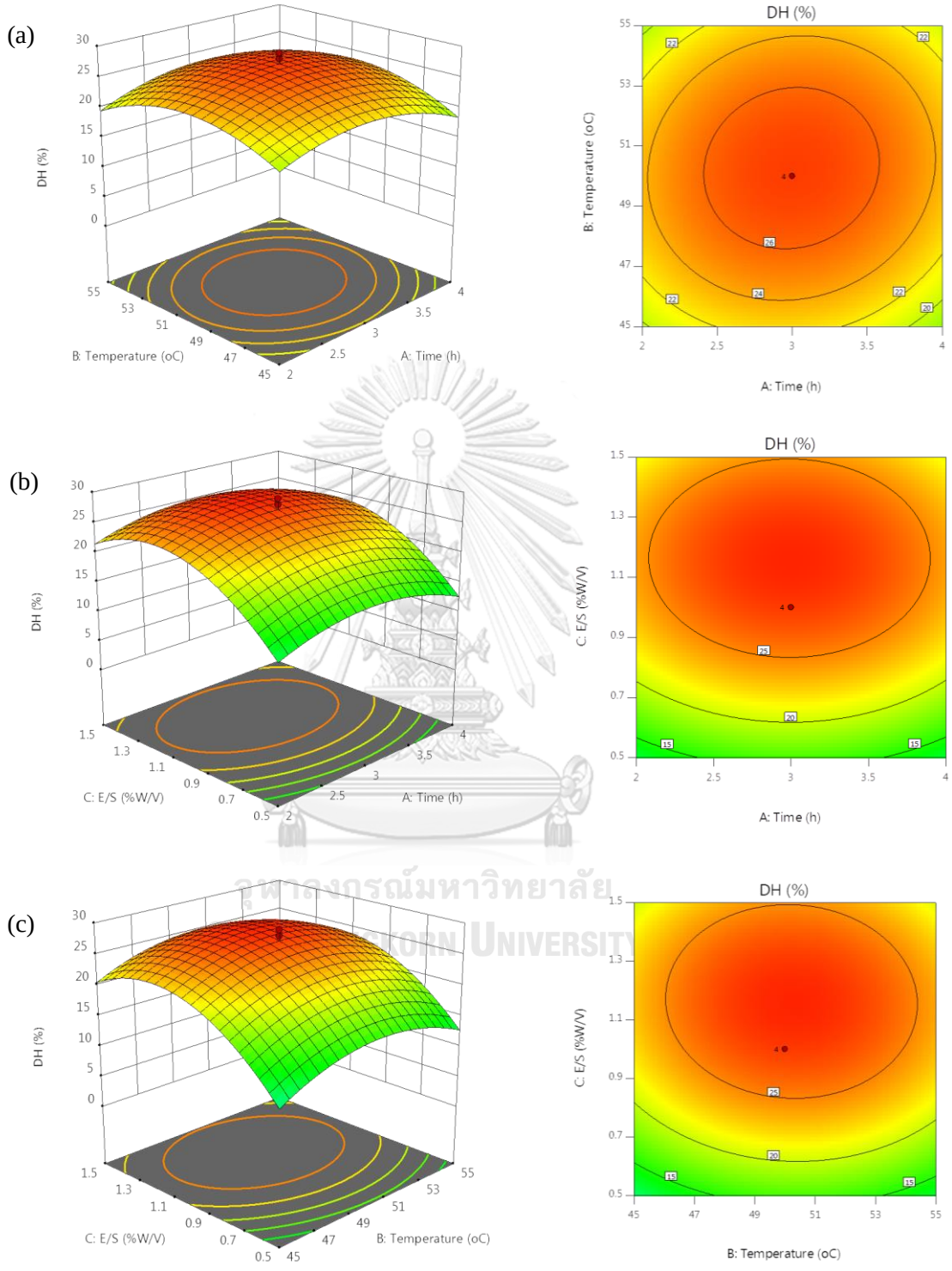


Figure 4.1 Response surface graphs for the %DH with the different factors. (a) The interactive effect of temperature and time. (b) The interactive effect of E/S and time. (c) The interactive effect of temperature and E/S.

4.2 Optimization of the ACE inhibitory activity of the peptides

The ACE inhibitory activity of the shitake mushroom hydrolysates are expressed as IC_{50} values and presented in Table 4.1. The shiitake mushroom hydrolysates had IC_{50} values in the range 0.39–11.90 μg/mL. The highest activity was shown with the hydrolysis conditions of 4 h, 45 °C, and an E/S of 0.5%. The ANOVA results for the ACE inhibitory activity of shiitake mushroom hydrolysates are shown in Table 4.3 The regression model was highly significant ($P < 0.0001$), and the lack of fit ($P = 0.0588$, > 0.05) was not significant. The R^2 value of this model indicated that 98.65% of the ACE inhibitory activity of the shiitake mushroom hydrolysate could be explained by this model. The Adjusted R^2 value was 0.9743 confirmed that the model could be used to accurately predict the ACE inhibitory activity of the shiitake mushroom hydrolysate. To study the parameters of the conditions on the ACE inhibitory activity, a second order polynomial model was obtained by multiple regression analyses as follows:

$$Y = 1.41-1.89A+0.27B+0.15C+2.04A^2+2.13B^2+0.81C^2+0.51AB+1.36AC-2.16BC$$

Where Y is the predicted response in real value (IC_{50} value for ACE inhibition); A is the coded value of variable time; B is the coded value of variable temperature; and C is the coded value of variable E/S.

For a verification test, an experiment was carried out to confirm the adequacy of the models at a time of 2.06 h, a temperature of 52.78 °C, and an E/S of 1.5%. The results showed that the experimental IC_{50} value for ACE inhibitory activity was 0.32 μg/mL, while the predicted value was 0.399 μg/mL. The model revealed that time was the most significant factor affecting the ACE inhibitory activity ($P < 0.0001$). The treatment temperature and the enzyme substrate ratio were not significantly related to the DH. The interaction terms AC and BC and the quadratic terms A^2 and B^2 were extremely significant ($P < 0.0001$) and the other terms were not significant ($P > 0.05$). The response surfaces for the effects of variables on the ACE inhibitory activity are shown in Figure 4.3

Table 4.3 Analysis of variance for the hydrolysate response of ACE inhibitory activity

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	220.93	9	24.55	80.93	< 0.0001
A-Time	48.72	1	48.72	160.63	< 0.0001
B-Temperature	0.97	1	0.97	3.18	0.1047
C-E/S	0.29	1	0.29	0.96	0.3494
AB	2.06	1	2.06	6.78	0.0263
AC	14.72	1	14.72	48.54	< 0.0001
BC	37.35	1	37.35	123.12	< 0.0001
A^2	59.88	1	59.88	197.42	< 0.0001
B^2	65.40	1	65.40	215.60	< 0.0001
C^2	9.37	1	9.37	30.88	0.0002
Residual	3.03	10	0.30		
Lack of Fit	2.50	5	0.50	4.64	0.0588
Pure Error	0.54	5	0.11		
Cor Total	223.96	19			
$P < 0.05$			$R^2 = 0.9865$		

The conditions for optimum DH and ACE inhibitory capacity in shiitake mushroom hydrolysates, determined herein, were not the same as have been reported by others. Guo et al. (2009) investigated the effect of process conditions on the ACE inhibitory activity of hydrolyzed whey protein concentrates using response surface methodology. This group found that the whey protein hydrolysate had a DH in the range 3.0%–49.5%, the optimal conditions to obtain high ACE inhibitory activity were close to 92.2%, and the DH was only 18.8%. These results indicated that a higher DH did not guarantee higher ACE inhibitory activity. The ACE activity might depend on the amino acids in the peptide sequence and the hydrolysis process might release a greater amount of these amino acids, which would otherwise contribute to the ACE inhibitory activity of the peptides.

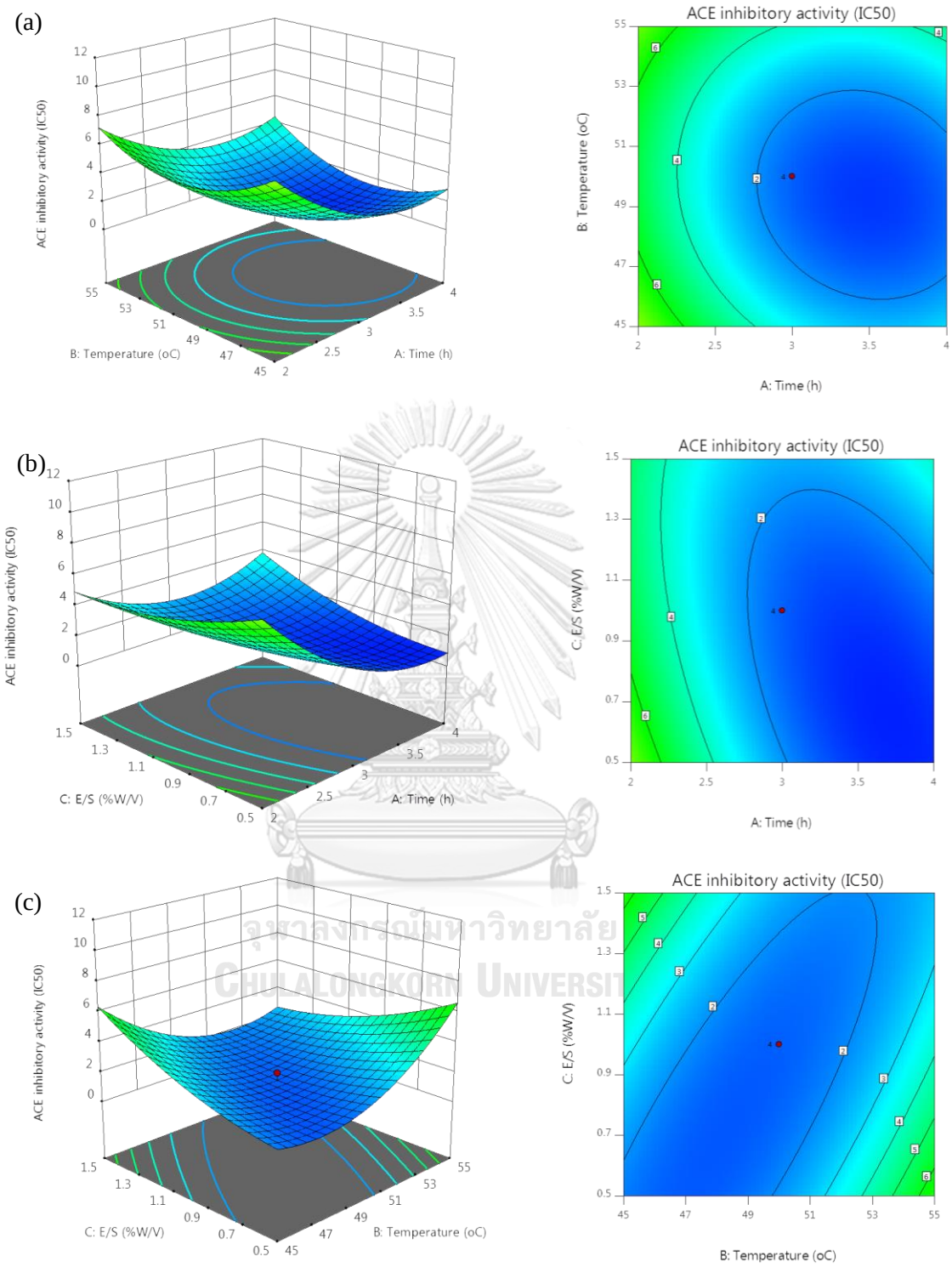


Figure 4.2 Response surface graphs for the ACE inhibitory activity with the different factors. (a) The interactive effect of temperature and time. (b) The interactive effect of E/S and time. (c) The interactive effect of temperature and E/S.

4.3 Purification of ACE inhibitory peptides

The shiitake mushroom sample that had demonstrated the highest ACE inhibitory activity under the optimal conditions was fractionated. Initially separation using a 10 kDa ultrafiltration membrane and then 5, 3, and 0.65 kDa membranes was used. The hydrolysate was sequentially fractionated into five individual fractions of > 10, 10–5, 5–3, 3–0.65, and < 0.65 kDa. The < 0.65 kDa fraction possessed the strongest inhibitory activity against ACE, while the fraction with molecular weight > 10 kDa was the least active, with an IC_{50} value of 8.17 μg/mL; the 10–5, 5–3, and 3–0.65 kDa fractions had IC_{50} values of 0.55, 0.071, and 0.26, respectively. These results suggested that the ACE inhibitory activity of the peptides is related to their molecular weight. In addition, the ultrafiltration of the shiitake hydrolysates improved the specific activity of the fractions. Considering that peptides with smaller molecular weights are absorbed easily by the living body, the < 0.65 kDa fraction was further separated to isolate ACE inhibitory peptides.

The molecular weight of peptides is also one of the factors that influence ACE inhibitory activity. The IC_{50} value of the shiitake mushroom hydrolysate fraction < 0.65 kDa was higher than for fractions with larger Mw values as reported by others (Mirzaei et al., 2015; Ngo et al., 2016; Salampessy et al., 2017; Wang et al., 2017). Ngo et al. (2016) investigated the ACE inhibitory activity of Pacific cod skin protein hydrolysates that were fractionated according to molecular weight using ultrafiltration membranes of 10, 5, and 1 kDa. Four fractions with different molecular weights of >10, 5–10, 1–5, and <1 kDa were obtained. The fraction < 1 kDa showed the strongest ACE inhibitory effect of 70%.

Rice bran protein has been hydrolyzed using trypsin and then separated by a membrane bioreactor system. The results for the ACE inhibitory activity of the fractions obtained corresponded with the molecular size, in which the fraction < 4 kDa exhibited the strongest ACE inhibitory activity with a relatively low IC_{50} value of 300 μg/mL, followed by the 4-6 and > 6 kDa fractions with IC_{50} values of 480 and 600 μg/mL, respectively (Wang et al., 2017). Toopcham et al. (2015) have studied tilapia mince hydrolyzed by *Virgibacillus halodenitrificans* SK1-3-7 proteinases. After ultrafiltration, the fraction < 5 kDa had the highest ACE inhibitory activity with a specific inhibitory activity of 87.74%, whereas the fraction with Mw > 30 kDa showed the lowest ACE inhibitory activity. These results confirmed that small peptides exhibit better ACE inhibition, and short peptides are the most efficient inhibitors because longer chain peptides typically bind less easily to ACE, resulting in lower inhibitory activity. Furthermore, small peptides may be easily absorbed in the digestive tract and exert biological effects.

4.4 Purifications of shiitake mushroom hydrolysates by RP-HPLC

The fraction with molecular weight < 0.65 kDa was purified by RP-HPLC as shown in Figure 4.3 After gradient elution over 55 min, the sample was fractionated into seven parts (1–7). After fractionation, the ACE inhibitory activity of each fraction was further evaluated using an *in vitro* ACE inhibitory assay to find the most effective fraction. Among the seven fractions, peak 1 with a retention time of 3.96 min had high ACE inhibitory activity (IC_{50} = 0.03287 μg/mL). Following activity analysis, the fraction with the retention time of 3.96 min (peak 1) was taken for determining the amino acid sequence.

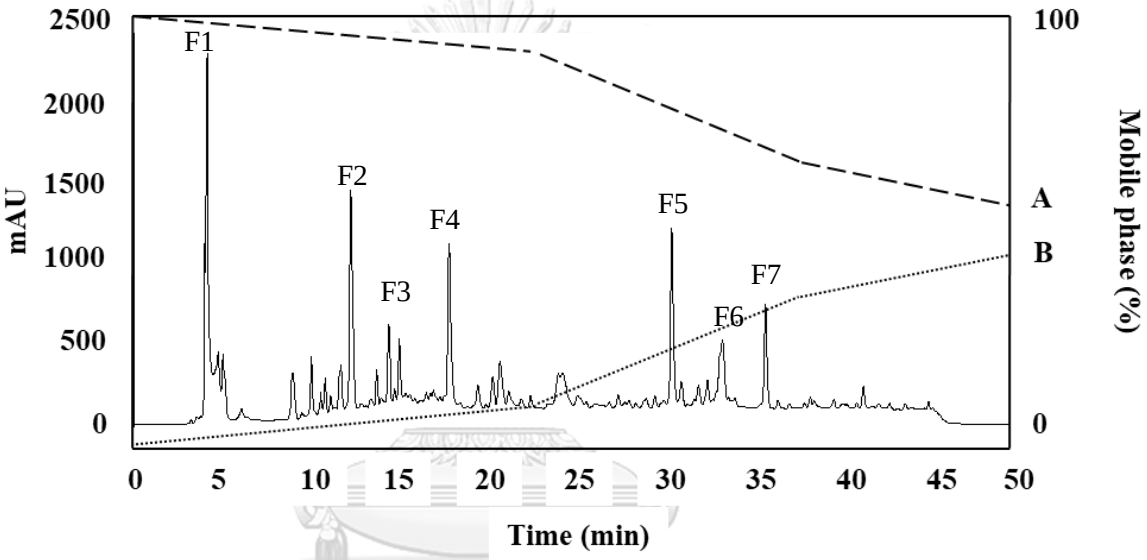


Figure 4.3 RP-HPLC profile of the active fraction (< 0.65 kDa) from shiitake protein hydrolysate. Eluent A: 0.1% trifluoroacetic acid (TFA), eluent B: 70% acetonitrile containing 0.05% TFA

4.5 Identification of peptide sequence

The amino acid sequence of the ACE inhibitory peptide as identified by Q-TOF2 was Lys-Ile-Gly-Ser-Arg-Ser-Arg-Phe-Asp-Val-Thr (KIGSRSRFDVT) Figure 4.4, with a molecular weight of 1265.43 Da. The fragment was aligned using NCBI GenBank and UniProt databases (*de novo* sequencing) to find the homologous region. The peptide sequence, KIGSRSRFDVT, revealed 63% (7/11) amino acid sequence similarity to the AFG1-like ATPase (*L. edodes*), but no exact match to the sequence of any known protein was found. The peptide was synthesized using the sequence as determined for the purified peptide. The IC_{50} value for the ACE inhibitory activity for the synthesized peptide was 0.3714 μM.

The ACE inhibitory properties of peptides are related to the composition, structure, size, sequence, and configuration of the peptide. The amino acids that are reported to have the most effect on the ACE inhibitory activity are tryptophan, tyrosine, proline, phenylalanine, and aliphatic amino acids. Koo et al. (2006) analyzed a hydrolysate of *Pholiota adipose* with distilled water and found the peptide sequence GEGGP with an IC_{50} value 0.25. Wang et al. (2017) isolated a peptide from rice bran with an ACE inhibitory activity IC_{50} value of 76 mM, a Mw of 395.0 Da, and the amino acid sequence YSK. According to several studies, the binding to ACE is strongly influenced by the amino acid sequence of the ACE inhibitor. It has been suggested that Trp, Tyr, Phe, Pro, and hydrophobic amino acids can contribute to the ACE inhibitory activity. In this study, the sequence of the peptide isolated from shiitake hydrolysate has Ile, Phe, and Val residues, which have been reported to have an effect on the ACE inhibitory activity. Therefore, the peptide KIGSRSRFDVT can be considered a potential bioactive peptide to treat hypertension.

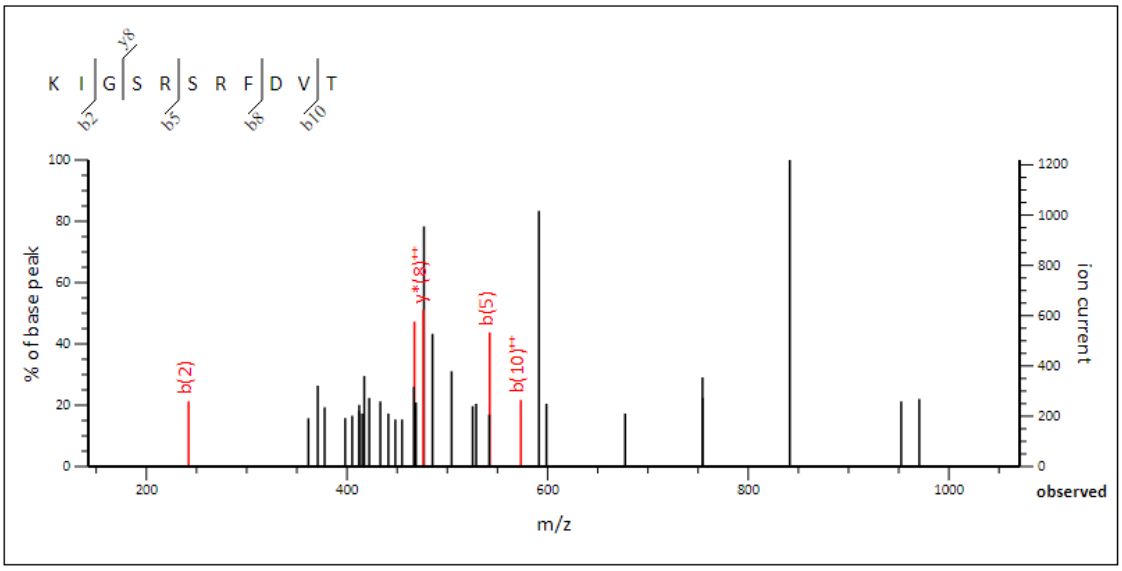


Figure 4.4 Identification of amino acid sequence and molecular mass of the ACE inhibitor peptide purified from the shiitake mushroom hydrolysate (peak 1 from RP-HPLC). Mass fragmentation spectrum of the ACE inhibitory peptide KIGSRSRFDVT.

4.6 Determination of the kinetic properties of ACE inhibition by KIGSRSRFDVT

To shed light on the mode of the ACE inhibition of KIGSRSRFDVT, the velocity of ACE, with different concentrations of HHL as the substrate, in the presence of fixed

concentrations of the peptide was analyzed. Figure 4.5 shows the ACE inhibition pattern of the purified peptide from shiitake mushroom hydrolysate analyzed by a Lineweaver-Burk plot using various concentrations of KIGSRSRFDVT (0–0.6 mg/mL). Four straight lines intersected at the same point on the 1/*S* axis of the Lineweaver-Burk plot, indicating the same maximum velocity regardless of the inhibitor concentration, whereas the slopes of these straight lines differed. Thus, it was concluded that the peptide KIGSRSRFDVT acted as a non-competitive inhibitor, suggesting that the binding of a substrate and ACE would not affect the inhibitory activity of this peptide. This ACE inhibitor from shiitake mushrooms may bind at a site on the ACE that is different from the active site where the substrate binds. The KIGSRSRFDVT peptide may combine with ACE molecules to produce a dead-end complex regardless of whether a substrate molecule is bound or not. The peptide may inhibit the enzyme by causing a conformational change, which inhibits the enzyme equally well at low and high concentrations of the substrate.

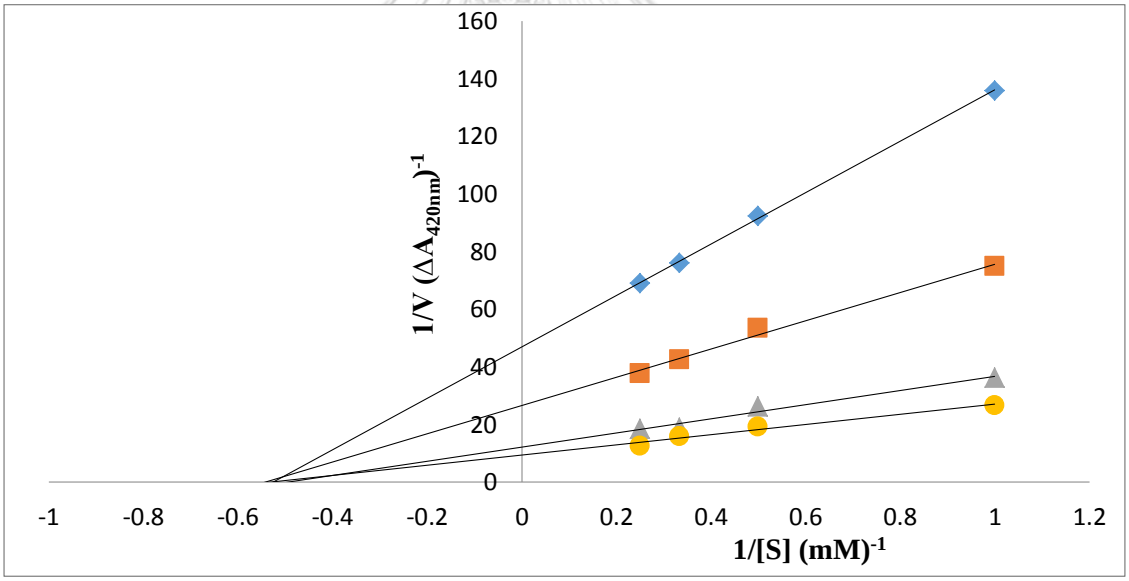


Figure 4.5 The Lineweaver-Burk plot of KIGSRSRFDVT ACE inhibition, No inhibitor (●), 0.4 mg/mL of peptide (▲), 0.6 mg/mL of peptide (■), and 0.8 mg/mL of peptide (♦).

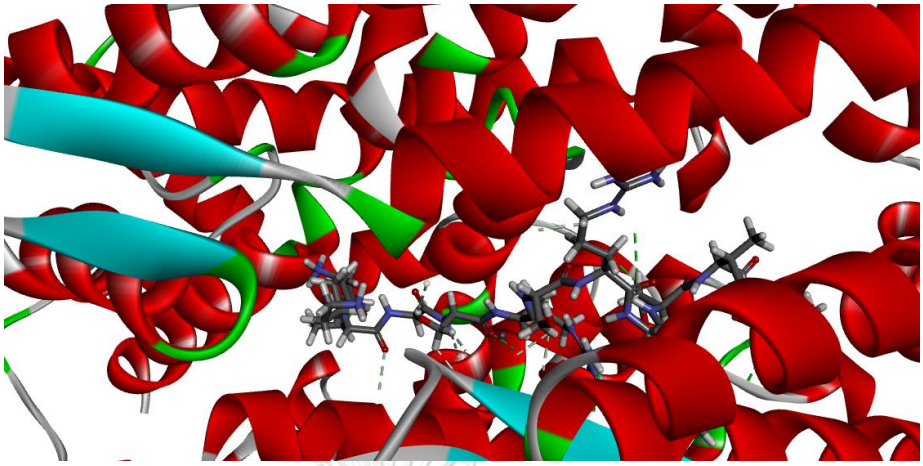
Recent studies have revealed that ACE inhibitory peptides can inhibit the activity of ACE via competitive, non-competitive, uncompetitive, and mixed-competitive inhibition patterns. Competitive ACE inhibitors have been frequently reported, such as WESLSRLLG from ostrich egg white (Asoodeh, Homayouni-Tabrizi, Shabestarian, Emtenani, & Emtenani, 2016), VVSLSIPR from pigeon pea (Nawaz et al., 2017), and TVGMTAKF and QLLLQQ from horse gram flour (Bhaskar et al., 2019). However, some non-competitive ACE inhibitor

patterns have also been reported. The ACE inhibitory activity of skate skin protein hydrolysates was investigated by Lee et al. (2011), they isolated the peptides PGPLGLTGP (975.38 Da) and ELGFLGPR (874.45 Da), which had IC_{50} values of 95 and 148 μM, respectively, and the Lineweaver–Burk plots suggested that these peptides acted as non-competitive inhibitors against ACE. Furthermore, the inhibition mode of several ACE inhibitory peptides has been revealed as noncompetitive, including PAFG from *Enteromorpha clathrata* (Pan et al., 2016); YAP, VIIF, and MAW from cuttlefish (Balti et al., 2010); AHIII from *Styela clava* flesh tissue (Ko et al., 2012); and VWDPPLFA from salmon (Ahn et al., 2012). Interestingly, the synthesis drugs including captopril, lisinopril, enalapril behave as competitive inhibitor of ACE *in vitro*. Moalli et al. (1985) investigated kinetics of captopril and enalapril type of inhibition toward ACE *in vivo* through nonlinear saturation model by utilizes data from indicator dilution pulmonary venous outflow curves. They found that both captopril and enalapril are non-competitive type of inhibition. Nonetheless, synthesis drugs may stronger against ACE than bioactive peptide; the bioactive peptides have higher tissue affinities and are subject to a slower elimination.

4.7 Molecular docking of KIGSRSRFDVT in the ACE binding site

Molecular docking is widely used to predict how an enzyme interacts with small molecules (ligands). Knowledge of the molecular mechanisms of the interactions between ACE and ACE inhibitory peptides has many potentially favorable consequences for further designing and synthesizing ACE inhibitors. The docking simulation between ACE and the peptide KIGSRSRFDVT was studied using the flexible docking tool of Hermes 1.10.1 software. In the docking study of the peptide KIGSRSRFDVT at the ACE non-active site, ligand A5 showed the best pose with an interaction score ASP fitness 30.8855 (RMSD 7.0137). The scoring results of a ligand–receptor combination can illustrate the potential stability of the interaction. The 3D interaction between ACE and the peptide KIGSRSRFDVT is shown in Figure 6(a). The 2D diagram in Figure 6(b) displays the interactions between the ACE residues and the peptide molecule. The peptide KIGSRSRFDVT established seven hydrogen bonds with the ACE residues Arg479, Asn31, Ala315, Glu345, Lys468, Tyr477, and His344 after docking. The residues in the ACE active site have been reported (M. Guo et al., 2017). ACE has three main active site pockets; S1, S2, and S1', which involve the residues Ala354, Glu384, Tyr523, Gln281, His353, Lys511, His513, Tyr520, and Glu162. Thus, the binding between KIGSRSRFDVT and ACE does not involve an active site pocket of ACE.

(a)



(b)

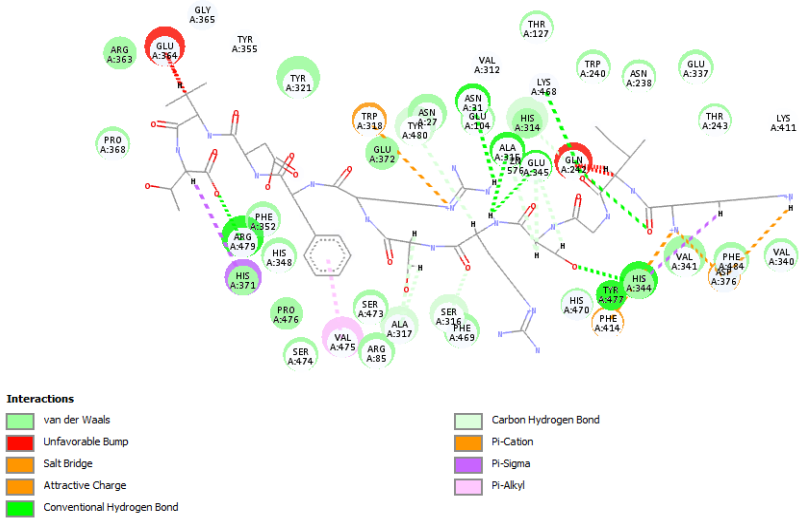


Figure 4.6 (a) The 3D diagram of the predicted interactions between the purified peptide (KIGSRSRFDVT) and ACE complex. (b) The 2D diagram of the predicted interactions between the purified peptide (KIGSRSRFDVT) and ACE molecule. Images obtained with Discovery studio 2019 software.

CHAPTER 5

CONCLUSION

An ACE inhibitory peptide generated from shiitake hydrolysate through enzymatic treatment using Alcalase was identified in this study. Central composite design and response surface methodology enabled the determination of the optimal operating conditions for obtaining greater DH and ACE inhibitory IC_{50} values. The validity of the model was proven by fitting the values of the variables to the model equation and by carrying out experiments using these values. Optimization of the analyzed responses indicated that the best results for production of a peptide, with strong ACE inhibitory activity (IC_{50} value of 0.32 μg/mL), was obtained with hydrolysis time: 3.46 h; temperature: 46.84 °C; and E/S: 0.59%. All points were located near the central point of the design. After fractionation by ultrafiltration, purification by RP-HPLC, and MS, KIGSRSRFDVT was identified as a potential ACE inhibitor, with an IC_{50} value of 0.37 μM, which acted in a non-competitive manner as determined from the Lineweaver-Burk plot. The binding site of the purified peptide KIGSRSRFDVT with ACE was predicted using a molecular docking study. The KIGSRSRFDVT binding site with ACE was a non-active site. In conclusion, this peptide isolated from shiitake mushroom protein hydrolysate is a potential bioactive peptide for the treatment of hypertension and might be a good candidate for future industrial production of a functional food containing a natural ingredient that mediates hypertension. However, further studies are necessary to investigate the cytotoxicity, allergenicity, and safety of the peptide before application in the food and medicinal industries.

APPENDICES

APPENDIX A

Buffer and reagents preparation

1. 20 mM Phosphate buffer, pH 7.2 (Hydrolysate buffer), 1 L

20 mM KH_2PO_4	2.721 g
20 mM K_2HPO_4	3.483 g
150 mM NaCl	8.766 g
Deionized water	1,000 mL

Protocol: Mix 100 mL of deionized water with all reagents. Adjust the pH to 7.2 by 6 M NaOH or 1 M HCl. Storage at 4 ˚C until use.

2. Bradford solution and protocol

2.1 1mg/mL bovine serum albumin (BSA), 10 mL

BSA	10 mg

10 mg BSA was dissolved by 10 mL deionized water and mix well for standard curve protein concentration. Storage at 4 ˚C until use.

2.2 Bradford Stock Solution, 650 mL

95% Ethanol	100 mL
88% Phosphoric acid	200 mL
Serva Blue G Dye	350 mg

Stable indefinitely at room temperature

2.3 Bradford Working Buffer, 500 mL

Bradford stock solution	30 mL
Deionized water	425 mL
95% Ethanol	15 mL
88% Phosphoric acid	30 mL

Note: before using, Bradford working solution must be filtrated through the Whatman No.1 paper. Bradford working solution is kept in a brown glass bottle at room temperature. Usable for several weeks but may need to be refiltered.

Bradford's protocol: 1. Pipet 100 µL of sample into 1.5 mL microtube.

2. Add 1 mL Bradford Working Buffer and mix well solution.

3. Pipet 200 µL of solution into 96-well plate.

4. Shake and read absorbance at 595 nm after 2 minutes but before 1 hour.

3. OPA solution, 200 mL

Step 1 Sodium tetraborate decahydrate 7.62 g

Sodium dodecyl sulfate (SDS) 200 mg

Dissolve in 150 mL of deionized water

Step 2 Phthaldialdehyde (Khuri & Mukhopadhyay)

160 mg

Dissolve in 4 mL ethanol

Step 3 Transfers solution from step 2 to solution from step 1

Step 4 Add 176 mg Dithiothreitol (DTT) in solution from step 3.

Add deionized water for making solution up to 200 mL.

4. Angiotensin I converting enzyme inhibitory activity solutions

4.1 0.01 M potassium phosphate buffer pH 7.0 containing 0.5 M NaCl (KPBS), 10 mL

KH_2PO_4 0.01361 g

K_2HPO_4 0.0174 g

NaCl 0.2772 g

Dissolve all chemicals in deionized water, mix well by vortex and adjust the volume to 1 L and the pH to 7.0 by 6 M NaOH or 1 M HCl Storage at 4 ˚C until use.

4.2 50 mM Potassium Phosphate buffer pH 8.3 containing 0.3 M NaCl (KPBS), 100 mL

KH_2PO_4 0.6805 g

K_2HPO_4 0.871 g

NaCl 1.6632 g

Dissolve all chemicals in deionized water, mix well by vortex and adjust the volume to 1 L and the pH to 8.3 by 6 M NaOH or 1 M HCl Storage at 4 ˚C until use.

4.3 ACE solution (200 mU/mL), 1 mL

ACE (≥ 2.0 units/mL) 100 µL

50 mM KPBS 900 µL

2 U/mL ACE diluted to 200 mU/mL in PPBS and fresh preparing.

4.4 5mM substrate HHL solution, 10 mL

Hippuryl-His-Leu acetate salt 0.021474 mg

Dissolve in deionized water 10 mL and mix well. Storage at 4 ˚C until use

4.5 200 mM Potassium Phosphate buffer, 300 mL

KH_2PO_4 8.166 g

K_2HPO_4 10.452 g

4.6 10 mM sodium sulfite, 200 mL

Na_2SO_4 0.2521 g

Dissolve in deionized water 200 mL and mix well. Storage at 4 ˚C until use

4.7 0.68 mM Picrylsulfonic acid (TNBS),

5% (W/V) Picrylsulfonic acid 0.8 mL

Dissolve in deionized water 9.8 mL and mix well. Storage at 4 ˚C until use

5. Mobile phase in RT-HPLC analysis

Eluent A: 0.1% trifluoroacetic acid (TFA), 1000 mL

Double deionized water 999 mL

TFA 1 mL

Mix and Filtrate through a cellulose acetate membrane

Eluent B: 70% acetonitrile containing 0.05% TFA, 500 mL

Step 1 Filtrate 350 mL acetonitrile through PTFE membrane

Step 2: Add 75 µL of TFA in 150 mL double deionized water. Then, filtrate though a cellulose acetate membrane. Add this solution into filtrated acetonitrile.

Both of eluent, before using, must be degassing by sonication 15 minutes.

APPENDIX B

Standard cure for determine the protein concentration by Bradford method

Bovine serum albumin (BSA) protein derived from cows, is used as a protein concentration standard in experiments. The standard curve remains linear from about 2.5 µg to 15 µg of BSA were prepared following

µg Protein (µg)	Standard solution (1 mg/mL BSA)	Experiment Buffer (µg)	Bradford Reagent (mL)	A_{595}
0	0	100	1	0.2569
2.5	2.5	97.5	1	0.3460
5	5	95	1	0.4326
7.5	7.5	92.5	1	0.4788
10	10	90	1	0.5694
12.5	12.5	87.5	1	0.6092
15	15	85	1	0.6719
17.5	17.5	82.5	1	0.7515
20	20	80	1	0.8296

Absorbance at 595 nm was plot as y-axis and BSA protein concentration was plot as x-axis.

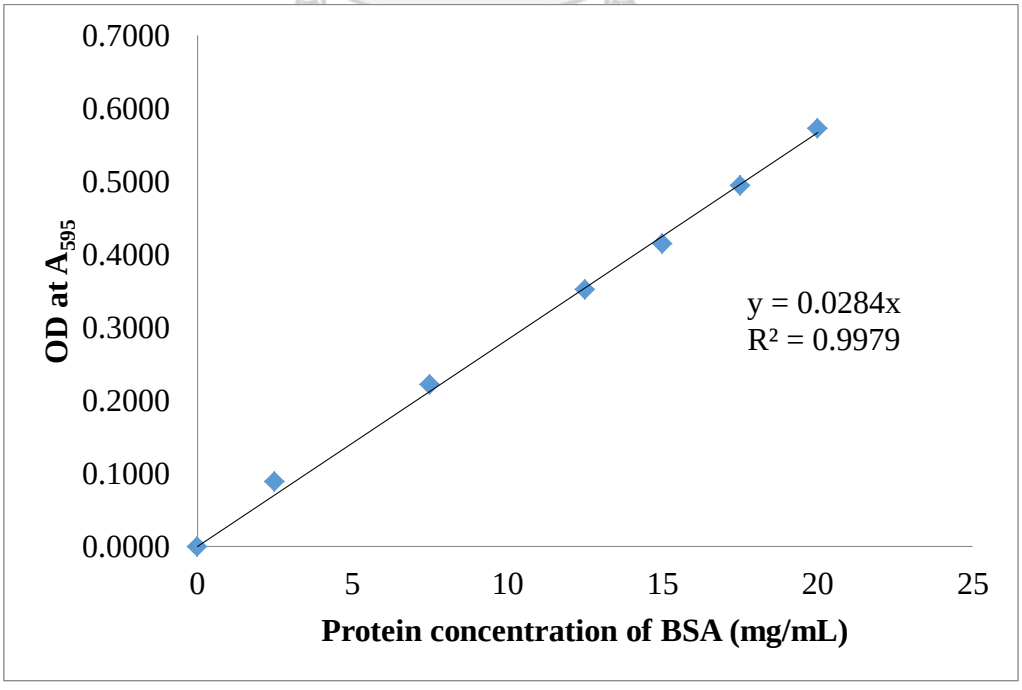


Figure B. 1 Standard curve of BSA concentration at the absorbance of 595 nm

APPENDIX C

Amino acid abbreviations

Amino acid	Three-letter code	One-letter code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid or aspartate	Asp	D
Cysteine	Cys	C
Glutamic acid or glutamate	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tryosine	Tyr	Y
Valine	Val	V

APPENDIX D

Peptide sequences identification by Q-TTOF mass spectrometry that was taken from the RP-HPLC. Sequences KIGSRSRFDVT was searched by Omphalotaceae (taxid: 72117) Family

organism	Query cover	Identity	accession
AFG1-like ATPase [*Lentinula edodes*]	63%	100%	gi|1139907730|GAW08606.1
hypothetical protein GYMLUDRAFT_158951 [*Gymnopus luxurians* FD-317 M1]	90%	64%	gi|751024085|KIK65785.1
hypothetical response regulator receiver protein [*Lentinula edodes*]	90%	75%	gi|1139910882|GAW05872.1
hypothetical protein GYMLUDRAFT_49772 [*Gymnopus luxurians* FD-317 M1]	90%	75%	gi|751010929|KIK52709.1
DNase I-like protein [*Lentinula edodes*]	63%	86%	gi|1139918014|GAV99052.1
DNase I-like protein [*Lentinula edodes*]	63%	86%	gi|1139918013|GAV99051.1
hypothetical protein GYMLUDRAFT_54378 [*Gymnopus luxurians* FD-317 M1]	63%	86%	gi|751025881|KIK67576.1
protein [Laccaria bicolor S238N-H82] XP_001878005 [*Lentinula edodes*]	54%	83%	gi|1139916473|GAW00584.1
alpha beta-hydrolase [*Lentinula edodes*]	72%	88%	gi|1139914313|GAW02585.1
hypothetical protein GYMLUDRAFT_358547 [*Gymnopus luxurians* FD-317 M1]	54%	83%	gi|751023838|KIK65538.1
alpha beta fold family [*Lentinula edodes*]	63%	78%	gi|1139906586|GAW09337.1

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