PRODUCTION OF ANGIOTENSIN I-CONVERTING ENZYME (ACE) INHIBITORY PEPTIDES DERIVED FROM CHICKEN BLOOD

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การผลิตเพปไทด์ที่มีสมบัติยับยั้งเอนไซม์เปลี่ยนรูปแองจิโอเทนซินจากเลือดไก่

นางสาววาสนา วงค์งาม

มหาวิทยาลัยเทคโนโลยีสุรนารี

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาเทคโนโลยีอาหาร
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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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วาสนา วงค์งาม : การผลิตเพปไทด์ที่มีสมบัติยับยั้งเอนไซม์เปลี่ยนรูปแองจิโอเทนซินจากเลือดไก่ (PRODUCTION OF ANGIOTENSIN I-CONVERTING ENZYME (ACE) INHIBITORY PEPTIDES DERIVED FROM CHICKEN BLOOD) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.จิรวัฒน์ ยงสวัสดิกุล, 119 หน้า.

วัตถุประสงค์ของงานวิจัยนี้คือ เพื่อเพิ่มมูลค่าเลือดไก่โดยผลิตเพปไทด์ที่มีสมบัติยับยั้งเอนไซม์เปลี่ยนรูปแองจิโอเทนซิน (angiotensin I-converting enzyme; ACE) ย่อยตัวอย่างเลือดไก่ส่วนต่างๆ ได้แก่ น้ำเลือด (whole blood; WB) พลาสมา (blood plasma; BP) และเม็ดเลือด (blood corpuscle; BC) โดยใช้เอนไซม์ชนิดต่างๆ ได้แก่อัลคาเลส (Alcalase) พาเพน (papain) เพปซิน (pepsin) เทอโมไลซิน (thermolysin) และเอนไซม์ที่ผลิตจากแบคทีเรียสายพันธุ์ *Virgibacillus* sp. SK1-3-7 โปรตีนไฮโดไลเสทจากเม็ดเลือด (blood corpuscle hydrolysate, BCH) ย่อยด้วยอัลคาเลสที่ระดับการย่อย (degree of hydrolysis; DH) 14.53% แสดงค่าการยับยั้งเอนไซม์ ACE ที่ร้อยละ 61.8 (ความเข้มข้นเพปไทด์ 0.5 มิลลิกรัม/มิลลิลิตร) จากวิธี response surface methodology (RSM) สภาวะที่เหมาะสมสำหรับการผลิตโปรตีนไฮโดรไลเสทจากเม็ดเลือด คือที่อุณหภูมิ 51.1 องศาเซลเซียส ด้วยเอนไซม์อัลคาเลสร้อยละ 4 (E/S) ควบคุมค่า pH ที่ 9.6 ตลอดการย่อย 6 ชั่วโมง โดยได้ระดับการย่อยที่ร้อยละ 35.8 และยับยั้งเอนไซม์ ACE ร้อยละ 37.7 (ความเข้มข้นเพปไทด์ 0.2 มิลลิกรัม/มิลลิลิตร) เพปไทด์ที่ผ่านกระบวนการอัลตราฟิวเตชั่น (ultrafiltration) ที่เยื่อกรองขนาด 1 กิโลดาลตัน (1-kDa permeate; BCH-III) แสดงประสิทธิภาพการยับยั้งเอนไซม์เพิ่มขึ้น 2.5 เท่าเมื่อเทียบกับโปรตีนไฮโดรไลเสททั้งโปรตีนไฮโดรไลเสท BCH และ ส่วนที่ผ่านเยื่อกรอง BCH-III มีองค์ประกอบกรดอะมิโนฟีนิลอะลานิน ไกลซีน อะลานิน วาลีน และลูซีนในปริมาณมาก หลังจากป้อนตัวอย่างโปรตีนไฮโดรไลเสท BCH และ ส่วนที่ผ่านเยื่อกรอง BCH-III ที่ความเข้มข้น 600 และ 200 มิลลิกรัม/กิโลกรัม ตามลำดับ ให้กับหนูความดันสูง (spontaneously hypertensive rat; SHR) พบว่ามีผลลดความดันซิสโทลิค (systolic blood pressure; SBP) ประมาณ -43.0 มิลลิเมตรปรอท และลดความดันไดแอสโทลิค (diastolic blood pressure; DBP) ได้สูงสุดที่ปริมาณการป้อนตัวอย่างโปรตีนไฮโดรไลเสท BCH-III ที่ความเข้มข้น 100 มิลลิกรัม/กิโลกรัม

แฟรคชั่น G3 ที่ได้จากไฮโดรไลเสทส่วนที่ผ่านเยื่อกรอง 1 กิโลดาลตัน ที่ผ่านการแยกโครมาโตกราฟฟีตามขนาด (size exclusion chromatography) แสดงกิจกรรมยับยั้งเอนไซม์ ACE สูงสุดด้วยค่า IC_{50} เท่ากับ 66 ไมโครกรัม/มิลลิลิตร และเมื่อนำไปทำบริสุทธิ์ต่อด้วยหลักการรีเวอร์สเฟสโครมาโตกราฟฟี (reverse-phase chromatography) พบว่ามี 2 พีคที่มีกิจกรรมยับยั้งเอนไซม์ ACE สูงสุดโดยมีค่า IC_{50} เท่ากับ 14.0 และ 18.0 ไมโครกรัม/มิลลิลิตร จากนั้นจึงนำทั้ง 2 พีควิเคราะห์ลำดับกรดอะมิโนด้วย LC-MS/MS tandem mass spectrometry พบว่าเพปไทด์ VSKRLNGDA หลัง

ผ่านการย่อยอาหารแบบจำลองแสดงกิจกรรมยับยั้งเอนไซม์ ACE สูงสุดโดยมีค่า IC_{50} เท่ากับ 26.46 ไมโครโมลาร์ และเมื่อให้เพปไทด์ในปริมาณ50 มิลลิกรัม/กิโลกรัมหนู กับหนูความดันสูงสามารถลดค่าความดันซิสโทลิคลงได้ -32.0 มิลลิเมตรปรอท ภายในเวลา 12 ชั่วโมง และสามารถลดความดันไดแอสโทลิคได้สูงสุดที่ระดับความเข้มข้นเพปไทด์ในช่วง 12.5-50 มิลลิกรัม/กิโลกรัม

เมื่อศึกษาผลการป้อนตัวอย่างโปรตีนไฮโดรไลเสทที่ผ่านเยื่อกรองขนาด 1 กิโลดาลตัน BCH-III และเพปไทด์ VSKRLNGDA ให้กับหนูความดันสูงทั้งในระยะสั้น (1 สัปดาห์) และระยะยาว (4 สัปดาห์) พบว่า หนูกลุ่มที่ป้อน VSKRLNGDA ปริมาณ 50 มิลลิกรัม/กิโลกรัม มีค่าความดันโลหิตลดลงสูงสุดเทียบเท่ากับหนูในกลุ่มที่ป้อนด้วยยาแคปโตพริล (captopril) ปริมาณ 10 มิลลิกรัม/กิโลกรัม หลังสัปดาห์ที่ 4 ผลของหนูในกลุ่มที่ป้อนเพปไทด์ VSKRLNGDA มีการแสดงออกของจีน (gene) เรนิน (renin) และ ตัวรับแองจิโอเทนซิน II ชนิดที่ I (angiotensin II type-1 receptor; AT-1) ลดลง และจีนอะดรีโนเซปเตอร์ชนิดเบต้า 3 (adrenoceptor β-3; AR-β3) และตัวรับเพอรอคซิโซมโปรลิเฟอเรเตอร์ที่ถูกกระตุ้นชนิดเดลต้า (peroxisome proliferator-activated receptor δ; Pparδ) เพิ่มขึ้น เมื่อวิเคราะห์จากระดับ mRNA ของเนื้อเยื่อไตด้วยวิธี real-time polymerase chain reaction (RT-PCR) ดังนั้นโปรตีนไฮโดรไลเสทจากเลือดไก่และเพปไทด์ VSKRLNGDAไม่เพียงมีผลยับยั้งเอนไซม์ ACE แต่ยังเกี่ยวข้องกับการแสดงออกของจีนดังกล่าวด้วยเช่นกัน ไฮโดรไลเสทจากน้ำเลือดไก่มีศักยภาพที่สามารถพัฒนาเป็นผลิตภัณฑ์อาหารสุขภาพที่มีคุณสมบัติการลดความดันสูงได้

มหาวิทยาลัยเทคโนโลยีสุรนารี

สาขาวิชาเทคโนโลยีอาหาร ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2561 ลายมือชื่ออาจารย์ที่ปรึกษา_____

WASANA WONGNGAM : PRODUCTION OF ANGIOTENSIN I-CONVERTING ENZYME (ACE) INHIBITORY PEPTIDES DERIVED FROM CHICKEN BLOOD. THESIS ADVISOR : ASSOC. PROF. JIRAWAT YONGSAWATDIGUL, Ph.D., 119 PP.

ANGIOTENSIN I-CONVERTING ENZYME (ACE)/ANTIHYPERTENSIVE PEPTIDE/CHICKEN BLOOD/SPONTANEOUSLY HYPERTENSIVE RATS (SHR)

The objective of this study was to increase the value of chicken blood by producing peptides with angiotensin I-converting enzyme (ACE) inhibitory activity. Chicken blood fractions, namely whole blood (WB), blood plasma (BP) and blood corpuscles (BC), were hydrolyzed using various enzymes, including Alcalase, papain, pepsin, themolysin and *Virgibacillus* sp. SK1-3-7 protease. Blood corpuscles hydrolysate (BCH) derived from Alcalase showed the highest degree of hydrolysis (DH) of 14.53% and ACE inhibition of 61.8% at 0.5 mg/mL peptide. Based on the response surface methodology (RSM), the optimized condition of BCH was at 51.1°C, 4% E/S, and pH 9.6 for 6 h, resulting in 35.8% DH and 37.7% ACE inhibition at 0.2 mg/mL peptide. After ultrafiltration using 1-kDa molecular mass cut-off membrane, the permeate of BCH (BCH-III) showed an increase in ACE inhibition about 2.5 folds compared to BCH. Both BCH and its permeate were rich in F, G, A, V, I and L. Oral administration of BCH and BCH-permeate to the spontaneously hypertensive rats (SHR) at 600 and 200 mg/kg body weight, respectively, lowered systolic blood pressure (SBP) up to around -43.0 mmHg. The maximum reduction of diastolic blood pressure (DBP) was observed at doses of permeate BCH of 100 mg/kg.

The fraction G3 obtained from size exclusion chromatography of BCH-III possessed the highest ACE inhibition with IC_{50} of 66 µg/mL. Further purification by reverse-phase chromatography resulted in 2 peaks exhibiting IC_{50} of 14.0 and 18.0 µg/mL, respectively. The *de novo* peptide sequencing by LC-MS/MS revealed VSKRLNGDA with high IC_{50} of 26.46 µM after gastrointestinal (GI) digestion and the peptide at 50 mg/kg showed reduction of SBP of -32.0 mmHg within 12 h. A maximum decrease in DBP was observed a dose ranging from 12.5 to 50 mg/kg.

The effect of short- (1 week) and long-term (4 weeks) intake of BCH, BCH-III and the VSKRLNGDA on blood pressure were investigated *in vivo*. The VSKRLNGDA at 50 mg/kg showed the greatest reduction in blood pressure similar to captopril (10 mg/kg). After the 4-week feeding trial, VSKRLNGDA resulted in down regulation of the renin and angiotensin II type-1 (AT-1) receptor genes, but up regulation of adrenoceptor β-3 (AR-β3) and peroxisome proliferator-activated receptor δ (Pparδ) genes as monitored by their mRNA levels in the kidney by real-time polymerase chain reaction (RT-PCR). Therefore, BCH and the VSKRLNGDA not only inhibited ACE but also involved in expression of those genes, which would be responsible for their antihypertensive effect. Hydrolysates from chicken blood corpuscles could be developed as a functional food exerting antihypertensive properties.

School of Food Technology Student's Signature _____

Academic Year 2018 Advisor's Signature _____

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LIST OF ABBREVIATIONS

Ala (A)	=	Alanine
Arg (R)	=	Arginine
Asn (N)	=	Asparagine
Asp (D)	=	Aspartic acid
AOAC	=	Association of Official Chemists
°C	=	Degree celsius
Cys (C)	=	Cysteine
BCH	=	Chicken blood corpuscles hydrolysate
DH	=	Degree of hydrolysis
GI	=	Gastrointestinal
Glu (E)	=	Glutamic acid
Gln (Q)	=	Glutamine
Gly (G)	=	Glycine
h	=	Hour
His (H)	=	Histidine
IC_{50}	=	Inhibitory concentration 50 (concentration of inhibitory agent required to inhibit the activity by 50 %)
Ile (I)	=	Isoleucine
kDa	=	kilo Dalton (10^3 Dalton)
LC-MS/MS	=	Liquid chromatography with tandem mass spectrometry
Leu (L)	=	Leucine

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LIST OF ABBREVIATIONS (Continued)

Met (M)	=	Methionine
µg	=	Microgram (10^{-6} gram)
µl	=	Microliter (10^{-6}L)
µM	=	Micromolar (10^{-6}mol L^{-1})
mg	=	Milligram (10^{-3}gram)
min	=	Minute
mL	=	Milliliter (10^{-3})
mM	=	Millimolar (10^{-3}mol L^{-1})
MS	=	Mass spectrometry
MS/MS	=	Tandem mass
Mw	=	Molecular weight
MWCO	=	Molecular weight cut off
m/z	=	Mass per charge ratio
Pro (P)	=	Proline
RP-HPLC	=	Reversed-phase high performance liquid chromatography
S	=	Second
SEC	=	Size exclusion chromatography
Ser (S)	=	Serine
Thr (T)	=	Threonine
TNBS	=	2, 4, 6-trinitrobenzenesulfonic acid
Trp (W)	=	Tryptophan
Tyr (Y)	=	Tyrosine

LIST OF ABBREVIATIONS (Continued)

U	=	Unit activity
UF	=	Ultrafiltration
Val (V)	=	Valine
w/v	=	Weight by volume
×g	=	Gravitational acceleration

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CHAPTER I

INTRODUCTION

1.1 Introduction

Thailand is one of the largest chicken exporters of the world. In 2018, it was estimated that chicken was produced for about 2.1 millions metric tons (MT), while domestic consumption was around 1.3 millions MT (Krungsri Research, 2018). Chicken blood is one of by-products constituting around 3-5% of body weight. Blood is composed of blood cells, which is about 40-45% of total weight, suspended in a liquid called blood plasma, representing up to 60% of the total weight (Parés, Saguer, and Carretero, 2011). Most of the chicken blood is processed into animal feed or coagulated blood for human consumption. Both forms are considered as low-value products. A means to increase utilization and value of chicken blood for human consumption should be sought.

It has been shown that protein hydrolysates possess various bioactive properties including antihypertensive, antioxidative in cell, opioid receptor (opioid agonistic), immunomodulatory, antimicrobial, prebiotic, mineral binding, antithrombotic and hypocholesterolemic effects (Arihara, 2006). Angiotensin I-converting enzyme (dipeptidyl carboxpeptidase, EC 3.4.15.1, ACE) belongs to the class of zinc metallopeptidase which acts as an exopeptidase and is activated by chloride with broad *in vitro* substrate specificit (Erdos and Skidgel, 1987). ACE is a key element in the rennin angiotensin system (RAS) responsible for the control of blood pressure which catalyzes the formation of the potent vasoconstrictive octapeptide (angiotensin II)

from decapeptide (angiotensin I) (Cushman, Cheung, Sabo, and Ondetti, 1977). ACE, a multifunctional enzyme, also inactivates the vasodilated peptide, bradykinin, a blood pressure lowering nonapeptide in the kallikrein-kinin system (Cushman, Cheung, Sabo, and Ondetti, 1977; Erdos and Skidgel, 1987). Both angiotensin II and degraded bradykinin affect the regulation of peripheral blood pressure, thus, inhibition of ACE is considered to be a useful therapeutic approach in the treatment of hypertension.

Researchers have used different individual or combined proteases (Alcalase, pepsin, trypsin, chymotrypsin, thermolysin, and Flavourzyme) to hydrolyze animal blood to obtain bioactive peptides. Bovine plasma hydrolysates obtained by Alcalase showed the highest ACE inhibiting activity with a DH of 6.7% (Sampedro and Montoya, 2014), while hydrolysate of red blood corpuscles obtained from a mixture of trypsin, chymotrypsin and thermolysin showed the highest ACE-inhibitory activity with IC_{50} of 0.58 mg/mL (Wei and Chiang, 2009). In addition, pepsin hydrolysate of porcine hemoglobin, at pH 2.0, 37°C for 6 h exhibited the highest ACE-inhibitory activity with IC_{50} of 0.02 mg/mL (Deng, Zheng, Zhang, Wang, and Kan, 2014). Extracellular proteinases from *Virgibacillus halodenitrificans* SK1-3-7, isolated from fish sauce fermentation showed catalytic activity of subtilisin-like alkaline serine proteinase and hydrolyzed fibrin to a greater extent than did plasmin (Montriwong, Kaewphuak, Rodtong, Roytrakul, and Yongsawatdigul, 2012). This enzyme can be used for production of protein hydrolysate. Enzyme cleaves the peptide linkage between amino acids of proteins, yielding a mixture of peptides of different molecular size and free amino acids. The ability of enzyme to hydrolyze proteins is highly variable. The selection of suitable enzymes for production of hydrolysates is critical. This is influenced by several factors, including substrate: enzyme ratio, incubation

time, temperature, and pH. The optimization of these parameters is imperative to achieve an economical process and high yield.

Amino acid sequences and characterization of individual peptides can be important for understanding mechanism of ACE-inhibitory peptides, especially the correlations of structure-activity. In addition, amino acid sequences of ACE-inhibitory peptides are critical for the peptide synthesis for either food or pharmaceutical industry. Bioactive peptides are typically purified through an ultrafiltration combined with series of chromatographic techniques including ion exchange, size exclusion and reversed-phase chromatography. Hydrolysates of bovine plasma and its isolated proteins, albumin and globulins, by Alcalase hydrolysis exhibited high ACE-inhibitory activity with molecular weights below 1000 Da (Hyun and Shin, 2000). The purified peptide, QELPG, from pepsin-hydrolyzed porcine hemoglobin showed IC_{50} for ACE-inhibition of 0.02 mg/mL (Deng, Zheng, Zhang, Wang, and Kan, 2014). In addition, two peptides, LGFPTTKTYFPHF and VVYPWT, derived from the 34-46 fragment of the α-chain and the 34-39 fragment of the β-chain showing ACE inhibitory activity of IC_{50} 4.92 and 6.02 μM, respectively (Yu, Hu, Miyaguchi, Bai, Du, and Lin, 2006). Moreover, purified hydrolysates of bovine serum albumin exhibited the highest inhibitory activity (IC_{50}) of 1.08 mg/mL with sequences of GYP, HL(I), HPY, HPGH, L(I)F, SPY, and YPH (Wanasundara, Ross, Amarowicz, Ambrose, Pegg, and Shand, 2002). Therefore, purification of blood hydrolysate leads to an increase in ACE-inhibitory activity and peptide identification.

Stability of ACE-inhibitory peptides during gastrointestinal (GI) digestion is an important parameter governing bioactivity of a peptide *in vivo*. When peptides pass through the GI tract, they would be modified through digestive proteases, which could

consequently alter ACE inhibitory activity (Hernandez-Ledesma, Amigo, Ramos, and Recio, 2004). Digesta obtained from other food proteins also showed ACE inhibitory activity, including Pacific Hake (Cinq-Mars, Hu, Kitts, and Li-Chan, 2008), chicken breast (Sangsawad, Roytrakul, and Yongsawatdigul, 2017) and soy protein isolate (Lo, Farnworth, and Li-Chan, 2006). Therefore, effects of *in vitro* GI tract model of ACE inhibitory peptides derived from chicken blood corpuscle should be monitored.

In the field of bioactive peptide, ACE inhibitor peptides have been widely studied in antihypertensive from other source such as cod protein hydrolysate, chicken skin protein hydrolysates, egg protein hydrolysates, oyster proteins hydrolysates (Girigh, Nwachukwa Hasan Fagbemi, Gil, and Aluko, 2015; Onuh, Girgih, Malomo, Aluko, and Aliani, 2015; Wang et al., 2008; Yu, Yin, Zhao, Chen, and Liu, 2014). However, nobody has been reported to study the peptide derived-chicken blood *in vivo* antihypertensive effect.

1.2 Research objectives

The objectives of this study were:

1. To determine the optimal blood protein substrate (whole blood (WB), blood plasma (BP) and blood corpuscles (BC)) and enzymes (Alcalase, pepsin, papain, thermolysin and *Virgibacillus* sp. SK1-3-7 protease) for producing hydrolysate with ACE-inhibitory activity.

2. To isolate, identify and characterize ACE-inhibitory peptide generated from chicken blood hydrolysate.

3. To study effect of chicken blood hydrolysates and ACE-inhibitory peptide derived from such hydrolysate on lowering blood pressure of spontaneously hypertensive rats (SHR) after short- and long-term oral administration.

1.3 Research hypotheses

Various protein fractions in blood yield hydrolysates with varied ACE inhibitory activities. The optimal condition of protein hydrolysate with ACE inhibitory activity varied with type and amount of proteases and pH. Such an optimal condition can be optimized through response surface methodology (RSM). In addition, partially-purified peptides prepared from BCH can act as ACE-inhibitory activity. ACE-inhibitory activity is likely to change upon GI digestion. Blood peptides can lower blood pressure in the SHR rat.

1.4 Scope of the study

The WB, PB and BC were hydrolyzed using five proteases; Alcalase, pepsin, papain, thermolysin and crude enzyme from *Virgibacillus* sp. SK1-3-7 under their optimal condition for 12 h. Hydrolysates were measured for degree of hydrolysis (DH) and ACE-inhibitory activity. Production of ACE-inhibitory hydrolysates under the optimal substrate and enzyme was optimized using response surface methodology (RSM). Three controllable variables, including temperature (X1: 50-60°C), enzyme concentration (X2: 2-4%E) and time (X3: 4-6 h), were selected for optimization. Hydrolysates obtained from RSM were evaluated for degree of hydrolysis (DH) and ACE-inhibitory activity. Model for production of ACE-inhibitory peptide was validated. In addition, the hydrolysate exhibiting the highest ACE-inhibitory activity

was selected for fractionation using sequential ultrafiltration (UF). The fraction showing the highest activity was selected for evaluating stability *in vitro* pepsin-pancreatin GI digestion and amino acid profiles. The chicken blood hydrolysate and ultrafiltrated fraction were tested for blood pressure lowering effect in SHR. Purification of antihypertensive peptides from chicken blood hydrolysate were carried out using series of chromatographic techniques including size exclusion and reversed-phase chromatography. ACE-inhibitory peptide was identified and its amino acid sequence was determined using tandem mass spectrometry (LC-MS/MS). Peptides obtained from *de novo* peptide sequencing were selected for synthesis. The synthesized peptides were evaluated for stability under *in vitro* pepsin–pancreatin GI digestion and *in silico* digestion. The inhibition kinetics of synthesized peptide was analyzed. Moreover, antihypertensive effect of the selected synthetic peptide was determined in SHR. the effect of the selected synthetic peptide on the gene expression of renin, angiotensin II type-1 receptor (AT-1), angiotensin-I-converting enzyme (ACE), adrenoceptor β-3 (AR-β3) and peroxisome proliferator-activated receptor δ (Pparδ) in the kidney was evaluated using real-time polymerase chain reaction (RT-PCR).

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1.5 Expected results

Results from this research would create value and utilization of chicken blood, which currently has relatively low value. The ACE inhibitory peptide with antihypertensive effect would be realized. This research would also lead to more understandings about the antihypertensive effect of peptides on the gene expression.

Ultimately, high protein ingredient with antihypertensive effect would be developed from chicken blood.

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CHAPTER II

LITERATURE REVIEWS

2.1 Characteristic of blood fractions

Blood is composed of blood cell fraction (blood corpuscles) and plasma. Blood corpuscles (BC) fraction is 40-45% of blood weight and is dispersed within the liquid fraction known as plasma which represents up to 55-60% of the total fraction (Parés, Saguer, and Carretero, 2011). The most important cellular elements are red blood cells (RBC or erythrocytes), white corpuscles (leukocytes), and platelets. The composition of blood and its fractions is shown in Table 2.1.

Table 2.1 Compositions of blood fractions.

Constituents	Blood	Plasma	BC
Water (%)	80	90.8	60.8
Salts (%)	0.9	0.8	1.1
Fat (%)	0.2	0.1	0.4
Protein (%)	17	7.9	35.1
Albumin (%)	2.8	4.2	-
Globulins (%)	2.2	3.3	-
Fibrinogen (%)	0.3	0.4	-
Hemoglobin (%)	10	-	30
Others (%)	1.1	0.4	2.6

From: Ockerman and Hansen (2000).

After bleeding, blood clots in 3-10 min (Ockerman and Hansen, 2000). This clotting is caused by thrombin which converts soluble fibrinogen into insoluble fibrin. Generally, blood is collected hygienically and added an anticoagulant such as ethylene diamine tetra acetic acid (EDTA) or sodium citrate concentrations of 0.1-1% (w/v) for blood clotting prevention (Ockerman and Hansen, 2000). Anticoagulants as sodium citrate and a number of phosphates at different concentrations have been used for blood collection as follows: sodium citrate solution (10%) for 30 ml/L, sodium pyrophosphate solution (10%) for 30 ml/L, sodium tripolyphosphate solution (10%) for 20 ml/L, 4.5% sodium citrate containing 5% sodium chloride solution for 90 ml/L (FAO, 1996).

BC and plasma are slightly alkaline pH (7.3-7.5) and can be easily separated by centrifugation. Within the RBC fraction, total protein content ranges from 28 to 38%, and hemoglobin (Hb) is the major protein component. Hb has a molecular weight as a tetramer of 68 kDa and consists of 4 subunits known as globin, of which the α- and β-chains are arranged in a spherical structure (Wismer-Pedersen, 1988; Parés, Saguer, and Carretero, 2011).

Plasma is a liquid with 6-8% of total protein content containing with a complex mixture of proteins. It can be classified into three major groups, namely albumin (up to 60%), globulins (40%) and fibrinogen (around 3%). When plasma proteins are to be used separately, the first fractionation step often consists of precipitating fribinogen from plasma. The liquid phase remaining after fibrinogen precipitation is named serum, and it is mainly composed of serum albumin ~66 kDa, with a pI ~4.8 – which shifts to ~5.3 when defatted (Peters, 1985), the major blood plasma protein, and a heterogeneous group of globulins (α, β and γ) with molecular weights ranging from 15

to hundreds of kDa (Parés, Saguer, Pap, Toldrà, and Carretero, 2012). Immunoglobulins, the most abundant proteins in the globulin fraction (~50%), display a symmetrical Y-shaped structure composed of 2 heavy chains (~50 kDa) and 2 light chains (~25 kDa), held together by both disulfide bonds and non-covalent interactions, but showing differences in types and/or number of interactions between molecules. Moreover, intrachain disulfide bonds are present (Coico and Sunhine, 2009. Fibrinogen is a fibrous glycoprotein of high-molecular weight (~340 kDa) with a complicated molecular structure consisting of two identical subunits. Each one is composed of three non-identical polypeptide chains (Aα, Bβ and γ), held together by 29 disulfide bonds (Doolittle, Goldbaum, and Doolittle 1978).

2.2 Hydrolysis of blood fractions

Various proteases (Alcalase, pepsin, trypsin, chymotrypsin, thermolysin and Flavourzyme) have been used to hydrolyze animal blood to obtain bioactive peptides. Both blood plasma and RBC/Hb have been investigated as potential sources of ACE inhibitory peptides. Alcalase (EC 3.4.21.62) is a commercial enzyme derived from *Bacillus licheniformis*, which is a serine endopeptidase with broad specificity, thus producing mainly small- and medium-size oligopeptides (Cumby, Zhong, Naczk, and Shahidi, 2008). pH optimum is 8-9 and the optimal temperature is 50-60°C. In addition, peptides obtained from Alcalase hydrolysis are likely to contain aromatic (F, W, and Y), acidic (E), sulfur-containing (M), aliphatic (L, I, V and A), hydroxyl (S), and basic (K) residues (Doucet, Otter, Gauthier, and Foegeding, 2003). Alcalase hydrolyzed bovine plasma, albumin and globulins showed ACE inhibitory activity (Hyun and Shin, 2000). In addition, Mito, Fujii, Kuwahara, Matsumura, Shimizu,

Sugano and Karaki (1996) demonstrated the *in vivo* effectiveness in lowering blood pressure in spontaneously hypertensive rats by oral administration of porcine hemoglobin hydrolysate prepared by Alcalase. Bovine albumin hydrolysate obtained from Alcalase showed the most active ACE-inhibitory activity (IC_{50} of 0.56 mg/mL) with a peptide yield of 69.2% (Hyun and Shin, 2000). Moreover, Sampedro and Montoya (2014) reported that bovine plasma hydrolysates obtained by Alcalase showed the highest ACE-inhibitory activity with DH of 6.7%.

Thermolysin (EC 3.4.24.27) is produced from *Bacillus thermoproteolyticus* and is a thermostable neutral metalloendopeptidase which requires one zinc ion for enzyme activity and four calcium ions for structural stability (Endo, 1962). It cleaves peptide bonds on the N-terminal side of hydrophobic amino acid residues towards F, I, L or Y (Matsubara and Feder, 1971). The pH optimum is 8.0 and the optimal temperature for activity is 70°C. Wei and Chiang (2008) found that porcine red blood corpuscles were hydrolyzed by a mixture of enzymes containing thermolysin, chymotrypsin and trypsin, resulting in high ACE-inhibitory activity.

Papain (EC3.4.22.2) from the latex of papaya (*Carica papaya* L.) is one of the widely used industrial enzymes. Papain, highly active endolytic cysteine protease of the peptidase C1 family, consists of a single polypeptide chain with three disulfide bridges and a sulfhydryl group necessary for activity of the enzyme. Papain cleaves peptide bonds of basic amino acids, L, or G (Ganapathy, Manolache, Sarmadi, and Denes, 2001). It also hydrolyzes esters and amides**.** It is a relatively heat resistant enzyme, with optimal temperature of 65-75^{o}C (Calkins and Sullivan, 2007). Papain was used for production of bovine whole plasm hydrolysate with ACE-inhibitory activity (Hyun and Shin, 2000).

Pepsin (EC 3.4.23.3) from porcine gastric mucosa is an aspartic protease which cleaves peptide bonds associated with the aromatic amino acids, F, W and Y at the NH group of peptide bond (Whitaker, 1994). Its optimal pH was about 2.0-4.0 and the optimal temperature is 37 °C. Deng, Zheng, Zhang, Wang, and Kan (2014) investigated that pepsin hydrolysate of porcine hemoglobin with a protein substrate–enzyme ratio of 100:3 (w/w) at pH 2.0, 37 °C for 6 h exhibited the highest ACE-inhibitory activity (IC_{50} = 0.02 mg/mL). In addition, Yu, Hu, Miyaguchi, Bai, Du, and Lin (2006) found that globin hydrolyzed by pepsin showed the highest ACE-inhibitory activity of IC_{50} of 1.19 mg/mL, while the lowest ACE-inhibitory activity was found in sample prepared from trypsin (IC_{50} = 8.79 mg/mL).

Degrees of hydrolysis (DH) varied with enzyme-to-substrate ratio and hydrolysis time. Wanasundara, Amarowicz, Ross, Pegg, and Shand (2002) found that defibrinated bovine plasma had a negligible amount of ACE-inhibitory activity, but defibrinated bovine plasma hydrolyzed by Flavourzyme for 15.5 h at 43% DH showed the highest ACE-inhibitory activity (IC_{50} = 1.08 mg/mL). In contrast, Sampedro and Montoya (2014) found that bovine plasma hydrolysates obtained from Alcalase 2.4 L showed the maximum ACE-inhibitory activity at 6.7% DH and activity began to decrease at higher DHs. Thus, the specificities of enzymes, DH, and the nature of released peptides (e.g., molecular weight and amino acid composition) are important parameters controlling ACE-inhibitory activity.

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2.3 Action of angiotensin I-converting enzyme (ACE) on renin angiotensin system (RAS)

Angiotensin I-converting enzyme (dipeptidyl carboxpeptidase, EC 3.4.15.1, ACE) belongs to the class of zinc metallopeptidase which acts as an exopeptidase and is activated by chloride with broad *in vitro* substrate specificity (Erdos and Skidgel, 1987). ACE has four functional amino acid residues of Y, R, E and K at the active site, and three hydrophobic binding subsites. The enzyme shows molecular weight of 140-

160 kDa and 90-100 kDa for the endothelial and the testicular, respectively, depending on the carbohydrate content of the molecule (Aldermann, Maddhavan, Ooi, Cohen, Sealey, and Laragh, 1991). The ACE is a key element in the rennin angiotensin system (RAS) responsible for the control of blood pressure which catalyzes the formation of the potent vasoconstrictive octapeptide (angiotensin II (Ang II), DRVYIHPF) from decapeptide (angiotensin I (Ang I), DRVYIHPFHL) (Cushman, Cheung, Sabo, and Ondetti, 1977). ACE, a multifunctional enzyme, also inactivates the vasodilative peptide, bradykinin, a blood pressure lowering nonapeptide in the kallikrein-kinin system (Cushman, Cheung, Sabo, and Ondetti, 1977; Erdos and Skidgel, 1987; Johnston and Franz**,** 1992). Both angiotensin II and degraded bradykinin affect the regulation of peripheral blood pressure, thus, inhibition of ACE is considered to be a useful therapeutic approach in the treatment of hypertension. The synthetic ACE-inhibitors, such as Captopril, Enalapril and Alacepril have been widely used in the clinical treatment against hypertension in human (Cushman, Noda, and Salans, 1981. However, the intake of these synthetic drugs can cause serious side effects such as the presence of cough, taste disturbances and skin rashes (Antonios and Macgregor, 1995). Thus, the development of safe and natural ACE inhibitors is necessary for prevention of hypertension.

2.3.1 ACE-inhibitory assay

ACE-inhibitory activity can be determined by several methods such as fluorometric (Friedland and Silverstein**,** 1976; Sentandreu and Toldra´, 2006), radiochemical (Ryan, Chung, Ammons, and Carlton, 1977), spectrophotometric (Cushman and Cheung, 1971; Hayakari, Kondo, and Izumi, 1979; Matsui, Matsufuji, and Osajima, 1992; Murray, Walsh, Fitz, and Gerald, 2004), capillary electrophoresis

methods (Chang, Chen, Huang, and Chang, 2001) and high-performance liquid chromatography (HPLC) (Wu, Aluko, and Muir, 2002; Piyadhammaviboon, Wongngam, Benjakul, and Yongsawatdigul, 2012). Hippuryl-L-histidyl-L-leucine (HHL) has been used as a sysnthetic substrate which is hydrolyzed by ACE to form hippuric acid (HA) and histidyl-leucine (His-Leu, HL) as products (Figure 2.1). The amount of HA can be measured at 228 nm using a spectrophotometer (Cushman and Cheung, 1977) or HPLC (Wu, Aluko, and Muir, 2002).

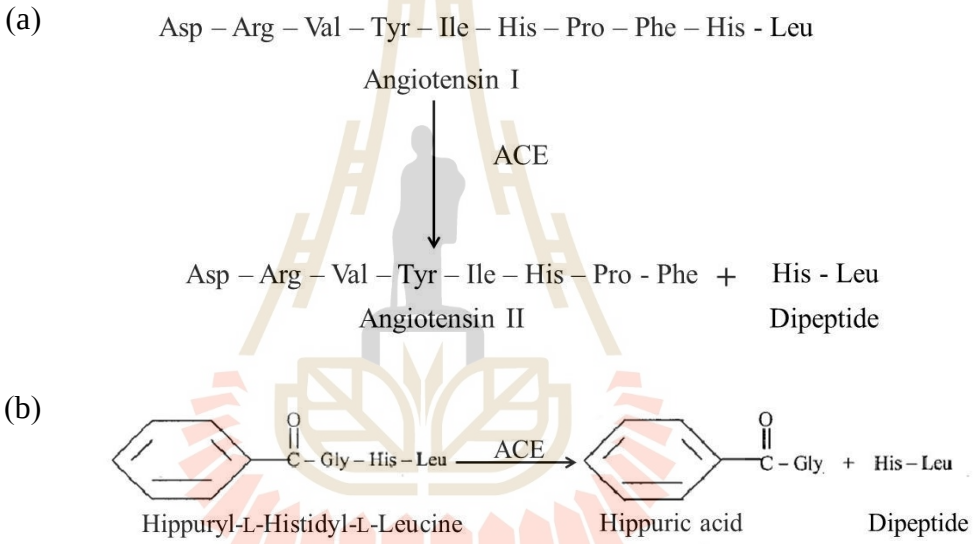

Figure 2.1 Angiotensin I-converting enzyme (ACE) activity on (a) angiotensin-I and (b) hippuryl-L-histidyl-L-leucine (HHL).

From: Cushman and Cheung (1971).

ACE can also hydrolyze N-(3-[2-furyl]acryloyl)-phenylalanylglycylglycine (FAPGG) into FAP and GG (Holmquist, Bunning, and Riordan, 1979). This leads to a decrease in absorbance at 345 nm (Udenigwe, Lin, Hou, and Aluko, 2009).

Alternatively, quantification of the released FAP can be determined by a reverse-phase HPLC detected at 305 nm (Lahogue, Réhel, Taupin, Haras, and Allaume, 2010).

In addition, the assay can be performed using a fluorescent substrate, o-aminobenzoylglycyl-p-nitro-L-phenylalanyl-L-proline (AbzGly-Phe-(NO_2)-Pro), as developed by Sentandreu and Toldra´ (2006). The action of ACE to the release of o-aminobenzoylglycine (Abz-gly) is measured at excitation and emission wavelengths of 355 and 405 nm, respectively. Ortho-aminobenzoic acid-phenylalanine-arginine-lysine-dinitrophenyl-proline [Abz-FRK(dnp)P-OH] is also used to determine ACE-inhibitory activity based on fluorometric method (van Elswijk, Diefenbach, van der Berg, Irth, Tjaden, and van der Greef, 2003). The arginine-lysine (RK) bond is cleaved by ACE, resulting in a remove of DNP quenching moiety. An increasing in fluorescence of the Abz moiety is measured using excitation at 320 nm and emission at 420 nm.

2.4 Action of angiotensin receptor blocker on renin angiotensin system

Ang II acts through two main receptors, angiotensin type 1 (AT-1) and type 2 (AT-2) receptors (Zhuo et al., 2013). Binding to AT-1 receptor causes vasoconstriction in vascular smooth muscle cells (VSMC). It also stimulates release of aldosterone to increase water and salt retention in the kidney, hypertrophic growth of cardiomyocytes, and collagen synthesis of cardiac fibroblasts resulting in cardiac remodeling. In pathogenic conditions involving tissue remodeling and vascular inflammation, AT-1 receptor is up regulated (Sriramula et al., 2011). On the other hand, AT-2 receptor presents in both endothelial and VSMC mediates vasodilation

upon activation, releases NO, and inhibits cell growth (Stankevicius, 2003). Therefore, AT-1 receptor mediates actions with potentially harmful consequences, whereas AT-2 receptor, mediated actions exhibits protective effects against hypertension. The synthetic angiotensin receptor blocker, such as losartan, valsartan and candesartan, has been widely used in the clinical treatment against hypertension in human (Cha et al., 2015).

2.5 Regulation of blood pressure via multiple pathways

Blood pressure (BP) not only regulation through RAS but involved via multiple pathways including endothelial dysfunction, sympathetic nervous system and vascular remodeling. Endothelial dysfunction is characterized by a shift of the properties of the endothelium toward reduced vasodilation, resulting in imbalanced production of relaxing, a proinflammatory, and prothrombic properties that it is responsible for various cardiovascular diseases including hypertension (Endemann and Schiffirin, 2004). Nitric oxide (NO) as a vasodilator and endothelin (ET-1) a potent vasoconstrictor are produced by endothelial cells that an imbalance between these two factors is a feature of endothelial dysfunction (Alonso and Radomski, 2003). Reduction of NO lead to reduce endothelial NO synthase (eNOS) activity and to decrease NO bioavailability because of the presence of excessive reactive oxygen species (ROS) such as superoxide anion (O_2^-) with formation of peroxynitrilte (Koppenol, Moreno, Pryor, Ischiropoulos, and Beckman, 1992). Peroxynitrite is a cytotoxic oxidant, causing damage to cell membrane while leads to cell death and/or inflammation and therefore endothelial function (Pennathur and Heinecke, 2007). Excessive formation of O_2^- will also result in reduction of tetrahydrobiopterin (BH_4)

as a cofactor for eNOS with increase in BH_2, leading to become uncoupled and paradoxically generated O_2^- instead of NO (Millatt, Abdel-Rahman, and Siragy, 1999). Reduction of NO bioactivity lead the consequent exaggeration of oxidant excess, stimulating pro-inflammatory pathways, deleterious effect on endothelial and vascular function, and resulting in increased blood pressure (Landmesser et al., 2003). In contrast, increase ET-1 plays a pathophysiologic role in various forms of cardiovascular disease and also stimulates the release of pro-inflammatory cytokine such as interleukin (IL)-1 and IL-2 that its cloud be as a potential marker of endothelial dysfunction (Bourque, Davidge, and Adams, 2011).

The sympathetic nervous system (SNS) is a part of the autonomic nervous response system and play an important pathophysiological role in hypertension (Wallin and Charkoudian, 2007). Activation of sympathetic nerves stimulate to release catecholamines (norepinephrine and epinephrine) leading to induce effects on the heart and vasoconstriction of renal efferent arterioles (Tsuru, Tanimitsu, and Hirai, 2002; Smith, Graham, Mackintosh, Stoker, and Mary, 2004). The SNS-activated also influences the RAS to release renin resulting in high blood pressure through the Ang II (Tsuru, Tanimitsu, and Hirai, 2002).

Vascular remodeling is an active process of structural change that it contributes to develop hypertension, end-organ damage in hypertension, and alterations of blood vessels (Intengan and Schiffrin, 2001). Hypertension associated with structural changes in the vessels has been called as remodeling (Baumbach and Heistad 1989). Apoptosis modulators in the vasculature are numerous and complex including ROS, NO, AT-2 receptors, and endothelial dysfunction that they are involved in the pathogenesis of hypertension (Cattaruzza, Dimigen, Ehrenreich, and Hecker, 2000).

Vascular inflammation can induce endothelial dysfunction resulting in vascular remodeling. The actions of Ang II are stimulated ROS production. The inflammatory and the excess oxidative stress can induce gene expression via the pathway initiated by the nuclear transcription factor κB (NF-κB), such as tumor necrosis factor-α (TNF-α) and IL-1β , leading to the expression of adhesion molecules (ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1), and the release of monocyte chemotactic protein-1 (MCP-1) (Nakane, Miller Jr., Faraci, Toyoda, and Heistad, 2000; Sack, 2002).

2.6 ACE-inhibitory peptides from blood fractions

There are many ACE substrates in various blood hydrolysates. Wei and Chiang (2009) demonstrated that hydrolysates of porcine red blood corpuscles with a mixture of trypsin, chymotrypsin and thermolysin showed the highest ACE-inhibitory activity (IC_{50} = 0.58 mg/mL). Protein hydrolysates show competitive, noncompetitive, uncompetitive, or even mixed-type inhibitors. ACE inhibitors usually contains g hydrophobic amino acid residues such as P, F, and Y at 3 positions from the C-terminal end (Cheung, Wang, Ondetti, Sabo, and Cushman, 1980). Peptides with ACE-inhibitory activities have been purified using series of chromatographic techniques including ion exchange, size exclusion and reversed-phase chromatography. Hyun and Shin (2000) reported that hydrolysate of bovine plasma and its isolated proteins, albumin and globulins, by Alcalase contained ACE inhibitory peptides with molecular weights below 1000 Da. In addition, Deng, Zheng, Zhang, Wang, and Kan (2014) purified pepsin hydrolysate of porcine hemoglobin with the highest ACE-inhibitory activity (IC_{50} = 0.02 mg/mL) with amino

acid sequence of QELPG. Moreover, GYP, HL(I), HPY, HPGH, L(I)F, SPY, and YPH sequence motifs corresponding to bovine serum albumin exhibited the highest activity of IC_{50} of 1.08 mg/mL (Wanasundara, Ross, Amarowicz, Ambrose, Pegg, and Shand, 2002). Yu, Hu, Miyaguchi, Bai, Du, and Lin (2006) purified and identified two peptides, LGFPTTKTYFPHF and VVYPWT, corresponding to the 34-46 fragment of the α-chain and the 34-39 fragment of the β-chain of porcine hemoglobin, which were obtained from pepsin hydrolysis. Four peptides from porcine hemoglobin hydrolysate by Alcalase exhibited ACE-inhibitory activity: E-1 (FQKVVA), E-2 (FQKVVAG), peptide 30-3 (FQKVVAK) and H-1 (GKKVLQ) with IC_{50} of 5.8, 7.4, 2.1 and 1.9 μM, respectively (Mito, Fujii, Kuwahara, Matsumura, Shimizu, Sugano, and Karaki, 1996). Plasma peptide after *in vitro* incubation with gastrointestinal proteases (pepsin, chymotrypsin and trypsin) exhibited subtle change in the ACE-inhibitory activity, suggesting that these peptides might be resistant to digestion in the gastrointestinal tract (Wanasundara, Ross, Amarowicz, Ambrose, Pegg, and Shand, 2002). Moreover, Yu, Hu, Miyaguchi, Bai, Du, and Lin (2006) found that two purified peptides, GFPTTKTYFPPHF and VVYPWT, from the pepsin hydrolysis of porcine globin were both competitively inhibited ACE and maintained inhibitory activity even after incubation with gastrointestinal proteases.

2.7 Antihypertensive peptides

Antihypertensive effect of protein hydrolysate has been evaluated using spontaneously hypertensive rats (SHR) model. Nwachukwu, Girgih, Malomo, Onuh, and Aluko (2014) reported that Thermoase-digested flaxseed protein hydrolysate (FPH) samples and ultrafiltration fractions were also effective in lowering systolic

blood pressure (SBP) in SHR at 200 mg/kg BW of the 1-3 kDa peptide fraction derived from 2.5% Thermoase-FPH and the 3-5 kDa fraction derived from 3% Thermoase-FPH. In addition, Feng, Dai, Zhang, Meng, Ye, and Ma (2015) reported that both SBP and diastolic blood pressure (DBP) were mildly decreased in the rats administered with ultrafiltrated hydrolysate (<3kDa) derived from Blue mussel (*M. edulis*) at 10 and 20 mg/kg BW for 1 month. Girgih, Nwachukwu, Hasan, Fagbemi, Gill, and Aluko (2015) founded that administration of reverse-phase chromatography of cod protein hydrolysate at 30 mg/kg showed the maximum reduction of SBP of -40 mmHg after 2 h while cod protein hydrolysate at a higher dose of 200 mg/kg BW led to a maximum SBP decrease of -19.1 mmHg at 2-h post-administration. Li, Qu, Wan, and You (2007) also found that rice protein hydrolysate prepared with Alcalase at a dose of 600 mg/kg of body weight (BW) showed maximum SBP reduction of -25.6 mmHg after 6 h administration in SHR.

Several tripeptides that inhibit ACE have been isolated from foods, including VPP and IPP derived from milk to reduce blood pressure in long-term administration in rats (Tuomilehto et al., 2004). In addition, single oral administration of TQVY derived from rice protein at a dose of 30 mg/kg of body weight also significantly decreased SBP of -40 mmHg in SHR (Li, Qu, Wan, and You, 2007).

2.8 Gene expression for hypertension

The underlying mechanisms of antihypertensive peptides have yet to be fully identified. Besides ACE in RAS, blood pressure is affected by gene expression including angiotensinogen (Ang), renin, angiotensin II type-1 receptor (AT-1) and type-2 (AT-2), adrenoceptor β-3 (AR-β3), interleukin 24 (IL-24), and peroxisome

proliferator-activated receptor δ (Pparδ). AR-β3 is an important regulator of the cardiovascular system and of endothelial cell function in particular that AR-β3 produces sustained peripheral vasodilation (Shen, Zhang, and Vatner, 1994). In addition, activation of the AR-β3 by nebivolol could enhance endothelial nitric oxide production and decrease the generation of reactive oxygen species, leading to pulmonary vasodilation, and attenuated vascular remodeling (Perros, Ranchoux, Izikki, Bentebbal, Happé, Antigny, et al., 2015). Pparδ activation has been reported to exert antihypertensive effects, to induce progressive systolic arterial blood pressure and heart rate reduction, to restore vascular structure and function, and to reduce the oxidative, proinflammatory, and proatherogenic statuses (Zarzuelo et al. 2011). Expression of various genes involved in hypertension have been studied. Yu, Zhao, Ding, Wang, Chen, and Liu (2017) demonstrated that antihypertensive effect of egg protein-derived peptide QIGLF (50 mg/kg) decreased in mRNA expression of ACE gene in the kidney. Antihypertensive effect in gene expression of AR-β3 and IL-24 genes were up-regulated in rats fed with ultrafiltrated hydrolysate (<3kDa) derived from Blue mussel (*M. edulis*) at 20 mg/kg BW group, whereas the Pparδ levels were up-regulated in the 10 mg/kg BW group (Feng, Dai, Zhang, Meng, Ye, and Ma, 2015). In addition, expression of AT-1 receptor was down-regulated in dose 10 and 20 mg/kg groups, while Ang and ACE not different with control that ultrafiltrated hydrolysate (<3kDa) derived from Blue mussel (*M. edulis*) had potential to reduce blood pressure in SHR (Feng, Dai, Zhang, Meng, Ye, and Ma, 2015). Moreover, antihypertensive effect of RVPSL (50 mg/kg) from egg protein showed that the expression of renin, ACE, AT-1 receptor genes were down-regulated while the AT-2 receptor was up-regulated (Yu, Yin, Zhao, Chen, and Liu, 2014).

2.9 References

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CHAPTER III

PRODUCTION OF CHIKCEN BLOOD HYDROLYSATE WITH ANTIHYPERTENSIVE EFFECT

3.1 Abstract

Chicken blood fractions, namely whole blood (WB), blood plasma (BP) and blood corpuscles (BC), were hydrolyzed using various enzymes, including Alcalase, papain, pepsin, themolysin and *Virgibacillus* sp. SK1-3-7 protease. Blood corpuscles hydrolysate (BCH) derived from Alcalase showed the highest degree of hydrolysis (DH) of 14.53% and angiotensin I-converting enzyme (ACE) inhibition of 61.8% at 0.5 mg/mL peptide. Based on response surface methodology (RSM) with central composite design, the optimized condition of BCH was 51.1°C, 4% E/S, and pH 9.6 for 6 h, resulting in 35.8% DH and 37.7% ACE inhibition at 0.2 mg/mL peptide. After ultrafiltration using 1-kDa molecular mass cutoff membrane, the permeate of BCH showed an increase in ACE inhibition about 2.5 folds compared to BCH. The permeate of BCH appeared to be resistant to *in vitro* gastrointestinal (GI) digestion, while BCH digesta exhibited an increase in ACE inhibitory activity. Both BCH and its permeate were rich in F, G, A, V, I and L. Oral administration of BCH and BCH-permeate to spontaneously hypertensive rat (SHR) at 600 and 200 mg/kg body weight, respectively, lowered systolic blood pressure (SBP) up to around -43.0 mmHg. The maximum reduction of diastolic blood pressure (DBP) was observed at administered doses of 100 mg/kg permeate BCH. Chicken blood could be revalorized

as a protein hydrolysate with antihypertensive effect.

Keywords: Angiotensin I-converting enzyme (ACE), Protein hydrolysates, Optimization, Simulated gastrointestinal digestion, Spontaneously hypertensive rat (SHR)

3.2 Introduction

Chicken meat is one of important sources of food protein with the production worldwide about 92.5 millions MT in 2018 (USDA, 2018). Blood is a by-product from a slaughterhouse, constituting around 3-5% of body weight. Whole blood (WB) is composed of blood cells and plasma accounting for 40-45% and 60% of total weight (Parés, Saguer, and Carretero, 2011). Majority of chicken blood is converted to low value animal feed or blood cubes as an inexpensive source of protein and iron in diet in Asian countries. A means to increase valorization of chicken blood for human consumption should be sought.

It has been shown that protein hydrolysates possess various bioactive properties, including antihypertensive, antioxidant, immunomodulatory, antimicrobial, prebiotic, mineral binding, antithrombotic and hypocholesterolemic effects (Arihara, 2006). Angiotensin I-converting enzyme (dipeptidyl carboxpeptidase, EC 3.4.15.1, ACE) catalyzes the formation of the potent vasoconstrictive octapeptide (angiotensin II, Ang II) from decapeptide (angiotensin I, Ang I) and inactivates the vsodilative peptide, bradykinin, a blood pressure lowering nonapeptide in the kallikrein-kinin system (Cushman, Cheung, Sabo, and Ondetti, 1977). Thus, ACE plays a key role in controlling blood pressure. Inhibition of ACE is considered to be one of therapeutic approaches in the treatment of hypertension. Peptides from various food sources have

been reported to exhibit ACE inhibitory activity including canola, flaxseed, rice, milk, chicken skin, cod, egg and oyster (Blanchard et al., 2014; Girigih, Nwachukwu, Hasan, Fagbemi, Gill, and Aluko, 2015; Li, Qu, Wan, and You, 2007; Onuh, Girgih, Malomo, Aluko, and Aliani, 2015; Udenigwe, Lin, Hou, and Aluko, 2009; Yamaguchi, Kawaguchi, and Yamamoto, 2009;Yu, Yin, Zhao, Chen, and Liu, 2014; Wang et al., 2008). Despite of high inhibition *in vitro* in some hydrolysates/peptides, they failed to show their efficacy *in vivo* (Cushman and Cheung, 1971; Marques, Amorim, Pereira, Pintado, Moura, Calhau, and Pinheiro, 2012). Therefore, animal model would be necessary to confirm potential of protein hydrolysate as a functional food for stabilizing blood pressure.

Type of proteases and substrates are critical factors controlling type of peptides generated and their ACE inhibition capacity. Blood corpuscles (BC) compose of various proteins with red blood cells (RBC or erythrocytes), white corpuscles (leukocytes), and platelets with approximately 35% protein and hemoglobin being the predominant protein in red corpuscles, while blood plasma (BP) contained more than 8% proteins with albumin and globulins being the predominant ones (Ockerman and Hansen, 2000). Optimal hydrolysis condition is varied with blood proteins and proteases applied. Sampedro and Montoya (2014) reported that Alcalase-hydrolyzed bovine plasma showed the highest ACE inhibiting activity with a degree of hydrolysis (DH) of 6.7%, while hydrolysate of RBC obtained from a mixture of trypsin, chymotrypsin and thermolysin showed the highest ACE-inhibitory activity with IC_{50} of 0.58 mg/mL (Wei and Chiang, 2009). In addition, pepsin hydrolysate of porcine hemoglobin at pH 2.0, 37°C for 6 h exhibited the highest ACE-inhibitory activity with IC_{50} of 0.02 mg/mL (Deng, Zheng, Zhang, Wang, and Kan, 2014). Selection of blood

proteins and enzymes for production of hydrolysates exhibiting ACE inhibitory activity is critical. Several factors, including substrate: enzyme ratio, hydrolysis time, temperature, and pH, also affect yield and bioactivity of hydrolysates. Response surface methodology (RSM) has been proposed as an effective tool to determine the optimization for production of hydrolysate with bioactivity (Vermeirssen, Camp, and Verstraete, 2002; Cinq-Mars and Li-Chan, 2007).

Stability of ACE-inhibitory peptides during gastrointestinal (GI) digestion is an important parameter governing bioactivity of a peptide *in vivo*. When peptides pass through the GI tract, they are modified through GI proteases, which could consequently affect ACE-inhibitory activity. Difference sources of protein hydrolysates showed varied ACE inhibition after simulated GI digestion. ACE inhibition of whey hydrolysates (<3 kDa), bovine plasma and porcine globin was not affected after *simulated* GI digestion (Hernandez-Ledesma, Amigo, Ramos, and Recio, 2004; Sampedro and Montoya, 2014; Yu, Hu, Miyaguchi, Bai, Du, and Lin, 2006). In contrast, peptides from Pacific hake (*Merluccius productus*) and whey hydrolysate exhibited less ACE inhibitory activity upon *simulated* GI digestion (Cinq-Mars, Hu, Kitts, and Li-Chan, 2008; Hernandez-Ledesma, Amigo, Ramos, and Recio, 2004). Therefore, changes of ACE-inhibitory activity of chicken blood hydrolysates after simulated GI digestion should be considered.

The objectives of this study were to determine a proper substrate (WB, BP, and BC) and protease (Alcalase 2.4L, papain, thermolysin, porcine pepsin and *Virgibacillus* sp. SK1-3-7) for production of hydrolsate with ACE inhibitory activity. In addition, hydrolysis condition was optimized using RSM. Antihypertensive effect

of chicken blood hydrolysate produced under the optimized condition was evaluated in vivo based on SHR model.

3.3 Materials and methods

3.3.1 Samples and chemicals

Chicken blood samples were obtained from a chicken slaughterhouse in Nakhon Ratchasima, Thailand. Blood was collected using sterile containers which included sodium citrate solution at 1% (w/v) as a coagulant. Samples were maintained at chill temperatures (5°C) until use. BP and BC were separated by centrifuging WB at 2530 ×*g* at 4°C for 15 min (Hitachi CR22GIII, Hitachi Koki Co. Ltd., Tokyo, Japan). BP and BC were lyophilized along with WB. Proximate composition of all blood fractions was determined according to AOAC (2000).

Alcalase 2.4L (EC 3.4.21.62) from *Bacillus licheniformis*, Subtilisin A from Novozymes (Bagsvaerd, Denmark), papain (EC 3.4.22.2, 10 units/mg) from papaya latex, thermolysin (EC3.4.24.27, 40 units/mg) from *Bacillus thermoproteolyticus rokko*, and porcine pepsin (EC3.4.23.1, 596 units/mg) were purchased from Sigma (Sigma-Aldrich Chemie Gmbh, Steinhem, Germany). Proteases from *Virgibacillus* sp. SK1-3-7 was prepared as described by Montriwong, Kaewphuak, Rodtong, Roytrakul, and Yongsawatdigul (2012). Trifluoroacetic acid (TFA) was purchased from Supelco (Bellefonte, PA, USA). An angiotensin converting enzyme (ACE), hippuryl histidyl leucine (HHL), and 2,4,6-trinitrobenzenesulfonic acid (TNBS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Other chemicals and reagents used were of analytical grade.

3.3.2 Enzymatic hydrolysis experiments

3.3.2.1 Selection of enzyme and chicken blood fractions

Each lyophilized powder of WB, BP and BC was dissolved in deionized water at 10 g/L. pH of suspension was adjusted to the optimum of each enzyme: pH 8 for Alcalase, *Vigibacillus* sp. SK1-3-7 and thermolysin; pH 7 for papain; and pH 2 for pepsin, using either 1 N NaOH or 1 N HCl solution. Enzymes were added at an enzyme/substrate ratio of 1:100. The mixtures were incubated at the optimum temperature of each proteinase: 60°C for Alcalase, *Vigibacillus* sp. SK1-3-7, and papain; 70°C for thermolysin; and 37°C for pepsin, in a shaking water bath. The enzymatic reaction was allowed to proceed for 12 h. pH of each reaction mixture was maintained at the optimal pH of the enzyme for every 15 min during hydrolysis using 1 N HCl for pepsin and 1N NaOH for others. To terminate enzymatic reaction, the mixtures were heated in a water bath at 95°C for 10 min and cooled immediately. After hydrolysis, pH of the solution was adjusted to 7 using either 1 N NaOH or 1 N HCl. The hydrolysates were centrifuged at 10,000 ×g for 15 min. The resultant supernatants were stored at -20°C until further use.

3.3.2.2 Experimental design of optimization

Alcalase-hydrolyzed BC exhibiting the highest ACE inhibition was selected for optimization using RSM. All experiments were performed in accordance with a rotatable central composite design (RCCD). Three controllable variables, including temperature (X_1: 50-60°C), enzyme concentration (X_2: 2-4%E) and time (X_3: 4-6 h), were selected for optimization. The selection of these factors was based on preliminary experiments. These variables were coded to five levels as -1.68, -1, 0, +1, and +1.68. Degree of hydrolysis (DH: Y_1) and ACE inhibition (Y_2) were used

as a response of the independent variables. In total, 20 experiments with six central points were performed. The experiment was performed in three replicates. The behavior of the system can be described with a quadratic model according to equation:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1} \sum_{j=1+1} \beta_i X_i X_j$$

where Y was the predicted response, β_0 was a constant, β_i was the linear coefficient, β_{ii} was the quadratic coefficient, β_{ij} was the interaction coefficient, and X_i and X_j were independent variables as shown in Table 3.1. The model was applied to characterize the effect of each independent variable to the response. To confirm the validity of the model, some conditions within the design space was tested. The predicted values were compared with the experimental ones.

3.3.3 Analysis of protein hydrolysates from chicken blood

3.3.3.1 DH determination

The DH was determined by the TNBS method (Adler-Nissen, 1979). Samples (0.5 mL) were added 0.5 mL of 0.2125 M sodium phosphate buffer (pH 8.2), and 0.5 mL of 0.05% (w/v) TNBS reagent. The reaction mixtures were incubated at 50°C for 60 min, and subsequently were stopped by 1.0 mL of 0.1 N HCl. Samples were allowed to cool at room temperature for 30 min, and absorbance was measured at 420 nm. L-Leucine (0-2.0 mM) was used as a standard. DH (%) was calculated using the following equation:

$$DH\ (\%) = [(h_s - h_o)/ h_{tot}] \times 100$$

where h_s was α-amino content of samples, h_o was α-amino content of sample at time 0, and h_{tot} was the total α-amino content obtained after hydrolyzing samples with 6 N HCl at 121°C for 24 h.

3.3.3.2 ACE inhibition

In vitro ACE inhibition was assayed using HHL as a substrate according to the method of Cushman and Cheung (1971) with slight modifications. The reaction mixture contained 50 µL of hydrolysates and 150 µL of ACE substrate (8.3 mM HHL in 50 mM sodium borate buffer containing 0.5 M NaCl, pH 8.3), and was pre-incubated at 37°C for 10 min. Subsequently, 50 µL of 25 mU/mL of ACE was added and further incubated at 37°C for 60 min in a shaking water bath. The reaction was terminated by 250 µL of 1 N HCl. Hippuric acid (HA) was extracted with 1.5 mL of ethyl acetate. The upper layer of ethyl acetate (1 mL) was dried at 80°C on a sand bath. The dried sample was dissolved in 1 mL of deionized water and the absorbance was read at 228 nm in a quartz cuvette. HA was used as an external standard. Blank was prepared by adding 0.1 N HCl before adding ACE. ACE inhibition (%) was calculated as follows:

$$ACE\ inhibition\ (\%) - \frac{(C_O - C_B) - (I_O - I_B)}{(C_O - C_B)} \times 100$$

where C_o was HA content of reaction of the control (without protein hydrolysates), C_B was HA content of the control blank, I_0 was HA content of reaction with protein hydrolysates and I_B was HA content of reaction blank.

Table 3.1 Central composite design with experimental and predicted values for degree of hydrolysis (DH) and angiotensin I-converting enzyme (ACE) inhibition.

	Independent variable						Response (Y_1)		Response (Y_2)	
	Coded			Actual			*DH (%)		*ACE inhibition (%)	
RunOrder	X_1 (°C)	X_2 (%E)	X_3 (h)	Temperature (°C)	Enzyme (%E)	Time (h)	Actual	Predicted	Actual	Predicted
1	-1	-1	-1	50	2	4	25.59	26.42	26.33	26.32
2	-1	-1	1	50	2	6	29.66	30.26	31.40	31.48
3	-1	1	-1	50	4	4	29.28	29.87	29.00	29.77
4	-1	1	1	50	4	6	33.80	34.32	36.27	36.14
5	1	-1	-1	60	2	4	21.80	22.16	22.66	23.32
6	1	-1	1	60	2	6	22.07	22.36	23.71	23.48
7	1	1	-1	60	4	4	25.12	25.40	25.59	26.04
8	1	1	1	60	4	6	26.17	26.21	26.86	27.41
9	0	0	0	55	3	5	31.18	31.42	31.95	32.20
10	0	0	0	55	3	5	30.98	31.42	33.62	32.20
11	0	0	0	55	3	5	31.95	31.42	34.51	32.20
12	0	0	0	55	3	5	32.48	31.42	31.44	32.20
13	0	0	0	55	3	5	32.00	31.42	30.32	32.20
14	0	0	0	55	3	5	29.74	31.42	31.20	32.20
15	0	-1.6817928	0	55	1.3182072	5	26.98	26.17	26.90	26.87
16	0	0	-1.6817928	55	3	3.3182072	28.71	27.90	28.45	27.60
17	0	0	1.6817928	55	3	6.6817928	32.24	31.81	32.99	33.09
18	0	1.6817928	0	55	4.6817928	5	32.74	32.31	33.79	33.08
19	-1.6817928	0	0	46.591036	3	5	29.29	28.21	29.48	29.32
20	1.6817928	0	0	63.408964	3	5	17.96	17.81	20.04	19.45

X_1: Temperature (°C), X_2: Enzyme (%E), X_3: Time (h). Actual values are expressed as mean (n = 3). Experimental conditions according to central composite design (CCD). *Actual and predict value are not different ($p>0.05$).

3.3.4 Ultrafiltration (UF)

BC hydrolysate (BCH) under the optimized condition was prepared and fractionated by ultrafiltration with membranes with molecular weight cut-offs (MWCO) of 30 and 1 kDa, respectively, in the sequential order (Pall, East Hills, NY, USA). Fractions were designated as follows: BCH-I, the retentate of the 30-kDa membrane (>30 kDa); BCH-II, retentate of the 1-kDa membrane obtained from the 30-kDa permeate (1-30 kDa); and BCH-III, the permeate of the 1-kDa membrane (<1 kDa). BCH and its ultrafiltered fractions were lyophilized and stored at -20°C until use. The α-amino group content of all fractions was determined using the TNBS method (Adler-Nissen, 1979). ACE inhibition and molecular weight distribution of all samples were subsequently determined.

3.3.5 Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF)

Peptide solution (2 µL) at 0.2 mg/mL was mixed with matrix solution (2 µL) composed of saturated α-cyano-4-hydroxy cinnamic acid (α-HCCA) in 30% (v/v) acetonitrile and 1% (v/v) TFA. The peptide mass fingerprint of the samples was determined by MALDI-TOF mass spectrometer (Ultraflex III TOF/TOF, Bruker Daltonik GmbH, Bremen, Germany) in positive reflector mode, with an accelerating voltage of 20kV, in the mass range of 1000-3200 Da and an extraction delay of 400 ns. Calibration was done using peptides calibration standard that covers the mass range 800-4000 Da.

3.3.6. *In vitro* gastrointestinal (GI) tract enzymatic digestion

The ultrafiltrated fraction showing the highest ACE inhibition (permeate 1 kDa; BCH-III) and unfractionated BCH was subjected to *in vitro* GI digestion using pepsin and pancreatin as described by Cinq-Mars, Hu, Kitts, and

Li-Chan (2008) with some modifications. The lyophilized powder (250 mg) was dissolved in deionized water (15 mL). pH was adjusted to 2.0 using 6 N HCl and pepsin was added at enzyme to substrate ratio of 1:35 and incubated at 37°C for 1 h. Subsequently, the pH was adjusted to 5.3 with saturated $NaHCO_3$ solution and further to pH 7.5 with 10 N NaOH. The mixture was further digested by porcine pancreatin at enzyme to substrate ratio of 1:25 (w/w) for 2 h at 37°C. The reaction was terminated by boiling for 10 min followed by centrifugation at 10,000×g for 10 min. α-amino group content, ACE inhibition and amino acid profiles of digesta were determined.

3.3.7 Amino acid analysis

Total amino acid (TAA) profiles of lyophilized powder of BCH and the 1-kDa permeate (BCH-III) before and after digesta were determined following AOAC 982.30 (AOAC, 2000). In brief, 25 mg of samples were digested with 50 mL of 6 N HCl containing 0.1% phenol at 110°C for 24 h. Hydrolysis under performic acid oxidation and sodium metabisulfite was carried out for determination of cysteine and methionine contents. Free amino acid (FAA) contents of samples were also determined by mixing lyophilized samples with 0.1 N HCl at sample to acid ratio of 1:9 (w/v), then centrifuging at 10,000×g for 20 min. Supernatant (1 mL) was mixed with 5% sulfosalicylic acid (1 mL) and left for 3 h before centrifuging at 10,000×g for 20 min. Quantitative analysis of amino acids was achieved using a Biochrom 30 amino acid analyzer (Biochrom Ltd., Cambridge Science Park, England). Norleucine was used as internal standard. The method involves a postcolumn derivatization of the amino acids with ninhydrin, which can be determined at 570 nm, except for proline at 440 nm.

3.3. 8 Antihypertensive effect

Spontaneously hypertensive rats (SHR) (9-12 week-old, male, 220-250 g body weight, blood pressure over 180 mmHg) were purchased from Charles River Japan, Inc. (Yokohama, Japan). SHRs were housed individually in steel cages with controlled room temperature (25 ± 1°C), humidity (55 ± 5%), and a 12-h light: 12-h dark cycle. They were allowed to freely access to the diet and water. The rats were acclimatized in the above conditions for about two weeks before the experiment. The experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The experimental protocols were approved by the Ethics Committee on Animal Experimentation of Shinshu University.

SHRs were randomly divided into 8 groups with 6 rats in each group. The rats were orally administered with BCH at 100, 300 and 600 mg/kg body weight (BW)/day, while BCH-III was administered at 50, 100 and 200 mg/kg BW/day. The negative control group was given distilled water, while captopril at 10 mg/kg BW/day was assigned for the positive control. The sample of each treatment was orally administered at 8:00 a.m, and the blood pressure of each rat was firstly measured at 11:00 am and every 3-h intervals onwards for 12 h by tail-cuff method with a tail measurement device (MK2000ST, Muromachi Kikai Co., Ltd., Tokyo, Japan). Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were recorded.

3.3. 9 Statistical analyses

Analysis of variance (ANOVA) was performed to determine the effect with various samples, enzymes and incubation time. Duncan's multiple range test (DMRT) was used to determine differences between mean at $p<0.05$. Statistical analysis was performed using the SPSS package (SPSS 17.0 for Windows, SPSS Inc., Chicago, IL, USA). The

response data for RSM were analyzed by Design Expert® v. 8.0.6.1 (Stat-Ease, Inc., Minneapolis, MN, USA).

3.4 Results and discussion

3.4.1 Selection of enzyme and chicken blood fractions

Crude protein content of lyophilized powder of WB, BP and BC was 75.55±0.12, 47.55±0.33 and 90.79±0.45% dry basis (db), respectively. All samples also contained relatively low-fat content of 0.45-0.67%db. DH and ACE inhibition of chicken blood hydrolysate varied with blood fractions and proteases applied (Figure 3.1). The highest DH values of 14% was found in hydrolysates prepared from BC hydrolyzed by either Alcalase or thermolysin ($p<0.05$). The Alcalase-hydrolyzed WB also showed comparable high DH of 13% (Figure 3.1a). In general, chicken plasma exhibited relatively low DH, particularly when hydrolyzed by Alcalase and papain. Blood corpuscles consist of hemoglobin as a major protein and serve as a main substrate of hydrolysis. Limited hydrolysis of blood plasma proteins could be partly attributed from endogenous protease inhibitors, like α_2-macroglobulin, serpins, α_1-antitrypsin, presenting in BP (Aubry and Bieth, 1976).

Alcalase is a known serine endopeptidase derived from *Bacillus licheniformis* with broad specificity (Cumby, Zhong, Naczk, and Shahidi, 2008), while thermolysin produced from *Bacillus thermoproteolyticus* showed specificity towards hydrophobic amino acid residues including F, I, L or Y (Matsubara and Feder, 1971). *Virgibacillus* sp. SK1-3-7 and pepsin were able to hydrolyze plasma proteins to a greater extent than papain and Alcalse. Montriwong, Kaewphuak, Rodtong, Roytrakul, and Yongsawatdigu (2012) reported that *Virgibacillus* sp. SK1-3-7 endopeptidases showed

rather broad specificity with high activity towards fibrin and fibrinogen, one of important proteins in BP. These results demonstrated that the extent of hydrolysis largely depends on both the nature of the substrate and the type of proteinase.

(a)

(b)

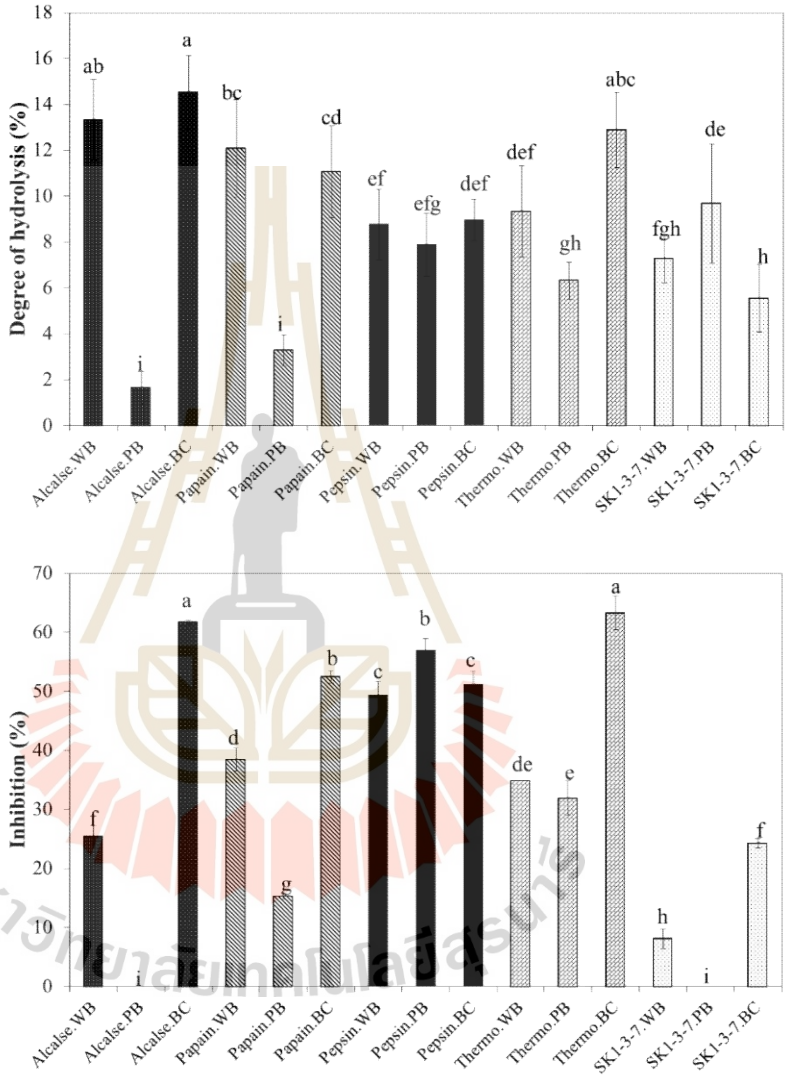

Figure 3.1 Degree of hydrolysis (DH) (a) and ACE inhibition 0.5 mg/mL peptide (b) of hydrolysate derived from various blood composition and proteases for 12h. Whole blood: WB; blood plasma: BP; blood corpuscle: BC; thermolysin: thermo; *Virgibacillus* sp. SK1-3-7: SK1-3-7. Data were expressed as mean value (n = 3).

The highest ACE inhibition of 61.8 and 63.8% was found in the BC hydrolyzed by Alcalase and thermolysin, respectively at 0.5 mg/mL peptide ($p<0.05$, Figure 3.1b), corresponding to the highest DH. These results suggested that smaller peptides exhibited higher ACE inhibitory activity. Deng, Zheng, Zhang, Wang, and Kan (2014) reported that porcine hemoglobin hydrolyzed by pepsin exhibited the highest ACE-inhibitory activity at 9.3%DH, while Sampedro and Montoya (2014) found that bovine plasma hydrolysates obtained from Alcalase 2.4 L showed the maximum ACE-inhibitory activity of 50.2% at 6.7% DH. Porcine blood corpuscles hydrolyzed by a mixture of thermolysin, chymotrypsin and trypsin, also showed high ACE-inhibitory activity of IC_{50} 0.58 mg/mL (Wei and Chiang, 2008). Blood proteins, particularly blood corpuscle, appeared to be a good source of substrate for ACE inhibitor peptides. BC and Alcalase were, thus, selected for optimization.

3.4.2 Optimization of the hydrolytic condition

Predicted and experimentally measured responses for 20 runs with 3 replications according to the experimental design are shown in Table 3.1. DH (Y_1) ranged from 17.96 to 33.80%, while ACE inhibition (Y_2) ranged from 20.04 to 36.27% (at 0.2 mg/mL peptide). ANOVA showed insignificant lack-of-fit ($p > 0.05$, Table 3.2), suggesting that the model significantly described the variation of the response. Models of DH (Y_1) and ACE inhibition (Y_2) were significant with the coefficient of determination (R^2) of 0.9719 and 0.9534, respectively, and can be described as:

$$Y_1 = -375.934 + 13.41011X_1 + 6.238374X_2 + 16.23549X_3 - 0.01021X_1X_2 - 0.18177X_1X_3 + 0.154559X_2X_3 - 0.11899X_1^2 - 0.11899X_2^2 - 0.77076X_3^2 \quad (1)$$

$$Y_2 = -377.091 + 12.92279X_1 + 7.047647X_2 + 21.04792X_3 - 0.03644X_1X_2 - 0.25047X_1X_3 + 0.302871X_2X_3 - 0.11043X_1^2 - 0.78526X_2^2 - 0.6548X_3^2 \quad (2)$$

where Y_1 *and* Y_2 are the predicted response of DH and ACE inhibition, respectively; X_1 is temperature (°C); X_2 is E/S ratio (%); X_3 is time (h).

DH gradually increased with the amount of enzyme added and reached the maximum at 4% and 6 h hydrolysis time, whereas increasing temperature up to 60°C appeared to lower DH (Figure 3.2a-b). ACE inhibition also showed the same trend as DH (Figure 2d-f). High correlation between DH and ACE inhibition was in agreement to van der Ven, Gruppen, Bont, and Voragen (2002) who reported that high DH led to high ACE inhibition of whey protein hydrolysates. Deng, Zheng, Zhang, Wang, and Kan (2014) also found that ACE inhibition of porcine hemoglobin hydrolyzed by Alcalase increased with DH. To confirm the validity of the quadratic regression model, some conditions within the design space were carried out to (Table 3.3). The obtained responses were comparable to predicated values ($p>0.05$), suggesting that models satisfactorily described ACE inhibitory activity and DH of chicken BC hydrolysate within reliability.

The maximum ACE inhibition and DH based on the optimized condition at 50.07°C, 4% E for 6 h was 37.70 and 35.83% respectively, at 0.2 mg/mL peptide (Table 3.3). When hydrolysis was carried out under such optimized condition with controlled pH at 9.6±0.2, ACE inhibition increased to 68.82%, which was a double increase at the same peptide concentration (Figure 3.3). The liberation of protons from

peptide cleavage leads to a decrease in pH of the reaction mixture, which falls below the optimal pH of Alcalase. The pH control process would help maintain enzyme activity during the course of hydrolysis, resulting in a significant increase in DH and ACE inhibition. Thus, the pH control process was recommended for further experiment.

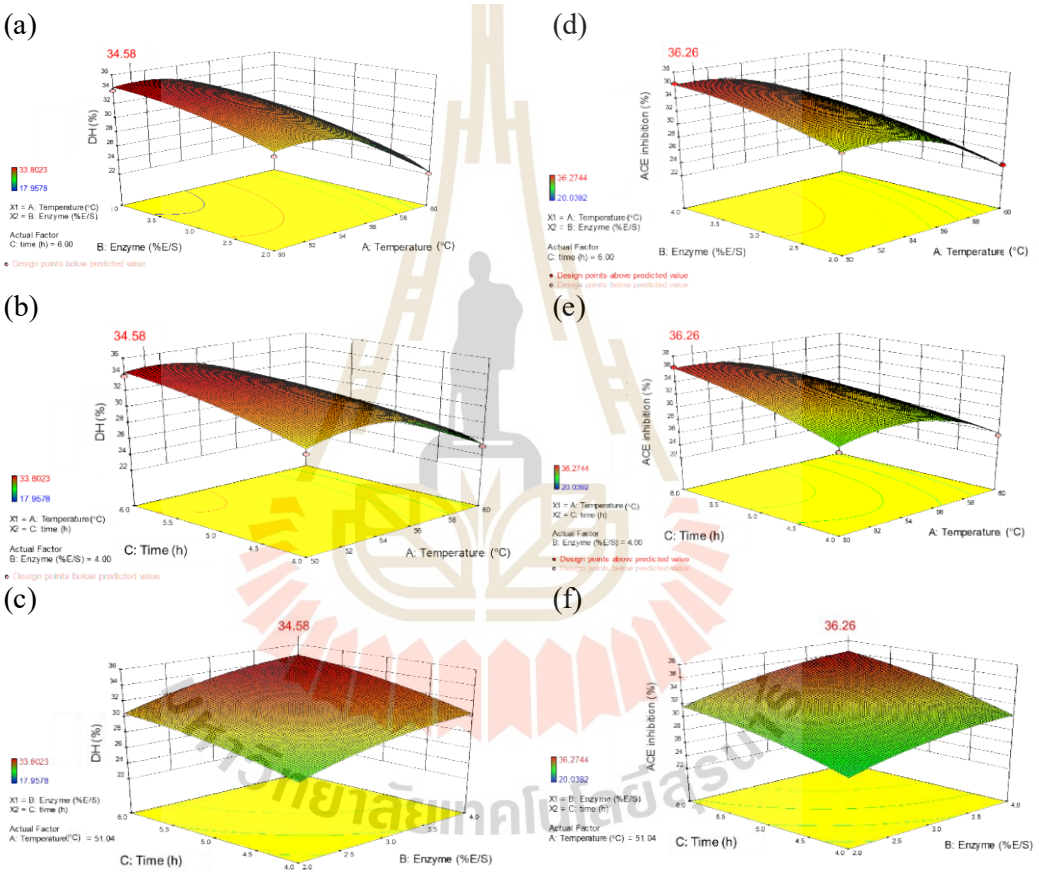

Figure 3.2 Response surface plots for degree of hydrolysis (DH) as a function of (a) temperature and enzyme, (b) temperature and time, and (c) enzyme and time. Plots of ACE inhibition as a function of (d) temperature and enzyme, (e) temperature and time, and (f) enzyme and time.

Table 3.2 ANOVA table of the model for DH (Y_1) and ACE inhibition (Y_2).

Source	Sum of squares		df		Mean square		F Value		p-value	
	Y_1	Y_2	Y_1	Y_2	Y_1	Y_2	Y_1	Y_2	Y_1	Y_2
Model	332.8242	329.2556	9	9	36.98046	36.58395	38.46411	23.2089	< 0.0001	< 0.0001
X_1-Temperature	130.5994	117.4803	1	1	130.5994	117.4803	135.839	74.52962	< 0.0001	< 0.0001
X_2-Enzyme	45.48405	46.53892	1	1	45.48405	46.53892	47.30885	29.52434	< 0.0001	0.0003
X_3-time	18.4169	36.39913	1	1	18.4169	36.39913	19.15578	23.09165	0.0014	0.0007
X_1*X_2	0.020854	0.265639	1	1	0.020854	0.265639	0.021691	0.168521	0.8858	0.6901
X_1*X_3	6.608028	12.54724	1	1	6.608028	12.54724	6.87314	7.95998	0.0255	0.0181
X_2*X_3	0.191109	0.733849	1	1	0.191109	0.733849	0.198776	0.465554	0.6652	0.5105
X_1*X_1	127.5316	109.8462	1	1	127.5316	109.8462	132.6481	69.68652	< 0.0001	< 0.0001
X_2*X_2	8.561435	8.886474	1	1	8.561435	8.886474	8.904916	5.637588	0.0137	0.039
X_3*X_3	4.423976	6.179048	1	1	4.423976	6.179048	4.601464	3.919994	0.0575	0.0759
Residual	9.614279	15.7629	10	10	0.961428	1.57629				
Lack of Fit	4.799902	3.219929	5	5	0.95998	0.643986	0.996993	0.256712	0.5013	0.9191
Pure Error	4.814377	12.54297	5	5	0.962875	2.508594				
Cor Total	342.4385	345.0185	19	19						

	Y_1	Y_2		Y_1	Y_2
Std. Dev.	0.9805	1.2555	R-Squared	0.9719	0.9543
Mean	28.4872	29.3271	Adj R-Squared	0.9467	0.9132
C.V. %	3.4420	4.2810	Pred R-Squared	0.8738	0.8747
PRESS	43.2228	43.2173	Adeq Precision	23.8099	18.7993

df: degree of freedom. X_1: Temperature (°C), X_2: Enzyme (%E), X_3: Time (h).

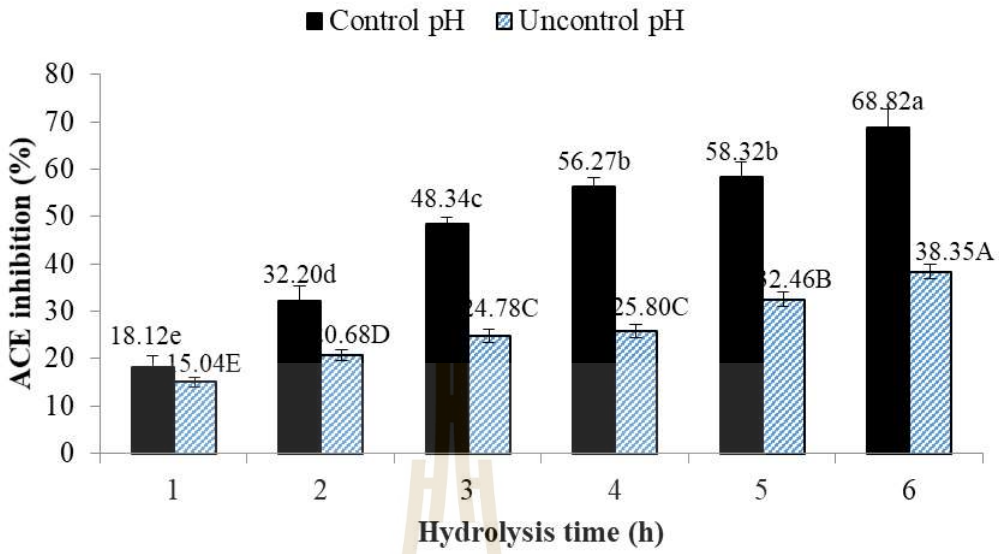

Figure 3.3 ACE inhibition of hydrolysates derived from blood corpuscles hydrolyzed by Alcalase for various times.

Table 3.3 Validation of the cubic models for DH and ACE inhibition within the design space.

Parameters			*DH (%)		*ACE inhibition (%)	
Temperature (°C)	Enzyme (% E/S)	Time (h)	Actual	Predict	Actual	Predict
50	4	6	34.55	34.32	37.01	36.31
50.31	4	6	34.64	34.42	36.85	36.38
50	3.77	6	33.96	34.16	35.84	36.03
51.07	4	6	35.83	34.58	37.70	36.50
50	2.78	6	32.33	32.58	33.77	34.09
52.22	3.95	5.99	34.82	34.53	36.15	36.33
51.81	3.97	5.95	34.91	34.56	36.51	36.36

*Actual and predict value are not different ($p>0.05$).

Actual values are expressed as mean (n = 3).

3.4.3 Ultrafiltration

BCH-III, the permeate of the 1-kDa membrane (<1 kDa), showed the highest ACE inhibition with IC_{50} of 0.138 mg/mL ($p<0.05$, Table 3.4). ACE inhibition potency of the permeate BCH-III increased by 2.5 folds compared to the crude BCH. Peptides with smaller molecular weight could be responsible for ACE inhibition. Cing-Mars and Li-Chan (2007) reported that hydroysate derived from Pacific hake (*Merluccius productus*) obtained from ultrafiltration also showed higher ACE inhibition than crude hydrolysate.

Table 3.4 IC_{50} value and yield of ACE inhibitory peptides from chicken blood corpuscle hydrolysate obtained from sequential ultrafiltration.

Fraction	*α*-amino content (mg)	IC_{50} (mg Leu eqv./mL)	Purification fold	Yield (%)
BCH	317.5	0.341[c]	1	100.0
BCH-I (>30 kDa)	46.1	0.323[c]	1.06	17.5
BCH-II (1-30 kDa)	299.3	0.259[b]	1.32	94.6
BCH-III (<1 kDa)	150.5	0.138[a]	2.47	47.4

Value are expressed as mean (n = 3). Different superscript letters at the column indicate significant difference ($p<0.05$).

Peptide mass fingerprint of crude BCH exhibited high abundance of peptides with mass-to-charge ratios (*m/z*) of 800-1080 and 1200-2000 (Figure 3.4a). The permeate fraction, BCH-III, apparently to exhibit smaller peptides with majority *m/z* of 800-1080 (Figure 3.4b). These results indicated that low MW peptides from BCH likely contributed to ACE inhibitory activity. It has been reported that peptides with

mass lower than 1000 Da, containing 2-13 amino acid residues, generally showed ACE inhibition potency (Hyun and Shin, 2000; Li, Le, Shi, and Shrestha, 2004).

(a)

(b)

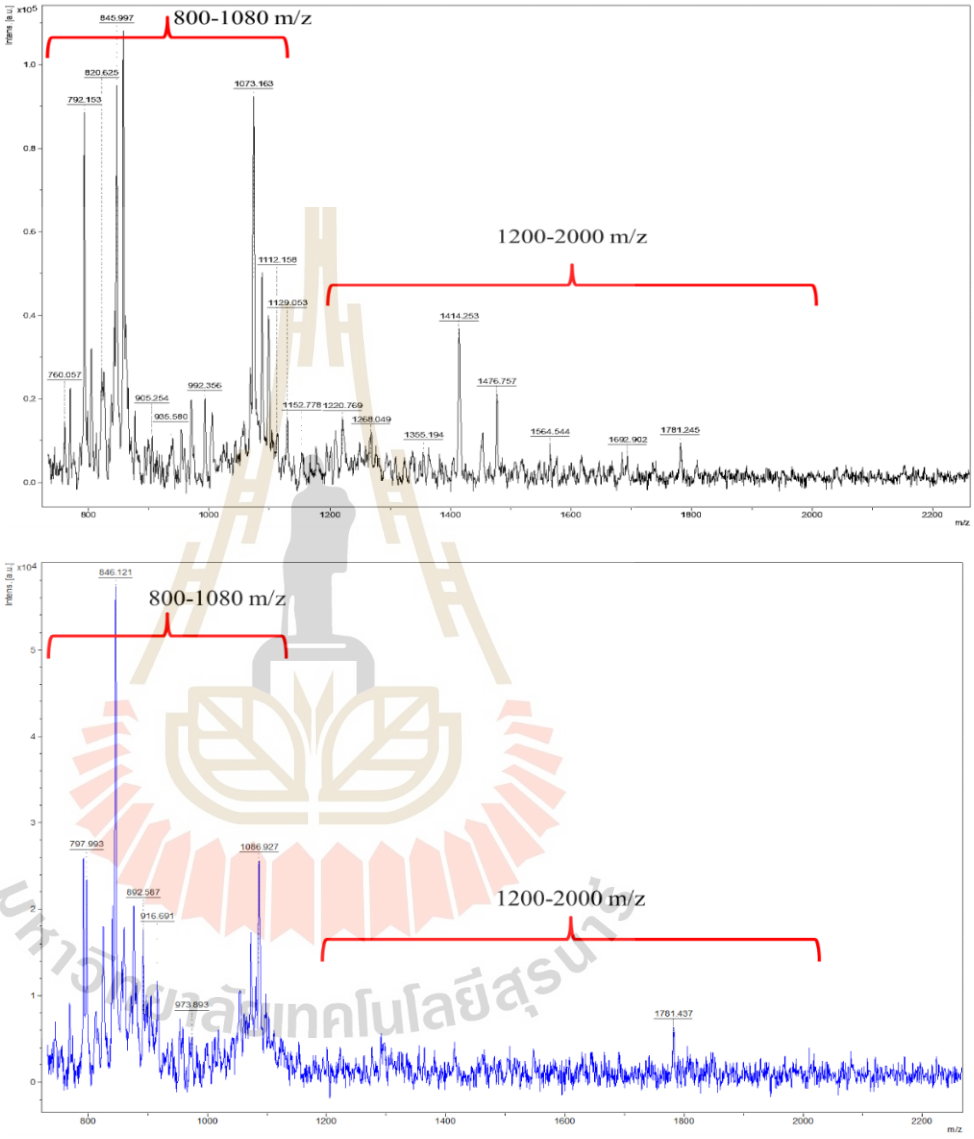

Figure 3.4 Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) spectra of hydrolysate of chicken blood corpuscle derived from Alcalase (a) crude blood corpuscle hydrolysate (BCH) and (b) <1 kDa ultrafiltrate (BCH-III).

GI digestion increased α-amino content of samples ($p<0.05$, Table 3.5). An increase in α-amino content of BCH-III was less than those of crude BCH. BCH-III contained smaller peptides, resulting lesser extent of digestion by GI enzymes. ACE inhibition of BCH digesta increased about 1.5 times ($p<0.05$), while that of permeate BCH-III digesta was comparable to its parent hydrolysates ($p>0.05$, Table 3.5). Smaller peptides generated from GI digestion appeared to possess ACE inhibitory activities. These results suggested that smaller peptides generated from GI digestion of crude BCH could play a vital role in ACE inhibition.

Table 3.5 IC_{50} value of ACE inhibitory peptides from chicken blood corpuscle hydrolysate *in simulated* gastrointestinal (GI) tract enzyme.

Treatment		**α-amino content (mg)**	**IC_{50} (mg Leu eqv./mL)**
BCH	undigest	4.31^{d}	0.362^{c}
	digesta	6.13^{b}	0.239^{b}
BCH-III	undigest	5.16^{c}	0.129^{a}
	digesta	7.01^{a}	0.113^{a}

Value are expressed as mean (n = 3). Different superscript letters at the column indicate significant difference ($p<0.05$).

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However, ACE inhibition of BCH-III digesta of IC_{50} 0.113 mg/mL showed higher than BCH digesta f IC_{50} 0.362 mg/mL ($p<0.05$). Efficacy of ACE inhibition of BCH-III was not negatively affected by GI proteases because BCH-III containing smaller peptides, which might be resistant to gastrointestinal enzymes. In addition, peptides generated from GI digestion might act as ACE inhibitors. ACE inhibitory peptides of BCH-III digesta could play an

important role in antihypertensive effect. Digesta of bovine plasma and porcine globin also showed comparable ACE inhibition to their parent hydrolysates (Sampedro and Montoya, 2014; Yu, Hu, Miyaguchi, Bai, Du, and Lin, 2006).

Crude BCH and BCH-III contained about 66-70% peptides and major amino acids in each sample were hydrophobic amino acids including F, G, A, V, I and L (Table 3.6). In general, amino acid profile of BCH-III was comparable to that of crude BCH, except that contents of A, L, and H were higher. These are common residues of ACE inhibition (Li, Le, Shi, and Shrestha, 2004; Ondetti and Cushman, 1982). Besides smaller size of BCH-III, specific amino sequences in BCH-III peptides could contribute to higher ACE inhibition.

After simulated *in vitro* GI digestion, peptides still remained in both digesta accounting for 50-60% of total weight (Table 3.6). Hydrophobic amino acids were the most abundant peptides of digesta, followed by polar amino acids, acidic and basic amino acids. However, BCH-III digesta (47.7%) contained more hydrophobic peptides than did BCH digesta (39.1%). These amino acids including F, Y, P, A, V, and L have been reported to be most favorable for the antepenultimate position (S1) for ACE inhibition (Ondetti and Cushman, 1982).

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Table 3.6 Amino acid composition of blood corpuscle hydrolysate (BCH) and the 1-kDa permeate (BCH-III) (g /100 g powder) between undigest and digesta.

AA in net peptide[1]	Undigest		Digesta	
	BCH	**BCH-III**	**BCH**	**BCH-III**
L-Proline (P)	8.11*	5.87	7.68	7.12
L-Glycine (G)	3.47	3.57	3.1	2.55
L-Alanine (A)	5.94	6.80*	4.19	6.47**
L-Valine (V)	5.80	5.28	4.23	4.10
L-Isoleucine (I)	2.77	2.27	2.30	2.01
L-Leucine (L)	7.28	8.57*	5.78	6.02
L-Methionine (M)	0.72*	0.62	0.54**	-
L-Threonine (T)	2.94	3.30	2.60	2.42
L-Serine (S)	2.51	1.87	1.75	1.56
L-Tyrosine (Y)	2.23	2.00	1.89	1.2
L-Phenylalanine (F)	4.18	3.82	2.78	2.44
L-Aspartic acid (D)	4.12*	3.50	3.37**	2.04
L-Glutamic acid (E)	6.74*	5.19	6.92**	3.68
L-Lysine (K)	6.10	6.67	5.02	4.02
L-Histidine (H)	3.64	4.35*	3.09	2.70
L-Arginine (R)	2.86	2.57	2.00	1.14
Hydrophobic	29.44	30.31	22.38	23.59
Yield (%)	*(42.4)*[2]	*(45.8)*	*(39.1)*	*(47.7)*
Polar	12.58	11.61	9.56	7.62
Yield (%)	*(18.1)*	*(17.5)*	*(16.7)*	*(15.4)*
Acidic	10.86	8.69	10.29	5.72
Yield (%)	*(15.7)*	*(13.1)*	*(18.0)*	*(11.6)*
Basic	12.60	13.59	10.11	7.86
Yield (%)	*(18.2)*	*(20.5)*	*(10.1)*	*(15.9)*
Net peptides total	**69.41**[a]	**66.25**[a]	**57.24**[b]	**49.47**[a]

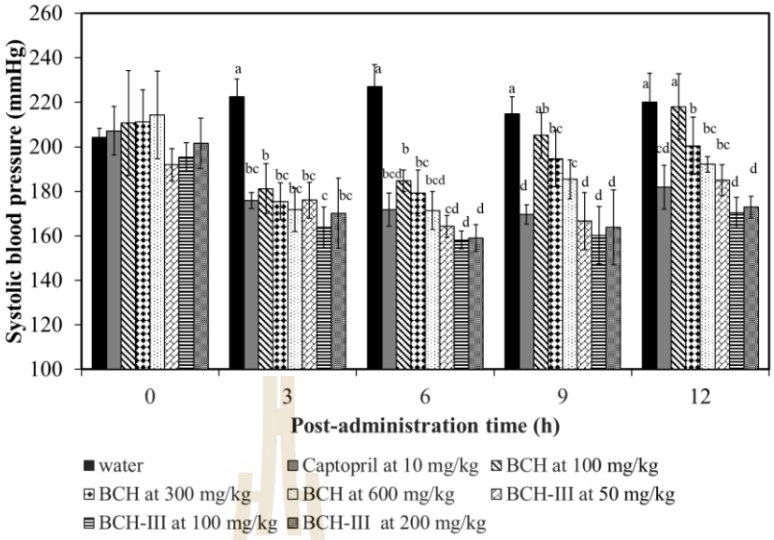
[1]Amino acid composition of peptides (total amino acids – free amino acids). [2]Numbers in parentheses indicated percentage of yield of amino acid in each R group based on net peptide total. *,**T-test was significant difference between BCH and BCH-III of parent hydrolysate and digesta ($p < 0.05$).

3.4.4 Antihypertensive effect

BCH and BCH-III at all studied concentrations decreased SBP during 3 to 6 h post-administration ($p<0.05$, Figure 3.5a). BCH-III appeared to be more effective in reduction of blood pressure than did BCH. The maximum reduction in SBP was found at a dose of 600 mg/kg of BCH and 100-200 mg/kg of BCH-III, which resulted in comparable SBP to the group fed with 10 mg/kg of captopril ($p>0.05$) at 6 h after oral administration. However, hypertensive effect of BCH reduced afterward, leading to an increase in blood pressure after 6 h. In contrast, BCH-III maintained lowest SBP throughout 12 h after oral administration of BCH-III at 100-200 mg/kg. The group fed with captopril also showed SBP reduction up to 12 h. These results confirmed that smaller peptides in BCH-III were likely to be more effective ACE inhibitors *in vivo*. High hydrophobic amino acid residues in BCH-III digesta appeared to correlate with its potent ACE inhibition.

The reduction of diastolic blood pressure (DBP) in all treatment groups was greater than that of the negative group at 3 h post-administration, except for 100 mg/kg BCH-treated group (Figure 3.5b, $p<0.05$). At 6h post-administration, the greatest decrease of DBP of -42.7 mmHg were found in the SHRs administered 100 mg/kg BCH-III, which was comparable to that of captopril-treated group ($p<0.05$). DBP value of the BCH-III-treated groups remained stable up to12 h post-administration. Therefore, oral administration of BCH-III at concentration as low as 100 mg/kg showed an effective reduction in blood pressure with longer lasting effects than the BCH ($p<0.05$).

(a)



(b)

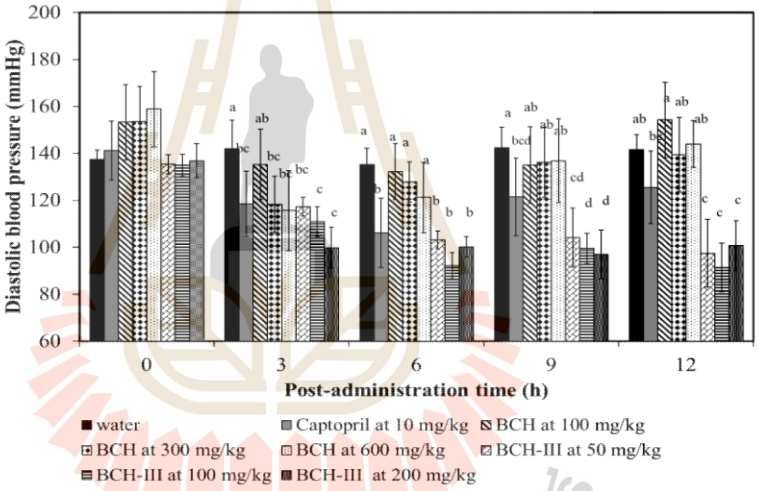


Figure 3.5 Changes of systolic blood pressure (a) and diastolic blood pressure (b) after oral administration of crude blood corpuscles hydrolysate (BCH) and ultrafiltrated hydrolysate (BCH-III) in spontaneously hypertensive rats (SHR). Data were expressed as mean value (n = 6). Different letters at the same time indicated significant difference ($p<0.05$).

This study demonstrated that BCH and BCH-III from chicken blood showed blood pressure-lowering ability up to 12 h, depending on dose of intake. Cod protein hydrolyate reduced SBP at dose of 200 mg/kg about -19 mmHg after 2 h of intake (Girgih, Nwachukwu, Hasan, Fagbemi, Gill, and Aluko, 2015). In addition, the membrane fractions of pea protein hydrolysate showed the highest SBP reduction of -36 mmHg after oral administration of 100 mg/kg for 4 h (Girigh, Nwachukwa, Onuh, Malomo, and Aluko, 2016). Moreover, flaxseed protein hydrolysate and its UF fraction were effective in lowering SBP about -30 mmHg in SHR at 200 mg/kg after 6h administration (Nwachukwu, Girgih, Malomo, Onuh, and Aluko, 2014). This is the first study demonstrating hypertensive effect of chicken BC hydrolysate. Identification of peptide(s) responsible for antihypertensive effect of BCH-III would be needed.

3.5 Conclusions

The optimum condition to yield chicken blood hydrolysate with ACE inhibition was to hydrolyze BC with Alcalase at 51.07°C, 4% enzyme, at controlled pH9.6 throughout the course of 6 h hydrolysis. The BCH-III, permeate of 1-kDa-membrane, exhibited the higher ACE inhibition than crude BCH. Both of BCH and BCH-III were rich sources of F, G, A, V, I and L. BCH was not effectively in DBP-lowering. The BP-lowering capacity indicated that the BCH-III at 200 mg/kg showed SBP reduction of -42.6 mmHg within 6 h after oral administration, demonstrating valorization as antihypertensive functional food ingredients.

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CHAPTER IV

CHARACTERIZATION AND IDENTIFICATION OF ANTIHYPERTENSIVE PEPTIDES FROM CHICKEN BLOOD CORPUSCLES

4.1 Abstract

Antihypertensive effect of Alcalase-hydrolyzed chicken blood corpuscles was evaluated. Fraction G3 obtained from size exclusion chromatography (SEC) of the 1-kDa permeate of blood corpuscles hydrolysate (BCH-III) possessed the highest angiotensin converting enzyme (ACE) inhibitory activity with IC_{50} of 66 µg/mL. Further purification by reverse-phase chromatography resulted in 2 peaks exhibiting IC_{50} of 14.0 and 18.0 µg/mL, respectively. The *de novo* peptide sequencing by LC-MS/MS revealed 3 potent inhibitor peptides of VSKRLNGDA, NVSTVLTMKKF and FPLCTPAFMTV, which showed the highest IC_{50} of 25.28-28.44 µM after *simulated* GI digestion. The VSKRLNGDA showed the blood pressure lowering effect on the spontaneously hypertensive rat model at 50 mg/kg with the maximum reduction of systolic blood pressure of -32.0 mmHg within 12h. A maximum decrease in diastolic blood pressure was observed at a dose of 12.5-50 mg/kg. Antihypertensive peptides derived from chicken blood corpuscle protein hydrolysate showed potential to be utilized as nutraceutical products for stabilizing blood pressure.

Keywords: angiotensin I-converting enzyme (ACE); antihypertensive peptide; blood pressure; chicken blood; spontaneously hypertensive rats (SHR)

4.2 Introduction

Hypertension is one of the primary risk factors of cardiovascular disease (CVD) leading to stroke, heart failure, and shortened life expectancy (Jensen, Eysturskarð, Madetoja, and Eilertsen, 2014). High blood pressure can be controlled through the renin-angiotensin system (RAS) in which angiotensin I-converting enzyme (ACE) is a key element in the RAS and catalyzes the formation of the potent vasoconstrictive octapeptide (angiotensin II) from decapeptide and inactivates the vasodilative peptide, bradykinin, a blood pressure lowering nonapeptide in the kallikrein-kinin system (Cushman, Cheung, Sabo, and Ondetti, 1977). Inhibition of ACE is considered to be one of therapeutic approaches in the treatment of hypertension.

It has been widely accepted that food proteins do not only provide nutritional value of amino acids but also peptides that exert biological activities (Sánchez and Vázquez, 2017). Peptides exhibiting ACE inhibitory activity has been widely studied. Hydrolysates from various protein sources, including porcine hemoglobin (Deng, Zheng, Zhang, Wang, and Kan**,** 2014), rapeseed protein (He, Aluko, and Ju, 2014), salmon byproducts (Ahn, Jeon, Kim, and Je, 2012) have been shown to inhibit ACE *in vitro*, while those prepared from pea protein (Girigh, Nwachukwa, Onuh, Malomo, and Aluko, 2016), chicken skin protein (Onuh, Girgih, Malomo, Aluko, and Aliani, 2015), egg protein (Onuh, Girgih, Malomo, Aluko, and Aliani, 2015) and oyster proteins hydrolysates (Wang et al., 2008) showed hypotensive effect in spontaneously

hypertensive rats (SHR) model. Previous results have been reported that chicken blood hydrolysate and its fraction showed high ACE inhibition and an effective reduction in blood pressure. Therefore, characterization and identification of peptides responsible for antihypertensive effect of protein hydrolysate derived from chicken blood would be investigated.

Proteolysis at gastrointestinal (GI) tract definitely modify structure and ACE inhibitory activity of protein hydrolysates and peptides. It is, therefore, necessary to evaluate ACE inhibitory capacity under GI digestion, even *in vitro*. Digesta obtained from various food protein hydrolysates from chicken breast and soy protein isolate showed an increase in ACE inhibitory activity (Lo, Farnworth, and Li-Chan, 2006; Sangsawad, Roytrakul, and Yongsawatdigul, 2017). In contrast, ACE inhibitory activity of peptides from Pacific hake (*Merluccius productus*) and whey hydrolysate decreased after *in vitro* GI digestion (Cinq-Mars, Hu, Kitts, and Li-Chan, 2008; Hernandez-Ledesma, Amigo, Ramos, and Recio, 2004). Selection of the potent inhibitor should also include residual activity after GI digestion. Objectives of this study were to isolate, identify, and characterize ACE-inhibitory peptides derived from chicken blood corpuscles hydrolysate. Antihypertensive effect of the purified peptide was evaluated based on spontaneously hypertensive rats (SHRs) model.

4.3 Materials and methods

4.3.1 Sample preparation

Chicken blood samples were obtained from a commercial chicken slaughterhouse in Nakhon Ratchasima, Thailand, using sterile containers including 1% (w/v) sodium citrate in the final volume as an anticoagulant. Samples were maintained

at chilled temperatures (5°C) until use. Plasma and blood corpuscles (BC) were separated by centrifuging the whole blood at 2530×*g* at 4°C for 15 min (Hitachi CR22GIII, Hitachi Koki Co. Ltd., Tokyo, Japan). Precipitated BC was collected, lyophilized and kept at -20°C throughout the study.

4.3.2 Preparation and fractionation of hydrolysate

Lyophilized BC (1 g) was added 100 mL deionized water and pH of suspension was adjusted to 9.6±0.5 using 1 N NaOH. The mixture was incubated at 51.1°C for 15 min in a shaking water bath before Alcalase 2.4L (2.4 Anson Unit/g, Sigma-Aldrich Chemie Gmbh, Steinhem, Germany) was added at 4%E/S. The enzymatic reaction was allowed to proceed for 6 h and pH of the reaction was controlled at 9.6±0.5 at 30-min intervals using 10 N NaOH. To terminate the reaction, the mixture was heated in a water bath at 95°C for 10 min and cooled. Subsequently, pH of the mixture was adjusted to 7 using 1 N HCl. The hydrolysate was centrifuged at 10,000×*g* for 15 min. The supernatant was referred to as BC hydrolysate (BCH).

The BCH was further fractionated using ultrafiltration with membranes having molecular weight cut-offs (MWCO) of 30 and 1 kDa in the sequential order (Pall, East Hills, NY, USA). Fractions were designated as follows: BCH-I, the retentate of the 30-kDa membrane (>30 kDa); BCH-II, retentate of the 1-kDa membrane obtained from the 30-kDa permeate (1-30 kDa); and BCH-III, the permeate of the 1-kDa membrane (<1 kDa). Crude BCH and all ultrafiltrated fractions were lyophilized and then stored at -20°C until use. The fraction showing the highest inhibitory activity was purified using size exclusion and reverse phase chromatography as described below.

Peptide content was determined by the 2,4,6-trinitrobenzenesulfonic acid (TNBS) method (Adler-Nissen, 1979). Triplicate aliquots (0.5 mL) were mixed with 0.5 mL of sodium phosphate buffer (0.2125 M, pH 8.2). Five hundreds microliters of 0.05% (w/v) TNBS was added and incubated at 50°C for 60 min. After incubation, the reaction was stopped by adding 1.0 mL of 0.1 N HCl. Samples were allowed to cool at room temperature for 30 min, and absorbance was measured at 420 nm. L-Leucine (0-2.0 mM) was used as a standard.

4.3.3 Determination of ACE inhibitions

The ACE inhibition was determined according to Holmquist, Bünning, and Riordan (1979) with some modifications. Briefly, the mixture of 1 mL of 0.5 mM N-[3-(2-Furyl)acryloyl]-Phe-Gly-Gly (FAPGG, Sigma Chemical Co. Ltd., St. Louis, MO, USA) dissolved in 50 mM Tris–HCl buffer containing 0.3 mM NaCl, pH 7.5, 20 µL of ACE (1 U/mL; final activity of 20 mU) and 200 µL of peptide sample in 50 mM Tris–HCl buffer were mixed. A decrease in absorbance was monitored at 340 nm at 2-min intervals for 10 min at room temperature (25°C). Tris–HCl buffer was used instead of peptide sample as a blank. The result was expressed as ΔA/min and ACE inhibition was calculated as follows:

$$ACE\ inhibition\ (\%) = 1 - \left(\frac{\Delta A/\ min\ _{(sample)}}{\Delta A/min_{(blank)}}\right) \times 100$$

where $\Delta A/min_{(sample)}$ and $\Delta A/min_{(blank)}$ were the reaction rates in the presence and absence of the peptide fraction, respectively. The IC_{50} value was defined as the concentration of inhibitor required to inhibit 50% of the ACE activity.

4.3.4 Peptide purification

4.3.4.1 Size exclusion chromatography (SEC)

The 1-kDa permeate exhibiting the highest inhibitory activity was purified using a Superdex Peptide 10/300 GL column (10×100 mm, GE Healthcare, Piscataway, NJ, USA). The lyophilized powder was dissolved in deionized water at 0.1 mg/mL and 100 µL of peptide solution was loaded onto a column. The elution was performed using a mobile phase of 0.1% (v/v) TFA containing 30% (v/v) acetonitrile at a flow rate of 0.5 mL/min. The eluate was monitored at 215 nm and collected in 1 mL-volume fractions. Yield (%) was calculated based on α-amino content. Fractions showing the highest ACE inhibition (IC_{50}) was pooled, lyophilized and further separated by reverse-phase high-performance liquid chromatography.

4.3.4.2 Reverse-phase fast protein liquid chromatography (RP-FPLC)

Lyophilized powder collected from a Superdex peptide column was resuspend in DI water to contain final concentration of 0.1 mg/mL and applied onto a SOURCE™ 5RPC ST 4.6/150 column (GE Healthcare, Piscataway, NJ, USA). Elution was performed at room temperature at a flow rate of 1 mL/min. The column was equilibrated with two column volumes of the equilibration solvent A (0.05% TFA in DI water). The elution gradient was started with 0–10% of mobile phase B (0.05% TFA in acetonitrile) for 2 CVs, 10-16%B for 11 CVs and 16–100%B for 10 CVs. The column was cleaned after each run with 100% B solvent for 1 CV. The absorbance was monitored at 214 nm, and 1-mL fractions were collected. Fractions showing the highest ACE inhibition were pooled, lyophilized and kept at -20°C until use for determination of amino acid sequences and peptide synthesis.

4.3.5 Peptide sequencing

Amino acid sequences of the highest ACE inhibitory peptide fraction obtained from RP-FPLC was carried out using the Ultimate 3000 LC System (Dionex Ltd., Surrey, UK) coupled to an ESI-Ion Trap MS (HCT Ultra PTM Discovery System, Bruker, Daltonics, Germany) with electrospray ionization. Peptide mass was performed in a positive ion mode using ESI-MS/MS and was separated on a nanocolumn (Acclaim PepMap 100 C18, 3 mm, 100A, 75 mm i.d. × 150 mm). Elution was performed using a gradient of mobile phase A (0.1% formic acid in water) and a 0-70% linear gradient of mobile phase B (80% ACN in water with 0.1% formic acid). Peptide was eluted at a flow rate of 300 nL/min for 13 min.

The *de novo* amino acid sequence was carried out using Pepnovo, which can be accessed through the URL http://proteomics.ucsd.edu/LiveSearch/. Sequences showing high PepNovo score (>60%) were chosen. NCBI Protein Blast (chicken taxid: 9031) for homology searches between the obtained sequences against the protein data bank proteins (pdb) database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

4.3.6 Peptide synthesis

Based on their Pepnovo scores and matched with chicken blood peptides, ten out of 19 peptides identified from LC-MS/MS were chosen and chemically synthesized using a solid phase peptide synthesis method (GL Biochem Ltd., Shanghai, China). Peptides, VNEDSGPFEDSTGATS, VSKRLNGDA, MMTCLAGMPNLF, ELNNLLNPALFFSA, ARCGSHCDYIKHWP, NVSTVLTMKKF, CSFDVPTGWASWTPL, FPLCTPAFMTV, NCVWSGSTFGNPRYSIG and VMKKSSRCTGFERLAGFNRNFEFA, were purified using an HPLC column to 95% purity. Molecular mass of synthetic peptides was verified by manufacturer using liquid chromatography coupled to a mass spectrometer

(LC-MS/ESI). The ACE inhibition was determined as described in 4.3.3. *In vitro* and *in silico* GI digestion of each synthetic peptide were also determined as described in 4.3.7.

4.3.7 *In vitro* and *in silico* gastrointestinal (GI) digestion

The *in vitro* GI digestion of synthetic peptides was carried out using pepsin and pancreatin as described by Cinq-Mars, Hu, Kitts, and Li-Chan (2008) with some modifications. Synthetic peptide solution (20 mg/mL) was adjusted to pH 2.0 using 6 N HCl and porcine pepsin (≥250 units/mg solid, Sigma Chemical Co. Ltd., St. Louis, MO, USA) was added at enzyme to substrate ratio of 1: 35 (w/w) and incubated at 37°C for 1 h. Subsequently, pH was adjusted to 5.3 with saturated $NaHCO_3$ solution and further to pH 7.5 with 5 N NaOH. The mixture was further digested by porcine pancreatin at E/S ratio of 1:25 (w/w) for 2 h at 37°C. Subsequently, the sample was boiled for 10 min followed by centrifugation at 10,000×*g* for 10 min. ACE inhibition of all peptide digesta was determined. *In silico* peptide digestion by pepsin, trypsin, chymotrypsin and pancreatic elastatse was carried out using the BIOPEP program (http://www.uwm.edu.pl/ biochemia/index.php/ pl/biopep).

4.3.8 Kinetics of ACE inhibition

The kinetics of ACE inhibition was evaluated at various concentrations of FAPGG (0.0625-0.5 mM) in the presence of the VSKRLNGDA peptide (0-0.08 mM). The mode of ACE inhibition was determined from the Lineweaver–Burk plots while kinetic parameters (V_{max}, K_m and K_i) were estimated using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA). All kinetics experiments were performed in duplicate.

4.3.9 Animal model

Male spontaneously hypertensive rats (SHR) at age of 9-12 weeks old and body weight of 220-250 g body weight (BW) and blood pressure over 180 mmHg, were purchased from Charles River Japan, Inc. (Yokohama, Japan). SHRs were housed individually in steel cages with controlled temperature (25 ± 1°C), humidity (55 ± 5%), and a 12-h light: 12-h dark cycle. They were allowed to have free access to the diet and tap water. Rats were acclimatized in the above conditions for about two weeks before the experiment. The experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The experimental protocols were approved by the Ethics Committee on Animal Experimentation of Shinshu University.

SHRs were randomly divided into 5 groups with 6 rats in each group. Rats were orally administered with the synthetic peptide, VSKRLNGDA, at 12.5, 25 and 50 mg/kg BW. The negative control group was given distilled water, while captopril at 10 mg/kg BW/day was assigned for the positive control. The sample in each treatment was orally administered. The blood pressure of each rat was measured at 0, 3, 6, 9 and 12 h after oral administration by a tail measurement device (model MK2000ST, Muromachi Kikai Co., Ltd., Tokyo, Japan). Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were recorded three times without anesthesia for each measurement.

4.3.10 Statistical analyses

Data was performed to determine by one-way analysis of variance (ANOVA). Duncan's multiple range test (DMRT) was used to determine differences

between mean at $p<0.05$. Statistical analysis was performed using the SPSS package (SPSS 17.0 for Windows, SPSS Inc., Chicago, IL, USA).

4.4 Results and discussion

4.4.1 Purification of ACE inhibitory peptides

After sequential ultrafiltration, the BCH-III fraction corresponding to MW < 1 kDa exhibited the highest ACE inhibitory activity with IC_{50} of 0.138 mg/mL, whereas crude BCH showed the lowest ACE inhibitory activity ($p<0.05$, Table 4.1). Inhibitory activity appeared to increase as size of peptides decreased, implying that potent inhibitors are among lower molecular weight peptides. The BCH-III fraction was, thus, selected for further purification.

Table 4.1 Purification fold and yield after a series of purifications of ACE inhibitory peptides of BCH.

Purification step	*α*-amino content (mg)	*IC_{50} (mg/mL)	Purification fold	Yield (%)
BCH	371.5	0.341^{d}	1.00	100.0
BCH-III (<1 kDa)	150.5	0.138^{c}	2.47	40.5
G3 from size exclusion	52.4	0.066^{b}	5.17	14.1
R2 from RPC	6.55	0.014^{a}	24.36	1.76
R3 from RPC	24.89	0.018^{a}	18.94	6.70

* IC_{50}: The concentration required to inhibit 50% of the ACE activity.

Value are expressed as mean (n = 3).

Size exclusion separated 4 fractions and the fraction G3 possessed the highest ACE inhibitory activity (IC_{50} 65.6 µg/mL, Figure 4.1). Purification fold of ACE inhibitor peptides achieved by size exclusion chromatography increased about 5 folds with yield of 14.1% (Table 4.1). It should be mentioned that ACE inhibitory peptides were also present in other fractions of size exclusion (G1, G2 and G4). But they were discarded due to relatively lower activity. This would be a main reason of lower peptide yield after size exclusion chromatography.

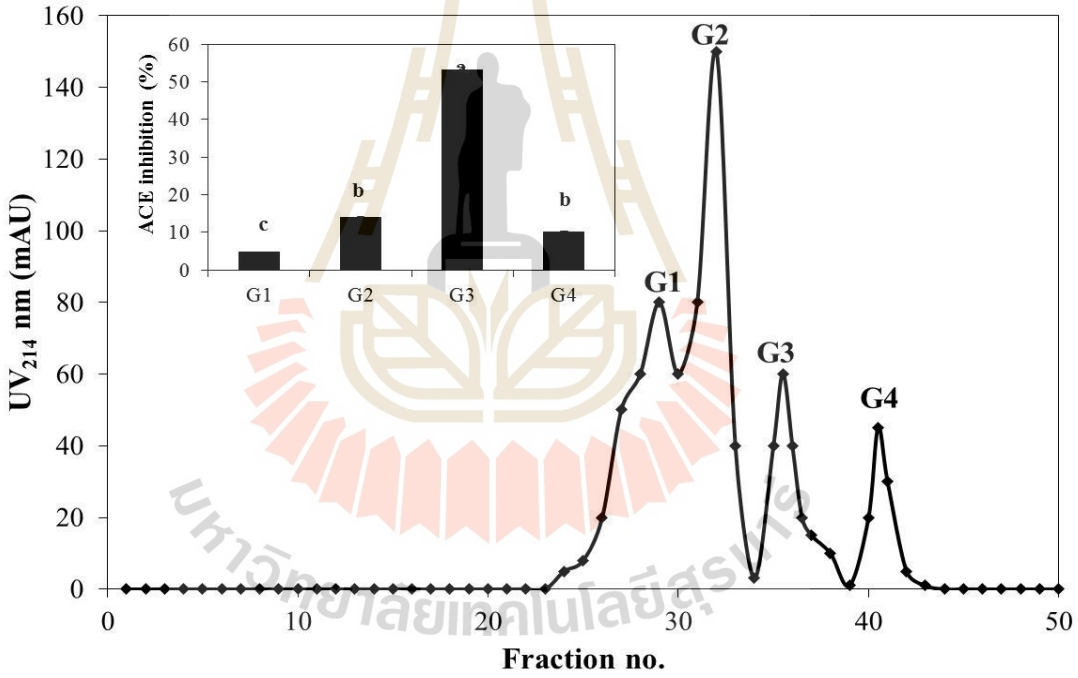

Figure 4.1 Superdex peptide 10/300 GL size exclusion chromatogram and ACE inhibition of chicken blood corpuscle hydrolysate derived from Alcalase with <1 kDa ultrafiltrate (BCH-III).

Reverse-phase chromatography of G3 yielded 2 fractions, namely R2 and R3, exhibiting the highest ACE inhibitory activity with IC_{50} of 14 and 18 µg/mL, respectively (Figure 4.2, Table 4.1). In addition, increased of purification fold of ACE inhibitor peptides by RP-HPLC was 19-24 folds with yield of 2-7% (Table 4.1). The separation was based on differences in hydrophobicity that the fraction with high hydrophobic was longer residence time on the column. The results indicated that R2 and R3 derived from BCH showed high ACE inhibition of novel bioactive peptide sequences based on hydrophobic properties of the amino acid residues. The results are higher than ACE inhibitory activity with IC_{50} of 130 µg/mL that was reported for RP-HPLC peptide fraction of cod protein hydrolysate as FAPGG substrate (Girgih, Nwachukwu, Hasan, Fagbemi, Gill, and Aluko, 2015). Moreover, based on hippuryl histidyl leucine (HHL) as substrate, purified ACE inhibitory peptides derived from porcine globin hydrolysate increased by 258 folds with IC_{50} of 4.6-7.7 µg/mL (Yu, Hu, Miyaguchi, Bai, Du, and Lin, 2006), while ACE-inhibitory peptides from porcine hemoglobin showed IC_{50} of 20 µg/mL (Deng, Zheng, Zhang, Wang, and Kan, 2014).

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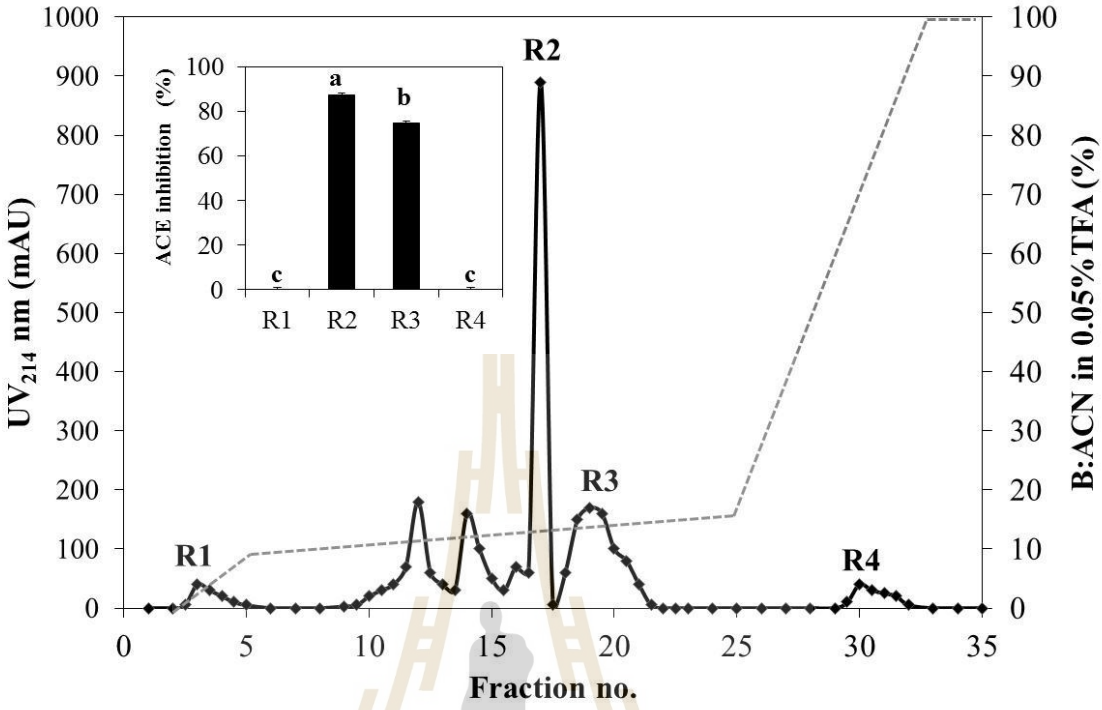


Figure 4.2 RP-HPLC SOURCE™ 5RPC ST 4.6/150 chromatogram eluted with ACN containing 0.05% TFA and ACE inhibition of the G3 fraction.

4.4.2 Peptide identification

Total of 19 peptides were identified, but only 10 peptides showed Pepnovo score of >60 and possess amino acid sequences of proteins derived from chicken blood (Table 4.2). Four out of 10 peptides were derived from the R2 fraction. The relatively long peptides containing 17-24 amino acid residues, ARCGSHCDYIKHWP and FPLCTPAFMTV showed the highest ACE inhibitory activity at the same IC_{50} of 29.17 µM while the lowest activity was found in MMTCLAGMPNLF, NVSTVLTMKKF and CSFDVPTGWASWTPL ($p<0.05$, Table 3). After simulated GI digestion, potency of ACE inhibition of some peptides reduced, while some remained the same and some increased. Peptides, VSKRLNGDA, NVSTVLTMKKF and

FPLCTPAFMTV showed an increase in ACE inhibitory activity after simulated GI digestion, and their digesta showed IC_{50} of 25.28-28.44 µM while lowest activity of VNEDSGPFEDSTGATS with IC_{50} of 114.04 µM ($p<0.05$, Table 4.2). A database search for sequence homology revealed that the VSKRLNGDA and NVSTVLTMKKF, respectively, showed sequence homology of a chain B, R-state form of chicken hemoglobin D, while FPLCTPAFMTV was a chain I, cytochrome Bc1 complex from chicken (Table 4.2).

Based on *in silico* peptide digestion, VSKRLNGDA, NVSTVLTMKKF, FPLCTPAFMTV yielded *in silico* digesta that contained di- or tri-peptides exhibiting ACE inhibitory activity (Table 4.2). Some of them have been reported as ACE inhibitory peptides as shown on BIOPEP database, especially VSKRLNGDA digesta costing of NG and DA. It should be note that digesta of NVSTVLTMKKF and FPLCTPAFMTV contained NV, TM, KKF and PL, CT, PA, TV, MT with high ACE inhibition of novel ACE inhibitory peptides.

In addition, digesta of VSKRLNGDA, NVSTVLTMKKF, FPLCTPAFMTV contained aromatic amino acids (F, Y, W) and hydrophobic amino acids (P, A, V, and L), which were favorable for the antepenultimate position (S1) (Ondetti and Cushman, 1982; Choi, Cho, Yang, Ra, and Suh, 2001). Moreover, digesta of VNEDSGPFEDSTGATS showed the lowest ACE inhibition. Peptides in digesta (VN, DSGP, EDS) have not been found to inhibit ACE.

Table 4.2 Amino acid sequences and IC_{50} values of purified peptides based on the *de novo* peptide sequencing algorithm and *in silico* of amino acid sequences.

Peptides	PepNovo score	Mass [M+H] (Da)	[w]Parent protein	[x]Database	IC_{50} (μM)		[z]*in silico* based on BIOPEP
					Undigest	[y]Digesta	
VNEDSGPFEDSTGATS	100	1612.66	Chain I, cytochrome Bc1 complex from chicken with designed inhibitor bound	NCBI id: PDB: 4U3F_I	35.56[c,*]	114.04[e]	VN, DSGP, EDS
VSKRLNGDA	64	959.53	Chain B, R-state form of chicken hemoglobin D	NCBI id:PDB: 1HBR_B	34.48[c]	26.46[a,*]	VSK, NG**, DA**
MMTCLAGMPNLF	91	1328.59	Chain C, cytochrome Bc1 complex from chicken	NCBI id:PDB: 1BCC_C	40.64[d]	35.56[b,*]	TCL, AGM, PN
ELNNLLNPALFFSA	100	1562.82	Chain D, chicken cytochrome Bc1 complex inhibited by an iodinated analogue of the polyketide crocacin-d	NCBI id:PDB: 3CWB_B	34.48[c,*]	47.42[c]	EL, PAL, SA
ARCGSHCDYIKHWP	100	1672.75	Chain B, chicken cytochrome Bc1 complex inhibited by an iodinated analogue of the polyketide crocacin-d	NCBI id:PDB: 3CWB_B	29.17[a,*]	35.56[b]	AR**, CG, SH, CD
NVSTVLTMKKF	72	1267.71	Chain A, R-state form of chicken hemoglobin D	NCBI id:PDB: 1HBR_A	40.64[d]	28.44[a,*]	NV, TM, KKF

Table 4.2 (Continued).

Peptides	PepNovo score	Mass [M+H] (Da)	[w]Parent protein	[x]Database	IC_{50} (μM)		[z]*in silico* based on BIOPEP
					Undigest	[y]Digesta	
CSFDVPTGWASWTPL	88	1666.76	Chain A, two fibronectin type-iii domain segment from chicken tenascin	NCBI id:PDB: 1QR4_A	39.24[d,*]	66.97[d]	CS, DV, PT**, WA**, WT, PL
FPLCTPAFMTV	100	1226.60	Chain I, cytochrome Bc1 complex from chicken	NCBI id:PDB: 1BCC_D	29.17[a]	25.28[a,*]	PL, CT, PA, TV, MT
NCVWSGSTFGNPR YSIG	100	1844.84	Chain A, crystal structure of native chicken fibrinogen	NCBI id:PDB: 1EI3_A	31.60[b,*]	39.24[b]	CVW, NCV, WS, CV, GN, PRY**, SIG, PR**, NPR
VMKKSSRCTGFERL AGFNRNFEFA	80	2795.39	Chain G, chicken cytochrome Bc1 complex inhibited by an iodinated analogue of the polyketide crocacin-d	NCBI id:PDB: 3CWB_G	51.73[e]	40.64[b,*]	VM, SSR, CT, ER, AGF, EF

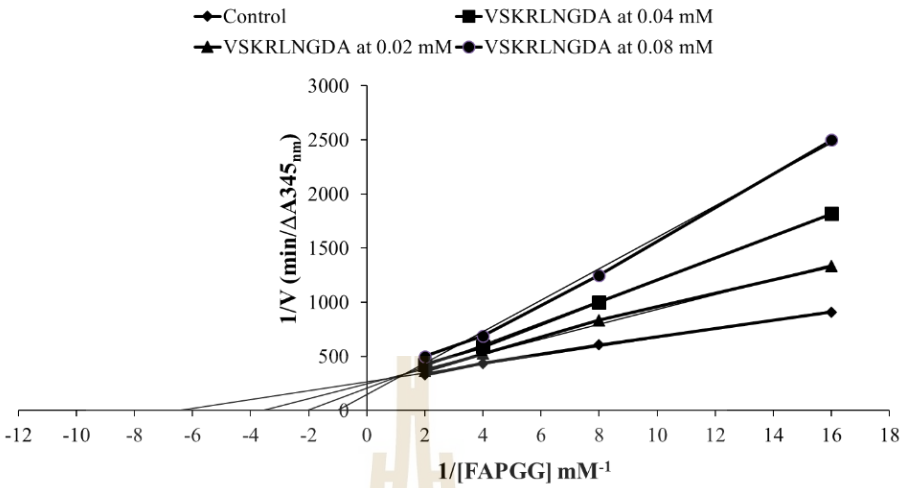
[w]PepNovo score match with reported parent protein (s).
[x]Databases used for assignment of peptide sequences.
[y]peptides were hydrolyzed by in vitro gastrointestinal (GI) enzyme.
[z]The *in silico* digestion by pepsin, trypsin, chymotrypsin and pancreatic elastatse.
IC_{50} value are expressed as mean (n = 3).
*T-test was signigicant to compare between undigest and digesta each peptide ($p<0.05$).
Small letter superscript at the same column had different ($p<0.05$).
**ACE inhibitor peptides reported in BIOPEPE database, http://www.uwm.edu.pl/biochemia/index.php/en/biopep.

ACE inhibition of VSKRLNGDA (IC_{50} of 34.48 μM) found in this study was higher than peptides derived from rapeseed protein, namely TF, LY and RALP with reported IC_{50} of 810, 107 and 648 μM, respectively (He et al., 2013). VSKRLNGDA was found to be a novel ACE inhibitory peptide derived from chicken blood corpuscles and showed strong inhibition after *simulated* GI digestion. Therefore, mode of inhibition of VSKRLNGDA was studied and its efficacy of on antihypertension was tested *in vivo*.

4.4.3 Mode of ACE-inhibitory kinetics

The double reciprocal plots of ACE-catalyzed reaction in the absence and presence of VSKRLNGDA inhibitor are shown in Figure 4.3. As peptide concentration increased, the apparent K_m values increased, while the apparent V_{max} values decreased. These results indicated a mixed type of ACE inhibition. The peptide likely bound to ACE and the ACE-FAPGG complex. Active site blockage by VSKRLNGDA prevented a substrate binding, leading to a limited activity. The VSKRLNGDA could also have other binding sites that induce conformational changes of ACE, altering substrate binding and reducing ACE activity.

Several peptides have been shown to exhibit a mixed mode inhibition, namely TF derived from rapeseed protein (He, Aluko, and Ju, 2014), TK, RMLGQTP derived from porcine troponin C (Katayama et al., 2004), FEDYVPLSCF and FNVPLYE derived from salmon byproduct protein (Ahn, Jeon, Kim, and Je, 2012).



Parameter	0 mM	0.02 mM	0.04 mM	0.08 mM
$V_{max}/V_{max,app}$ (A.min^{-1})	0.004194	0.00478	0.004319	0.003854
$K_m/K_{m,app}$ (mM)	0.1873	0.3616	0.3966	0.4446
K_i (mM)	0.04545			
FAPGG as substrate.				

Figure 4.3 Lineweaver–Burk plot of the inhibition kinetics of ACE by VSKRLNGDA and the control (without peptide), at varying concentrations of FAPGG (0.0625-0.5 mM). V is the initial rate of reaction. V_{max} is maximum reaction velocities in the absence and presence of inhibitor, respectively. K_m is Michaelis constants in the absence and presence of inhibitor, respectively. K_i is enzyme-inhibitor dissociation constant.

K_i is a measure of peptide affinity for the target enzyme. Inhibitor binding strength to ACE indicates that the K_i of VSKRLNGDA was estimated to be 0.04545 mM. This binding affinity was higher than RALP and TF obtained from rapeseed proteins with Ki of 0.1041-12.2727 mM (He, Aluko, and Ju, 2014). There, the VSKRLNGDA derived from chicken blood corpuscles is the first report

demonstrating to be characterized as mixed type of ACE inhibition and showed inhibitor binding strength.

4.4.4 Antihypertensive effect of VSKRLNGDA

All studied concentrations of VSKRLNGDA decreased SBP at 3-12 h post-administration when compared to the distilled water group ($p<0.05$, Figure 4.4a). Oral administration of VSKRLNGDA at 12.5-50 mg/kg BW led to highest SBP reduction of -28.9 to -44.9 mmHg after 3-9 h. At 12 h post-administration, the maximum reduction in SBP was found at a dose of 50 mg/kg BW (-32.0 mmHg), which was similar to the group administered captopril (-36.8 mmHg) at 10 mg/kg BW. After 12 h post-administration, reduction of SBP was maintained in all sample groups.

The reduction of DBP in all treatment groups was greater than that of the negative group at 3 h post-administration ($p<0.05$, Figure 4.4b). At 3h post-administration, the maximum reduction of DBP (-28.7 mmHg) were found in the SHRs administered 12.5 mg/kg BW of VSKRLNGDA, which showed greater reduction than the group received captopril (-19.3 mmHg). After 12 h post-administration, reduction of DBP was maintained in rats of all sample groups. Therefore, oral administration of 12.5-50 mg/kg BW showed effective BP reduction with long lasting effect.

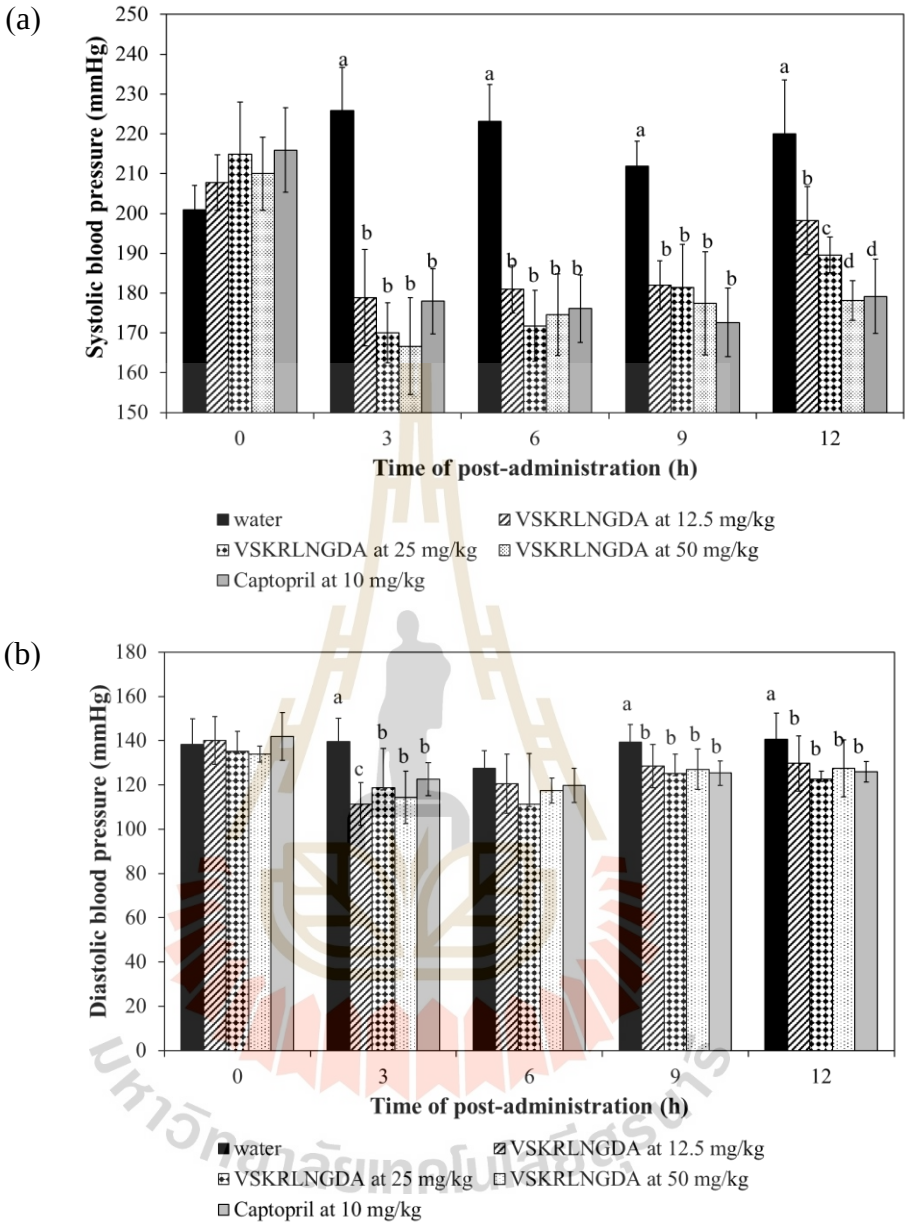


Figure 4.4 Changes in (a) systolic blood pressure and (b) diastolic blood pressure after oral administration of VSKRLNGDA in spontaneously hypertensive rats (SHR). Data were expressed as mean value (n = 6). Letters within the same time indicated differences ($p<0.05$).

This short-term experiment indicated that the VSKRLNGDA showed antihypertensive effect at 3-h post-administration and lasted up to 12 h. The VSKRLNGDA at 12.5 mg/kg with SBP-lowering of -26.8 mmHg appeared to be more effective than the VELYP derived from cuttlefish (*Sepia officinalis*) muscle at 10 mg/kg with a reduction of SBP of -20 mmHg after 6 h of administration (Balti et al., 2015). However, it was difficult to do a comparative analysis with previous works because there are almost no reported *in vivo* studies with peptide derived from blood in the literature. Based on our knowledge, the VSKRLNGDA is the first chicken blood peptide showing antihypertensive effect in SHR model. Thus, it could be developed as a functional ingredient stabilizing blood pressure.

4.5 Conclusions

The VSKRLNGDA was identified as a novel peptide derived from chicken blood corpuscles that showed antihypertensive effect in SHR model. After simulated GI digestion, VSKRLNGDA, NVSTVLTMKKF and FPLCTPAFMTV showed an increase in ACE inhibitory activity with IC_{50} of 25.28-28.44 µM. The effective dose in reducing SBP and DBP of SHR was 50 and 12.5 mg/kg, respectively. The VSKRLNGDA showed a mixed type inhibition. This study demonstrated that valorization of chicken blood as a raw material for antihypertensive peptides is feasible. Long-term effect on antihypertensive properties of this peptide should be further investigated.

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CHAPTER V

ACE-INHIBITORY PEPTIDES FROM CHICKEN BLOOD CORPUSCLES MODULATE EXPRESSION OF ANTIHYPERTENSIVE-RELATED GENES

5.1 Abstract

This study evaluated the antihypertensive effect of short- and long-term intake of chicken blood corpuscle hydrolysate (BCH), ultrafiltrated fraction with MW < 1 kDa (BCH-III) and VSKRLNGDA in spontaneously hypertensive rats (SHRs). The systolic blood pressure (SBP) and the diastolic blood pressure (DBP) of rats were measured by the tail-cuff method on 0, 1, 2, 3, 4, 5, 6 and 7 days for short-term study and 1, 2, 3 and 4 weeks for long-term trial. The SBP and DBP decreased in all treatments. After 7days, reduction of blood pressure (BP) were maintained in rats of all treatment groups, especially groups fed with either captopril (10 mg/kg) or VSKRLNGDA (50 mg/kg). Subsequently, the effect of VSKRLNGDA on relative expression of renin, angiotensin II type-1 receptor (AT-1), angiotensin-I-converting enzyme (ACE), adrenoceptor β-3 (AR-β3) and peroxisome proliferator-activated receptor δ (Pparδ) gene extracted from were detected by real-time polymerase chain reaction (RT-PCR) after the fourth week administration. The peptide VSKRLNGDA acected the expression of major renin angiotensin system (RAS) components by down-regulating the renin and AT-1 receptor, while up-regulating the AR-β3 and Pparδ.

Therefore, antihypertensive effect of VSKRLNGDA involved various mechanisms, including renin angiotensin system (RAS), endothelial dysfunction (ED), sympathetic nervous system (SNS) and vascular remodeling (VR).

Keywords: angiotensin I-converting enzyme (ACE); antihypertensive peptide; hypertension; chicken blood; spontaneously hypertensive rats (SHR); real time-polymerase chain reaction (RT-PCR)

5.2 Introduction

Hypertension is a key risk factor of cardiovascular disease (CVD), leading to stroke, heart failure, and shortened life expectancy (Jensen, Eysturskarð, Madetoja, and Eilertsen, 2014). Hypertension can be controlled through the renin-angiotensin system (RAS) that regulates the key enzymes of renin and angiotensin I-converting enzyme (ACE). Angiotensinogen can be converted into angiotensin I by renin (Cushman, Cheung, Sabo, and Ondetti, 1977). ACE catalyzes the formation of the potent vasoconstrictive octapeptide (angiotensin II) from decapeptide (angiotensin I) and also inactivates the vasodilative peptide, bradykinin, a blood pressure lowering nonapeptide in the kallikrein-kinin system (Cushman, Cheung, Sabo, and Ondetti, 1977; Erdos and Skidgel, 1987; Johnston and Franz**,** 1992). Angiotensin II acts through two main receptors, angiotensin type 1 (AT-1) and type 2 (AT-2) receptors. Binding to AT-1 receptor causes vasoconstriction in vascular smooth muscle cells, while AT-2 receptor mediates vasodilation upon activation, releases nitric oxide (NO), and inhibits cell growth (Zhuo, Ferrao, Zheng, and Li, 2013; Stankevicius, Kevelaitis, Vainorius, and Simonsen, 2003).

Various protein hydrolysates and peptides have been reported to exert blood pressure lowering effect in spontaneously hypertensive rats (SHRs). Reduction of blood pressure of -40 mmHg was evident by purified cod protein hydrolysate with a dose of 30 mg/kg body weight (BW) after 2 h (Girgih, Nwachukwu, Hasan, Fagbemi, Gill, and Aluko, 2015). Hemp seed protein hydrolysate (at 1%, w/w of feed) reduced systolic blood pressure (SBP) of -31.0 mmHg after 3h (Girgih, Alashi, He, Malomo, and Aluko, 2014), while silkworm pupae hydrolysates with a dose of 60 mg/kg BW showed blood pressure reduction of -25.0 mmHg after 6 h (Wang, Wang, and Zhang, 2014). The peptide, TQVY, derived from rice protein (30 mg/kg BW) after 6 h and RVPSL peptide derived from egg protein (50 mg/kg BW) after 4-week reduced SBP of -42 mmHg and -5.0 mmHg, respectively (Li, Qu, Wan and You, 2007; Zhipeng, Yongguang, Wenzhu, Feng, and Jingbo, 2014). Our previous study revealed that the antihypertensive effect of chicken blood hydrolysate (BCH), permeate 1-kDa (BCH-III) and VSKRLNGDA with single oral administration in SHR up to 12 h. However, their effect on changes of blood pressure in short-term (1 week) and long-term (4 week) has not been established.

Many hydrolyastes are often reported to have antihypertensive effects. However, the underlying mechanisms have yet to be fully identified. Besides ACE in RAS, blood pressure is affected by gene expression including angiotensinogen (Ang), renin, angiotensin II type-1 receptor (AT-1) and type-2 (AT-2), adrenoceptor β-3 (AR-β3), interleukin 24 (IL-24), and peroxisome proliferator-activated receptor δ (Pparδ). AR-β3 is an important regulator of the cardiovascular system and of endothelial cell function in particular that AR-β3 produces sustained peripheral vasodilation (Shen, Zhang, and Vatner, 1994). Moreover, Pparδ activation has been reported to exert antihypertensive effects, to induce progressive systolic arterial blood pressure and

heart rate reduction, to restore vascular structure and function, and to reduce the oxidative, proinflammatory, and proatherogenic statuses (Zarzuelo et al. 2011). Feng, Dai, Zhang, Meng, Ye, and Ma (2015) studied antihypertensive effect in gene expression that AR-β3 and IL-24 genes were up-regulated in rats fed with ultrafiltrated hydrolysate (<3kDa) derived from Blue mussel (*M. edulis*) at 20 mg/kg BW group, whereas the Pparδ levels were up-regulated in the 10 mg/kg BW group. In addition, AT-1 receptor was down-regulated in dose 10 and 20 mg/kg BW groups, while Ang and ACE not different with control that ultrafiltrated hydrolysate (<3kDa) derived from Blue mussel (*M. edulis*) had potential to reduce blood pressure in SHR (Feng, Dai, Zhang, Meng, Ye, and Ma, 2015). Moreover, Yu, Yin, Zhao, Chen, and Liu (2014) studied the expression of renin, ACE, AT-1 receptor genes were down-regulated while the AT-2 receptor was up-regulated with oral administration of RVPSL (50 mg/kg) from egg protein after the fourth week. However, their effect of peptide-derived chicken blood on changes of gene expression has not been established.

The objective of this study was to evaluate the short- and long-term intake of chicken blood corpuscle hydrolysate on blood pressure of spontaneously hypertensive rats. In addition, to explore the expression of renin, AT-1 receptor, ACE, AR-β3 and Pparδ mRNA levels in the kidney after long-term intake of the peptide, VSKRLNGDA, derived from chicken blood corpuscle.

5.3 Materials and methods

5.3.1 Reagents and hydrolysate preparation

Trifluoroacetic acid (TFA) was purchased from Supelco (Bellefonte, PA, USA). An angiotensin converting enzyme (ACE), N-[3-(2-Furyl)acryloyl]-Phe-

Gly-Gly (FAPGG), and 2,4,6-trinitrobenzene sulfonic acid (TNBS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Other chemicals and reagents used were of analytical grade.

Chicken blood samples were obtained from an industrial slaughterhouse in Nakhon Ratchasima, Thailand. Sodium citrate solution was added upon collection as anticoagulant to attain a final concentration of 1% (w/v). Samples were maintained at 5°C until further processed. Blood corpuscles (BC) fraction were obtained by centrifuging whole blood at 2530 ×*g* at 4°C for 15 min (Hitachi CR22GIII, Hitachi Koki Co. Ltd., Tokyo, Japan). BC was lyophilized and kept at -20°C throughout the experiment.

Chicken blood corpuscles hydrolysate (BCH) and sequential ultrafiltration were prepared as described in Chapter IV (4.3.2). The peptide, VSKRLNGDA, was synthesized to achieve 95% purity as detailed in 4.3.6.

5.3.2 Animal model

SHR with age of 9-12 weeks old (male, 220-250 g body weight (BW), over BP 180 mmHg) were purchased from Charles River Japan, Inc. (Yokohama, Japan). SHRs were housed individually in steel cages with controlled room temperature (25±1°C), humidity (55±5%), and a 12-light: 12-h dark cycle. They were allowed free access to diet and tap water. The rats were acclimatized in the above conditions for about two weeks before the experiment. The experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (the experimental protocols were approved by the Ethics Committee on Animal Experimentation of Shinshu University).

SHRs were randomly divided into 8 groups with 6 rats in each group. The rats were orally administered with BCH, BCH-III and VSKRLNGDA at 600, 100 and 50

mg/kg BW/day, respectively. The negative control group was given distilled water, while captopril at 10 mg/kg BW/day was assigned for the positive control. For short term experiment, sample in each treatment was orally administered at 8:00 am., and the BP of each rat was measured at 2 pm for every day by tail-cuff method. For long term experiment, BP was measured every week for 4 weeks. At each measurement, systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured three times without anesthesia using a BP monitors m (Model MK2000ST, Muromachi Kikai Co., Ltd., Tokyo, Japan).

After 4-week feeding period, all rats were killed under pentobarbital anesthesia (30 mg/kg). Blood was collected from the aorta ventralis and centrifuged at 3500 g for 30 min to obtain the plasma. Kidneys were removed and plasma angiotensin I-converting enzyme (ACE) activity was measured. These organs are freeze-clamped in liquid nitrogen and stored at -80°C until use.

5.3.3 Determination of plasma ACE activity

Plasma ACE activity was determined according to Girgih, Alashi, He, Malomo, and Aluko (2014). One mL of 0.5 mM FAPGG (dissolved in 50 mM Tris-HCl buffer containing 0.3 M NaCl, pH 7.5) was mixed with 20 μL plasma or ACE (final unit of 0.0313, 0.0625, 0.125, 0.25, 0.5 U/mL) and 200 μl of 50 mM Tris-HCl buffer. Rate of decrease in absorbance was monitored at 345 nm and recorded at 20 intervals for 2 min at 23°C. The result was expressed as ΔA/min and plotted against ACE enzyme concentration to obtain a standard curve. ACE activity (U/mL) of the plasma was obtained by linear regression using the standard curve.

5.3.4 RNA extraction

Kidney tissues were homogenized using a tissue homogenizer under liquid nitrogen. The RNA purity and concentration were determined by the ratio of A260/A280

ratio using a Nano drop 2000 spectrophotometer (Thermo scientific, Waltham, Massachusetts, USA).

5.3.5 Single-strand cDNA synthesis

Transcription first-strand cDNA was synthesized from 1 μg of RNA using the First Strand cDNA synthesis kit for RT-PCR. One μg portion of total RNA was added to the thin-wall tube, and then 4 μL of 4x DN master mix (Toyobo Co., Ltd., Ozaka, Japan) was added. RNA-free water was added to a total volume of 16 μL. Mixture was incubated at 37°C for 5 min and then 4 μL of 5x DN master mix was added. The reaction mixtures were incubated at 37°C for 15 min followed by 50°C for another 5 min. The reaction was stopped by heating at 95°C for 5 min and placed on ice and stored at −20°C until further analysis.

5.3.6 Quantitative real-time polymerase chain reaction (RT-PCR)

Quantification of relative gene expression was performed by RT-PCR for the mRNA levels of renin, ACE, angiotensin II type-1 receptor (AT-1), peroxisome proliferator-activated receptor δ (Pparδ) and adrenoceptor beta 3 (AR-β3), using a StepOne Real-time PCR System (Applied BioSystems, Foster City, CA, USA). These genes were randomly selected, and the β-actin was used as internal reference gene to carry out using the Kapa SYBR Fast qPCR kit (Kapa Biosystems, Woburn, MA, USA). Quantitative PCR was carried out using the thermal cycling program, which consisted of one cycle at 95°C for 30 s, 40 three-segment cycles (95°C for 5 s and 60°C for 30 s), and a final dissociation cycle (95°C for 15 s and stepwise increase from 60 to 95°C). The relative expression of target genes was calculated using the comparative Ct method. The primer sequences were as follows in Table 5.1.

5.3.7 Statistical analysis

Effect of treatments was analyzed by one-way analysis of variance (ANOVA). Duncan's multiple range test (DMRT) was used to determine differences

between mean at $p<0.05$. Statistical analysis was performed using the SPSS package (SPSS 17.0 for Windows, SPSS Inc., Chicago, IL, USA).

Table 5.1 Primers used for RT-PCR.

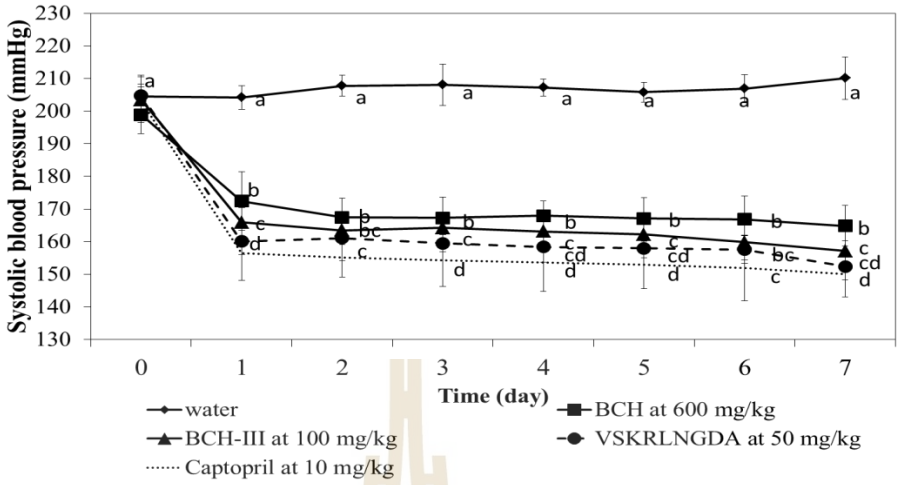
Assay gene name	Forward Primer (5' to 3')	Reversed Primer (5' to 3')	PCR product length (bp)
Renin	GTCCTGTGGGTGT GTATA	GAGCAAGATTCGT CCAAA	175
Angiotensin I-converting enzyme (ACE)	CACCGGCAAGGTC TGCTT	CTTGGCATAGTTTC GTGAGGAA	55
Angiotensin II type-1 receptor (AT-1)	CGGCCTTCGGATA ACATGA	CCTGTCACTCCACC TCAAAACA	67
Peroxisome proliferator-activated receptor δ (Pparδ)	AACATCCCCAACT TCAGCAG	GACGATGGAGGCC AGCATGG	99
Adrenoceptor β-3 (AR-β3)	ATCTTAGCCAGGA TTGAGGTGGAG	AGTTACCCGGAGA CACATGAGGA	85
β-actin	ATTGTTACCAACT GGGACGACATG	CAGCCTGGATGGC TACGTACATG	193

5.4 Results and discussion

5.4.1 Short-term effect

After 6-h administration, SBP of rats administered samples were decreased throughout 7 days, while the negative control (water) was ineffective (Figure 5.1a, $p<0.05$). At day 1, rats administered VSKRLNGDA showed the highest reduction of SBP of -44.7 mmHg, which was comparable to that of the captopril group with the reduction of -46.6 mmHg (Figure 5.1a). After day 7, reduction of SBP was maintained and VSKRLNGDA and captopril showed more effectiveness than BCH and BCH-III. DBP of

(a)



(b)

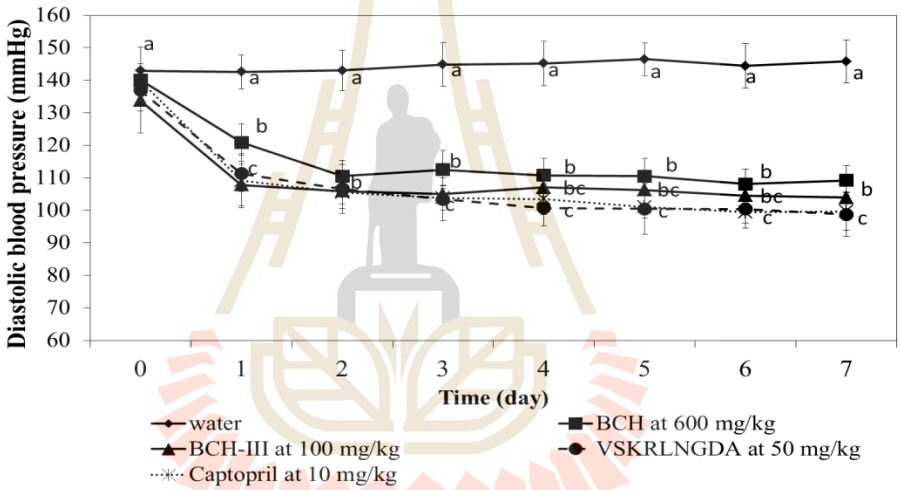


Figure 5.1 Changes of systolic blood pressure (a) and diastolic blood pressure (b) of spontaneously hypertensive rats administered of crude chicken blood corpuscles hydrolysate (BCH), fraction obtained from sequential ultrafiltration (BCH-III), and VSKRLNGDA, for 7 days. Data were expressed as mean value (n = 6). Different letters in the same time indicated significant difference ($p<0.05$).

sample groups also decreased during 7-day trail (Figure 5.1b, $p<0.05$). Captopril and VSKRLNGDA were the most effective in DBP reduction. BCH and BCH-III also showed BP lowering power, but to a lower extent than the purified peptides. After day 7, reduction of DBP was maintained in rats of all sample groups, especially in rats fed with VSKRLNGDA and captopril with DBP reduction of -39.1 and -40.1 mmHg, respectively.SBP-reduction observed in short-term study has been reported in TQVY derived from rice protein and purified cod protein hydrolysate with a reduction of -42 mmHg (Li, Qu, Wan and You, 2007) and -40 mmHg (Girgih, Nwachukwu, Hasan, Fagbemi, Gill and Aluko, 2015), respectively.

5.4.2 Long-term effect

The antihypertensive peptides derived from chicken blood corpuscles were then tested for potential ability to provide long-term relief from hypertension. During the long-term (4 weeks) oral feeding trial, the peptide VSKRLNGDA showed better SBP-reducing effect than crude hydrolysate (BCH) and the ultrafiltration fraction (BCH-III), which is consistent with results obtained from the short-term experiment. The results demonstrated that BCH and BCH-III showed potential to reduce BP for both short- and long-term and the peptide VSKRLNGDA was more effective than hydrolysate samples. SBP decreased throughout 4 weeks of study in all treatments (Figure 5.2a, $p<0.05$). At the 1st week, the highest reduction of SBP was found in the rat fed with VSKRLNGDA and captopril ($p<0.05$). At the 4th week, reduction of blood pressure was maintained in rats of all sample groups, especially captopril and VSKRLNGDA with the SBP reduction of -71.5 and -62.5 mmHg, respectively. In addition, BCH-III and BCH showed reduction of -50.4 and -40.6 mmHg, respectively ($p<0.05$). Reduction in DBP was also evident (Figure 5.2b,

p<0.05). At the 1st week, the highest reduction of DBP was observed in the captopril and VSKRLNGDA groups (p<0.05) with the reduction of -66.3

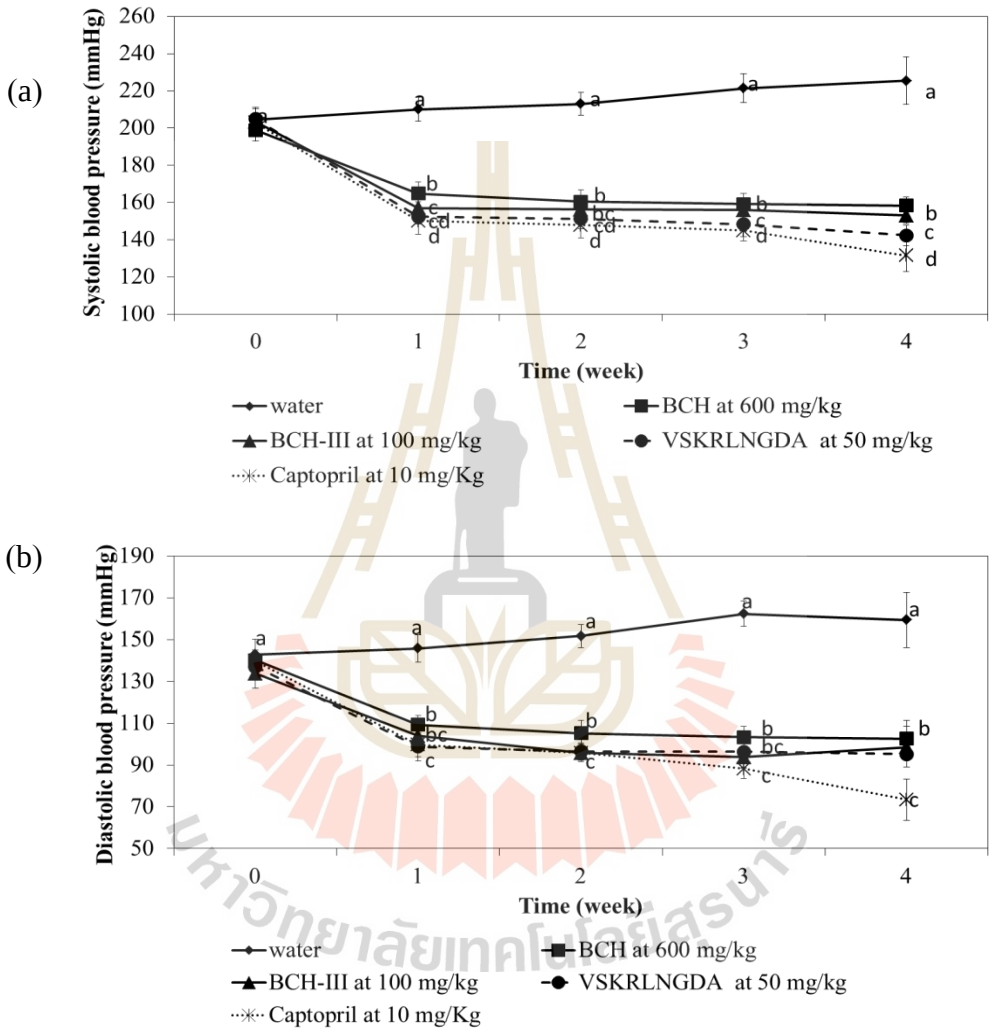


Figure 5.2 Changes of systolic blood pressure (a) and diastolic blood pressure (b) of spontaneously hypertensive rats administered of crude chicken blood corpuscles hydrolysate (BCH), fraction obtained from sequential ultrafiltration (BCH-III), and VSKRLNGDA, for 4 weeks. Data were expressed as mean value (n = 6). Different letters in the same time indicated significant difference (p<0.05).

and -41.5 mmHg, respectively. DBP remained stable throughout 4-wk period in rats fed with hyrolysates and peptides.The VSKRLNGDA showed more SBP-reduction in long-term than 1% hemp seed protein hydrolysate intake of -31.0 mmHg (Girgih, Alashi, He, Malomo, and Aluko, 2014). Silkworm pupae hydrolysates at 60 mg/kg showed a reduction of -25.0 mmHg (Wang, Wang and Zhang, 2014). The purified peptide from egg protein, RVPSL, at 50 mg/kg reduced SBP only -5.0 mmHg after 4th week intake (Zhipeng, Yongguang Wenzhu, Feng, and Jingbo, 2014), while well-known VPP and IPP derived from milk reduced SBP about -15.9 mmHg (Tuomilehto et al., 2004).

It could be speculated that smaller peptides generated from GI digestion of VSKRLNGDA could play an important role in BP reduction. Conversion of some inactive peptides in the BCH, BCH-III and VSKRLNGDA to active peptides after passage through the GI tract which is typical characteristics of prodrug peptides (Fujita, Yokoyama, and Yoshikawa, 2000). In addition, strong reduction of BP implied that the active peptides in BCH, BCH-III and VSKRLNGDA were efficiently absorbed from the GI tract into the blood circulatory system. Thus, the BCH, BCH-III and VSKRLNGDA peptides showed potential to be developed as a nutraceutical product for stabilizing blood pressure.

5.4.3 Plasma activity

ACE activity of plasma of rats fed with hydrolysates (BCH, BCH-III), the peptide (VSKRLNGDA), and captopril increased when compared to the negative control group fed with water ($p<0.05$, Figure 5.3). This could be due to differences in the mechanisms responsible for the observed hypotensive effects. An increase in circulating ACE levels during the treatment of rats with ACE inhibitors was associated to an increase in ACE gene transcription and ACE synthesis in somatic cells (Costerousse, Allegrini, Clozel, Ménard, and Alhenc-Gelas, 1998). Our results were in

agreement with those of Miguel, Manso, Martín-Álvarez, Aleixandre, and López-Fandiño (2007) who reported that ACE activity in plasma significantly increased after the long-term treatment with egg white hydrolysed by pepsin at a dose of 0.5 g/kg/day. However, several studies reported that ACE activities in plasma of SHR decreased upon the oral administration of egg yolk (Yoshi et al., 2001), soya protein hydrolysates (Yang, Yang, Chen, Tzeng and Han, 2004) and soluble cocoa fiber (Sánchez, Quiñones, Moulay, Muguerza, Miguel, and Alexiandre, 2010).

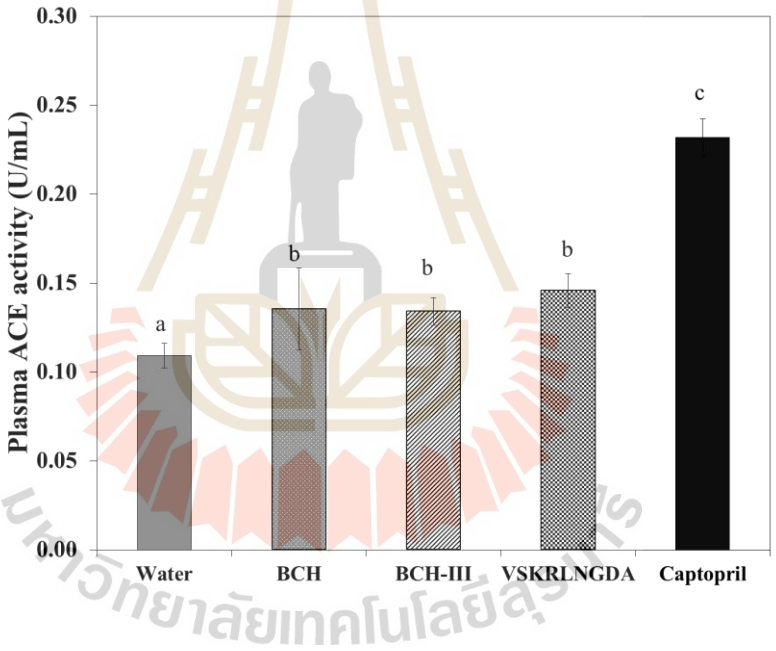


Figure 5.3 Blood plasma ACE activity of spontaneously hypertensive rats (SHR) after oral administration of crude chicken blood corpuscles (BCH), BCH-III fraction obtained from sequential ultrafiltration (BCH-III) and the VSKRLNGDA. Data were expressed as a mean value (n = 6). Different letters indicate significant difference ($p<0.05$).

5.4.4 Effects of synthetic peptide on gene expression

The expression of genes involved in hypertension was analyzed after the 4 weeks of feeding trial of VSKRLNGDA (Figure 5.4). Renin and AT-1 were down-regulated in rats fed with the VSKRLNGDA, whereas the AR-β3 and Pparδ levels were noticeably up-regulated, when compared to the negative control group. In addition, the relative expression of ACE was no difference between these 2 groups ($p>0.05$).

Antihypertensive peptides reduce BP via multiple pathways including; blockage of the AT-1 receptor, inhibition of ACE and renin through renin angiotensin system (RAS) and increase expression of endothelial nitric oxide synthase to release nitric oxide (Turpeinen, Jarvenpaa, Kautiainen, Korpela, and Vapaatalo, 2013; Majumder, Liang, Chen, Guan, Davidge, and Wu, 2015). A decrease in mRNA expression of ACE in the kidney could be related to BP-lowing effect. However, expression of ACE was comparable with the negative group. This could be due to an adaptative response to the inhibition of the enzyme.

A decrease in expression of renin implied that VSKRLNGDA affected renin production in the kidneys. Renin can cleave angiotensinogen to form angiotensin I, which is later converted to angiotensin II by ACE (Cushman, Cheung, Sabo, and Ondetti, 1977). Angiotensin II raises blood pressure by stimulating vasoconstriction, increasing aldosterone secretion, sympathetic tone, and vascular remodeling (Majumder and Wu, 2015). A decrease of renin may contribute to low production of Angiotensin I and Angiotensin II, exerting blood pressure lowering effect. Yu, Yin, Zhao, Chen, and Liu (2014) also reported that the expression of renin reduced in the SHR orally administrated RVPSL at 50 mg/kg) from egg protein.

Angiotensin II acts through two main receptors, angiotensin type 1 (AT-1) and type 2 (AT-2) receptors (Peah, 1977; Zhuo, Ferrao, and Zheng, 2013). Binding to AT-1 receptor causes vasoconstriction in vascular smooth muscle cells. In pathogenic conditions involving tissue remodeling and vascular inflammation, AT-1 receptor is up regulated (Millatt, Abdel-Rahman, and Siragy, 1999; Liu, 2009; Sriramula, Cardinale, Lazartigues, and Francis, 2011). This study indicated that the VSKRLNGDA suppressed expression of AT-1, which could partly contribute to a reduction of blood pressure.

AR-β3 is an important regulator of the cardiovascular system and of endothelial cell function in particular. AR-β3 produces sustained peripheral vasodilation that is predominant in skin and fat (Shen, Zhang, and Vatner, 1994). Atef, Lafontan, Doublé, Hélary, Ktorza, and Pénicaud (1996) reported that AR-β3 caused a vasodilation of microvessels in the islets of Langerhans. In addition, activation of the AR-β3 by nebivolol could enhance endothelial nitric oxide production and decrease the generation of reactive oxygen species, leading to pulmonary vasodilation, and attenuated vascular remodeling (Perros, Ranchoux, Izikki, Bentebbal, Happé, Antigny, et al., 2015). An increase of expression of AR-β3 by the peptide VSKRLNGDA may contribute to its blood pressure lowering effect.

The increased BP is also associated with changes in the vascular structure and function. The vascular smooth muscle cells (VSMCs) directly drive the contraction of the vascular wall and hence regulate the BP. Pparδ activation has been shown to suppress both the proliferation and the inflammation of VSMCs, which thereby exerted beneficial effects in preventing vascular remodeling (Liu et al., 2013; Galatou, Kelly and Lazou, 2014). Moreover, Pparδ activation has been reported to exert antihypertensive effects, to induce progressive systolic arterial blood pressure and heart rate reduction, to restore vascular structure and function, and to reduce the

oxidative, proinflammatory, and proatherogenic statuses (Zarzuelo et al. 2011). This study showed that the VSKRLNGDA increased expression of Pparδ, resulting in a decrease in blood pressure. Feng, Dai, Zhang, Meng, Ye and Ma (2015) also reported that reduction of BP correlated with an increased expression of Pparδ and AR-β1 in SHR with orally administrated ultrafiltrated hydrolysate (<3kDa) derived from Blue mussel (*M. edulis*) at dose of 10 and 20 mg/kg. This study demonstrated that VSKRLNGDA derived from chicken blood corpuscle hydrolysate showed blood pressure lowering effect via various mechanisms, including renin angiotensin system (RAS), endothelial dysfunction (ED), sympathetic nervous system (SNS) and vascular remodeling (VR).

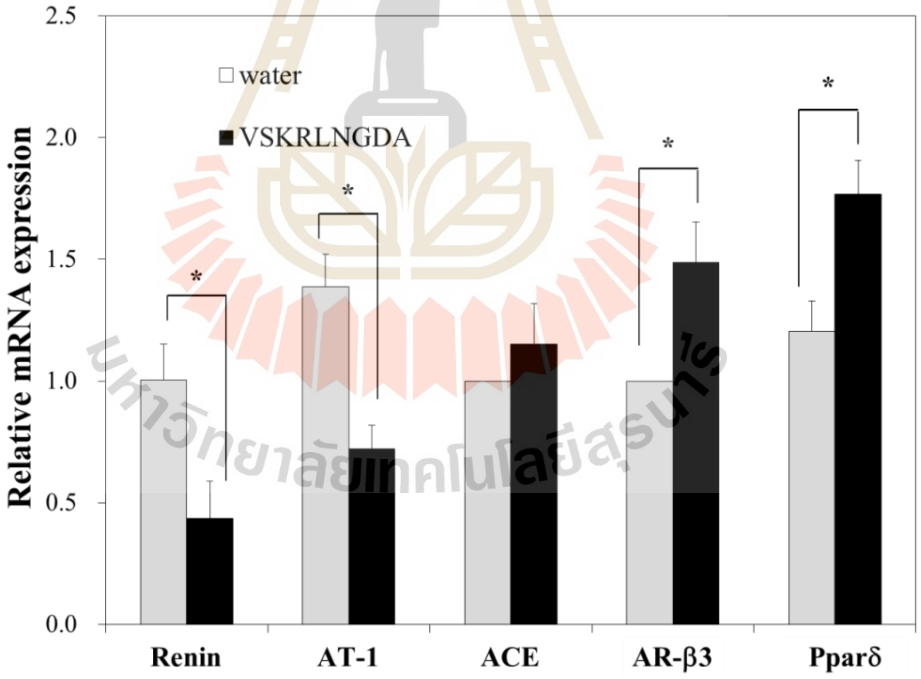


Figure 5.4 Gene expression of renin, angiotensin II type-1 receptor (AT-1), angiotensin I-converting enzyme (ACE), adrenoceptor β-3 (AR-β3) and peroxisome proliferator-activated receptor δ (Pparδ) in kidneys of SHR

fed with VSKRLNGDA. Data were expressed as mean value (n = 6). An asterisk (*) indicates significant difference by t-test ($p<0.05$).

5.5 Conclusions

BP-reduction was maintained in rats fed with BCH (600 mg/kg) and fractionated BCH (100 mg/kg) and the peptide VSKRLNGDA (50 mg/kg) for both short- and long-term oral administration. The peptide VSKRLNGDA controlled blood pressure not only inhibiting ACE, but also down-regulated expression of renin and AT-1 receptor, while up-regulated the AR-β3 and Pparδ.

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CHAPTER VI

SUMMARY

Chicken blood corpuscles are a potential source for production of protein hydrolysate with ACE inhibitory activity. BC was the optimum substrate for producing chicken blood hydrolysate with ACE inhibition based on the hydrolytic condition of 4% Alcalase at 51.1°C at controlled pH9.6 throughout 6 h. The BCH-III, permeate of 1-kDa-membrane, exhibited the higher ACE inhibition than crude BCH .Both of BCH and BCH-permeate were rich sources of F, G, A, V, I and L. Oral administration of BCH and BCH-permeate to the spontaneously hypertensive rat (SHR) at 600 and 200 mg/kg body weight, respectively, lowered systolic blood pressure up to around -43.0 mmHg .The maximum reduction of diastolic blood pressure was observed at administered doses of permeate BCH of 100 mg/kg.

VSKRLNGDA was identified as a novel peptide derived from chicken blood corpuscles that showed hypotensive effect in SHR model. After simulated GI digestion, VSKRLNGDA, NVSTVLTMKKF and FPLCTPAFMTV showed an increase in ACE inhibitory activity with IC_{50} of 25.28-28.44 µM. VSKRLNGDA showed mix type of ACE inhibition .The VSKRLNGDA showed the blood pressure lowering effect on SHR at 50 mg/kg with the maximum reduction of systolic blood pressure of -32.0 mmHg within 12h. A maximum decrease in diastolic blood pressure was observed at a dose of 12.5-50 mg/kg.

The experiments were subjected to investigate short- and long-term effect of peptide. BP-reduction was maintained in rats fed with BCH (600 mg/kg) and fractionated BCH (100 mg/kg) and the peptide VSKRLNGDA (50 mg/kg). Subsequently gene expression in the kidney was detected by RT-PCR after the fourth week .The peptide VSKRLNGDA affected the expression of major RAS components by down regulating the renin and AT-1 receptor while up regulating the AR-β3 and Pparδ. Chicken blood hydrolysate and the VSKRLNGDA not only inhibited ACE but involved expression of renin, AT-1 receptor, Pparδ and AR-β3 genes. Therefore, this research could enhance value-added utilization of chicken blood use as active ingredients to formulate antihypertensive functional foods and nutraceuticals.

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BIOGRAPHY

Wasana Wongngam was born in November 30th, 1980 in Ubon Ratchathani, Thailand. She studied for high school at Benjamamaharat School (1993-1995). In 2000, she received the degree of Bachelor of Food Technology with first class honor from Khon Kaen University, Khon Kaen. In 2009, she received the degree of Master of Food Engineering, King Mongkul University of Technology Thonburi from Bangkok and she obtained a the 2nd place winning in poster contest award (Food Innovation Asia Conference) in title "The effect of soaking time on phosphate uptake, soaking yield and cooking yield of cooked tailed-on shrimp" at Food Innovation Asia Conference 2008, (TRF) Grant number MRG-OSMEP505 E138. In 2013-2015, she received the Thailand Research and Researcher for Industry (RRi) Scholarship (Grant no. PHD56I0024) and a Research Grant from the Agricultural Research Development Agency (Public Organization; ARDA) to study for the degree of Doctor of Philosophy (Food Technology) at Suranaree University of Technology. During her graduate study, she also published her researched work under the title as 1) "Chemical parameters for traceability of raw material freshness of tropical surimi" in Journal Food Chemistry Journal of Aquatic Food Product Technology; DOI:10.1080/ 10498850.2014.964433 in 2016, 2) "Antioxidant and angiotensin-converting enzyme inhibitory activities of protein hydrolysates prepared from threadfin bream (*Nemipterus* spp.) surimi by-products" in Journal of Aquatic Food Product Technology. 2012 ;21: 265-278. and 3) "Gelation characteristics of mince and washed mince from small scale mud carp and common carp" in Journal of Aquatic Food Product Technology. 2012.