เปปไทด์ที่แสดงฤทธิ์ทางชีวภาพจากการย่อยในระบบย่อยอาหารจำลองเนื้อไก่ปรุงสุก

นายปภังกร ส่างสวัสดิ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต

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BIOACTIVE PEPTIDES DERIVED FROM *IN VITRO* GASTROINTESTINAL DIGESTION OF COOKED CHICKEN MEAT

Papungkorn Sangsawad

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Food Technology

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BIOACTIVE PEPTIDES DERIVED FROM *IN VITRO*

GASTROINTESTINAL DIGESTION OF

COOKED CHICKEN MEAT

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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ปภังกร ส่างสวัสดิ์ : เปปไทด์ที่แสดงฤทธิ์ทางชีวภาพจากการย่อยในระบบย่อยอาหารจำลองเนื้อไก่ปรุงสุก (BIOACTIVE PEPTIDES DERIVED FROM *IN VITRO* GASTROINTESTINAL DIGESTION OF COOKED CHICKEN MEAT) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร. จิรวัฒน์ ยงสวัสดิกุล, 193 หน้า.

วัตถุประสงค์ของงานวิจัยนี้เพื่อศึกษาผลของสภาวะการให้ความร้อนต่อกิจกรรมการออกฤทธิ์ทางชีวภาพของเปปไทด์ที่เกิดขึ้นหลังจากการย่อยเนื้อไก่ในระบบย่อยอาหารจำลอง ตลอดจนระบุลำดับกรดอะมิโนของเปปไทด์ที่มีฤทธิ์ในการยับยั้งเอนไซม์ที่เปลี่ยนรูปแองจิโอเทนซิน หรือเอซีอี (Angiotensin converting enzyme, ACE) ในส่วนของโปรตีนที่ถูกย่อยแล้ว (digest) และศึกษาความสามารถในซึมผ่านเซลล์มะเร็งลำไส้ (Caco-2) ของเปปไทด์ที่เกิดจากการย่อย อกไก่เนื้อโคราช (Kc) และไก่เนื้อทางการค้า (Br) ที่ถูกทำให้สุกที่สภาวะต่างๆ คือ การให้ความร้อนที่อุณหภูมิ 70 องศาเซลเซียส เป็นเวลา 0.5 (H-0.5) และ 24 ชั่วโมง (H-24) และ การให้ความร้อนภายใต้ความดันที่อุณหภูมิ 121 องศาเซลเซียส เป็นเวลา 15 (AC-15) และ 60 นาที (AC-60) จากนั้นย่อยต่อด้วยระบบย่อยอาหารจำลอง ผลการทดลองพบว่า เปปไทด์ที่มีคุณสมบัติออกฤทธิ์ต้านอนุมูลอิสระและยับยั้งเอซีอีเกิดขึ้นหลังการย่อย Kc และ Br ที่ได้รับความร้อนภายใต้สภาวะ H-0.5 ส่งผลให้ได้เปปไทด์ที่มีสมบัติจับโลหะ (metal chelating) และยับยั้งเอซีอีสูงสุด ในขณะที่เปปไทด์ที่ได้จากการย่อยเนื้อไก่ภายใต้สภาวะ AC-15 แสดงความสามารถในการรีดิวซ์เฟอร์ริกไอออน และมีฤทธิ์ปกป้องเซลล์ตับที่ถูกเหนี่ยวนำให้เกิดออกซิเดชันจากอนุมูลอิสระสูงสุด เปปไทด์ท่อนใหม่ที่มีฤทธิ์ยับยั้งเอซีอีได้สูงสุด คือ KPLLCS, ELFTT และ KPLL ซึ่งมีค่า IC_{50} เท่ากับ 0.36 6.35 และ 11.98 ไมโครโมลาร์ ตามลำดับ โดย ELFTT และ KPLL มีความทนทานต่อการถูกย่อยด้วยเอนไซม์พลาสมิน

ความสามารถการซึมผ่านเซลล์ Caco-2 ซึ่งเทียบเคียงได้กับเซลล์ลำไส้เล็ก ของเปปไทด์ส่วนที่ผ่านอัลตราฟิลเตรชั่นขนาดเมมเบรน 1 กิโลดาลตัน จากตัวอย่างที่เกิดจากการย่อยในระบบย่อยอาหารจำลองของตัวอย่าง Kc/H-0.5 และ Kc/AC-60 พบว่า ตัวอย่าง Kc/H-0.5 แสดงความสามารถในการซึมผ่านไปได้และแสดงฤทธิ์การยับยั้งเอซีอีสูงกว่าส่วนของเปปไทด์จาก Kc/AC-60 ในบรรดาเปปไทด์ที่ซึมผ่านจากตัวอย่าง Kc/H-0.5 นั้น พบ 9 เปปไทด์ที่มีฤทธิ์ยับยั้งเอซีอีโดยที่ APP เป็นท่อนเปปไทด์จากไมโอซินและแสดงค่าการยับยั้งเอซีอีได้สูงที่สุด ด้วยค่า Ki เท่ากับ 0.93 ไมโครโมลาร์

KPLLCS และ KPLL มีความไวต่อการย่อยสลายด้วยโปรตีเอส ที่ผิวเซลล์ของลำไส้เล็ก (brush border peptidases) ในขณะที่ ELFTT ถูกย่อยเพียงเล็กน้อยแต่ไม่สามารถซึมผ่านได้ เปปไทด์ที่ซึมผ่านไปได้สูงสุดคือ KPLL ซึ่งแสดงค่าการยับยั้งเอซีอีได้สูงที่สุด โดยที่ KPLL บางส่วนถูกย่อยเป็น KP และ LL ก่อนการซึมผ่านผนังลำไส้ (apical) และเปปไทด์ทั้งสามสามารถซึมผ่านไปได้และแสดงค่า K_i เท่ากับ 0.09 3.86 และ 56.74 ไมโครโมลาร์ ตามลำดับ

ผลการศึกษานี้แสดงให้เห็นว่าค่ากิจกรรมการออกฤทธิ์ทางชีวภาพของเปปไทด์ที่ได้รับจากการย่อยไก่เนื้อโคราชสุกจะขึ้นอยู่กับวิธีการให้ความร้อน การให้ความร้อนระดับปานกลางที่สภาวะ H-0.5 กับอกไก่เนื้อโคราชส่งผลให้ค่ากิจกรรมการต้านอนุมูลอิสระและกิจกรรมการยับยั้งเอซีอีของเปปไทด์เกิดจากการย่อยอาหารในสภาวะจำลอง เปปไทด์ที่แสดงกิจกรรมการยับยั้งเอซีอีสามารถซึมผ่านไปได้และมีการเปลี่ยนแปลงระหว่างการซึมผ่าน นอกเหนือจากคุณค่าทางโภชนาการแล้ว อกไก่เนื้อโคราชที่ถูกทำให้สุกภายใต้สภาวะเหมาะสมยังแสดงกิจกรรมทางชีวภาพอีกด้วย

สาขาวิชาเทคโนโลยีอาหาร ลายมือชื่อนักศึกษา Papongkorn Sangsanad

ปีการศึกษา 2559 ลายมือชื่ออาจารย์ที่ปรึกษา

PAPUNG KORN SANGSAWAD : BIOACTIVE PEPTIDES DERIVED FROM *IN VITRO* GASTROINTESTINAL DIGESTION OF COOKED CHICKEN MEAT. THESIS ADVISOR : ASSOC. PROF. JIRAWAT YONGSAWADIGUL, Ph. D., 193 PP.

MUSCLE PROTEIN/POUTRY/GASTROINTESTINAL DIGESTION/BIOACTIVE PEPTIDE/ANTIOXIDANT/ANGIOTENSIN CONVERTING ENZYME/ PERMEABILITY

The objectives of this study were to investigate the effect of thermal treatments on bioactivities of peptides generated from *in vitro* gastrointestinal (GI) digestion of cooked chicken meats and to identify the angiotensin converting enzyme (ACE) inhibitory peptides presented in digests, and to determine their permeability through Caco-2 cell monolayer model. Breast of Korat chicken (Kc) and commercial broiler (Br) were cooked under various conditions, namely heated at 70°C for 30 min (H-0.5) and 24 h (H-24), autoclaved (AC) at 121°C for 15 min (AC-15) and 60 min (AC-60), and then subjected to *in vitro* GI digestion. Antioxidant and ACE inhibitory peptides were released upon *in vitro* GI digestion of both KC and Br. H-0.5 promoted metal chelating and ACE inhibitory activity of digest, while AC-15 enhanced FRAP value and cytoprotective effect. Novel potent ACE inhibitor peptides were identified to be KPLLCS, ELFTT, and KPLL with IC_{50} of 0.37, 6.35, and 11.98 µM, respectively, while ELFTT and KPLL were resistant to hydrolysis of plasmin.

Permeability of Kc/H-0.5 and Kc/AC-60 digests permeated through a 1-kDa ultrafiltration membrane was investigated using monolayers of human colorectal

carcinoma cell line (Caco-2) cells. Permeate of Kc/H-0.5 digest revealed higher permeability and ACE inhibitory activity than did the Kc/AC-60 counterpart. Among transported peptides of Kc/H-70 digest, nine peptides showed ACE inhibitory activity, and a tripeptide, APP, derived from myosin, exhibited the highest ACE inhibition with the K_i value of 0.93 μM.

KPLLCS and KPLL were susceptible to hydrolysis by brush border peptidases, while ELFTT was slightly hydrolyzed but could not be transported. The transported peptides of KPLL showed the highest ACE inhibitory. KPLL was partially hydrolyzed to KP and LL in the apical side. KPLL, KP and LL, were transported across Caco-2 cells with the K_i values of 0.09, 3.86, and 56.74 mM, respectively.

These results revealed that the bioactivity of GI digests derived from cooked Korat chicken muscles would greatly depend on the applied thermal treatment. Mild thermal treatment at 70˚C for 30 min of Kc improved antioxidant and ACE inhibitory activity of digest. ACE inhibitory peptides can be transported and structural changes occurred during permeation. Besides the nutritional value of cooked chicken, optimally cooked Kc breast also provided multifunctional health benefits.

School of Food Technology Student's Signature Papungkorn Sangsanad

Academic Year 2016 Advisor's Signature

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LIST OF ABBREVIATIONS

°C	=	Degree Celsius
d	=	Day
rpm	=	Revolutions per minute
RP-HPLC	=	Reverse phase high performance chromatography
g	=	Relative centrifugal fields
kDa	=	Kilodalton
M	=	Molar
Mg	=	Milligram
µg	=	Microgram
µl	=	Microliter
ml	=	Milliliter
mM	=	Millimolar
mU	=	Milliunit
U	=	Unit activity
Min	=	Minute
mm	=	Millimeter
µm	=	Micrometer
MW	=	Molecular weight
Sec	=	Second
MS	=	Mass spectrometry

LIST OF ABBREVIATIONS (continued)

SDS-PAGE	=	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
MTT	=	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
DMSO	=	Dimethyl sulfoxide
M	=	Methionine
I	=	Isoleucine
L	=	Leucine
F	=	Phenylalanine
R	=	Arginine
N	=	Asparagine
Q	=	Glutamine
D	=	Aspartic acid
K	=	Lysine
H	=	Histidine
P	=	Proline
E	=	Glutamic acid
G	=	Glycine
V	=	Valine
A	=	Alanine
C	=	Cysteine
T	=	Threonine
Y	=	Tyrosine

LIST OF ABBREVIATIONS (continued)

HAT	=	Hydrogen atom transfer
HBSS	=	Hank's balanced salt solution
HepG2	=	Human hepatoma carcinoma
H_2O_2	=	Hydrogen peroxide
Hyp (O)	=	Hydroxyproline
IC_{50}	=	50% inhibition of the enzyme activity
GI	=	gastrointestinal digestion
ICP-OES	=	Inductive Coupled Plasma-Optical Emission Spectroscopy
kDa	=	kilo Dalton (10^3 Dalton)
LC-MS/MS	=	Liquid chromatography with tandem mass spectrometry
M	=	mol l^{-1}
MWCO	=	Molecular weight cut off
Kc	=	Korat chicken
Br	=	Commercial broiler
Th	=	thigh
H-0.5	=	heating at 70˚C for 30 min
H-24	=	heating at 70˚C for 24 h
AC-15	=	heating at 121˚C for 15 min
AC-60	=	heating at 121˚C for 60 min
H-70	=	heating at 70˚C for 30 min
H-100	=	heating at 100˚C for 30 min
H-121	=	heating at 121˚C for 30 min

LIST OF ABBREVIATIONS (continued)

ACE	=	angiotensin converting enzyme
MWCO	=	molecular weight cutoff
RT-10	=	retentate of MWCO 10 kDa
RT-10-3	=	retentate of MWCO 10-3 kDa
RT-3-1	=	retentate of MWCO 3-1 kDa
PM-1	=	permeate of MWCO ≤1 kDa
Caco-2	=	colorectal adenocarcinoma
PepT1	=	peptide transporter 1
DPP-IV	=	dipeptidyl-peptidase-IV
HHL	=	hippuryl-histidyl-leucine tetrahydrate
HA	=	hippuric acid
ACN	=	acetonitrile
TFA	=	trifluoroacetic acid
HBSS	=	Hank's balanced salt solution
EMEM	=	Eagle's minimum essential medium
FBS	=	fetal bovine serum
UF	=	ultrafiltration
HPLC	=	high performance liquid chromatography
RP-HPLC	=	reversed phase high performance liquid chromatography
LC-MS/MS	=	Liquid chromatography-tandem mass spectrometry
AHTPDB	=	Anti-Hypertensive Inhibiting Peptide database
LC-MS/ESI	=	liquid chromatography coupled to a mass spectrometer

CHAPTER I

INTRODUCTION

1.1 Introduction

Muscle proteins is a valuable source of energy and essential amino acids, which are needed for growth and maintenance of physiological functions. They can be hydrolyzed by proteolytic enzymes into peptides and amino acids (Korhonen and Pihlanto, 2006). Bioactive peptides have been defined as "food derived components that exert a physiological effect in the body" (Bouglé and Bouhallab, 2017; Ryan, Ross, Bolton, Fitzgerald, and Stanton, 2011). Most studies have focused on the production of bioactive peptides using commercial or new sources of proteases (Udenigwe and Aluko, 2012). Peptides originating from digests in the size of 2–20 amino acids can display various biological activities, such as antihypertensive and antioxidative (Escudero, Toldrá, Sentandreu, Nishimura, and Arihara, 2012; Qian, Jung, Byun, and Kim, 2008; Tagliazucchi, Shamsia, and Conte, 2016). But their bioactivities have not been well elucidated as compared to hydrolysates produced from commercial enzymes. It could be assumed that consumption of muscle foods not only provides nutritional value in the form of amino acids but also yields bioactive peptides.

Peptides have been shown to possess antioxidant activity in human, which can protect cellular damage caused by reactive oxygen species (ROS) or free radicals and reduces the risk of diabetes, cancer, cardiovascular diseases, and Alzheimer's disease

(Quirós, Dávalos, Lasunción, Ramos, and Recio, 2008). In addition, ROS radicals are very unstable and react rapidly with other groups or substances in the body, leading to cell or tissue injury (Halliwell, Aeschbach, Löliger, and Aruoma, 1995). Most of *in vitro* antioxidant activity assays includes 2,2′-Azinobis (3-ethyl-benzothiazoline-6-sulfonate (ABTS) radical-scavenging, Hydroxyl radical scavenging, ferric reducing antioxidant power (FRAP), metal chelating and β-carotene bleaching ability assays, which are conducted under non-physiological condition and might not necessarily correlate with biological responses. A step closer to *in vivo* is the use of the *in vitro* cellular models. Determination of antioxidant activity in cell culture model represents a more biologically relevant method than chemical assays of antioxidant activity because it accounts for some aspects of the uptake, metabolism, and location of antioxidants within cells (Carrasco-Castilla et al., 2012). Antioxidant peptides generated from GI digestion has been reported only in cooked egg (Remanan and Wu, 2014). It is important to investigate antioxidant activity of digest of cooked chicken meat as it would illustrate more benefits of chicken meat consumption in addition to its nutritional value.

High blood pressure or hypertension can lead to many serious diseases, including disorders of the heart and blood vessels, kidney disease, arteriosclerosis, myocardial infarction, and stroke, affecting 30 % of the adult population in the world (Norris and FitzGerald, 2013; World Health Organization, 2017). ACE is one of the main regulators of blood pressure through its action on two body systems. Firstly, ACE forms part of the renin-angiotensin system (RAS), converting angiotensin I to a potent vasoconstrictor, angiotensin II, which also induces the release of aldosterone and

therefore, increases the sodium concentration and blood pressure. ACE also takes part of the kallikrein-kinin system as it hydrolyzes bradykinin, which has a vasodilator action (Bhat, Kumar, and Bhat, 2017; Hernández-Ledesma, del Mar Contreras, and Recio, 2011). ACE inhibitors derived from peptide are stable and have minimal side effects compared with synthetic ACE inhibitor drugs such as captopril, enalapril, lisinopril (Lee and Hur, 2017; Salvetti, 1990). Digests of raw pork and fish have been reported to have ACE inhibitory activity (Escudero et al., 2012; Matsufuji et al., 1994; Nazeer, Kumar, and Ganesh, 2012). However, these studies had no relevance to health benefits since raw meat is not commonly consumed. ACE inhibitory peptides generated from the GI digestion of cooked meat should be systematically elucidated.

Chicken meat is a great source of dietary protein and widely consumed all over the world. Consumption of chicken meat has been increasing every year, it is estimated to reach 91.6 pounds per capital worldwide in 2017 (The National Chicken Council). Korat crossbred chicken (Kc) is a new crossbreed between the male line of Thai indigenous chickens and the female line of broiler (Br). Kc offers a firmer and chewier texture and contains less fat and higher quantities of collagen (Maliwan, Khempaka, and Molee, 2017). Muscle composition of Br and Kc is expected to be different due to differences in age (45 days for Br vs. 70 days for Kc) and breed (pure line vs. hybrid). In addition, breast and thigh exhibit different composition with breast containing the higher proportion of muscle fiber type II than thigh, and thigh containing a higher level of collagen than breast muscle (Jaturasitha, Srikanchai, Kreuzer, and Wicke, 2008). Thus, it is reasonable to assume that the digests from different types and muscles of chicken would contain different peptides which could lead to varied antioxidant and ACE inhibitory activity.

Thermal process is mandatory for muscle foods preparation. It has been applied to improve palatability and safety (Woods and Church, 1999). Time and temperature at which meat is cooked greatly affect the extent of protein denaturation. This would, in turn, influence the accessibility of GI enzymes to break down proteins into varied bioactive peptides. Muscle proteins compose of sarcoplasmic, myofibrillar and stroma proteins. Mild thermal treatment (60–80 °C) induces protein unfolding, thus enhancing protease susceptibility (Bax et al., 2012; Soladoye, Juárez, Aalhus, Shand, and Estévez, 2015). In contrast, extreme thermal heating (>100 °C) leads to irreversible unfolding, aggregation, disulfide interchanges, protein fragmentation and chemical modifications of side chains, including sulfoxidation, carbonylation, hydroxylation, nitrosylation, and glutathiolation (Davis and Williams, 1998; Soladoye et al., 2015). Modifications of primary protein structures would lead to alteration of the enzyme recognition sites, which would lower digestibility and produce peptides with reduced bioactivity (Swaisgood, 1993). It can assume that bioactivity of peptides varies with the type of heating regime used to cook meat. In addition, the release of bioactive peptides derived from cooked muscle meat upon GI digestion has not been investigated.This would shed some light on the optimal thermal treatment for generation of bioactive peptides derived from GI digestion.

Oligopeptides in digest are further hydrolyzed by brush border membrane peptidases during permeation through intestinal epithelial cells. It has been reported that small (di- and tripeptides) and large (10-51 amino acids) peptides generated from digestion can be absorbed through intestines and produce biological effects at the tissue level (Roberts, Burney, Black, and Zaloga, 1999). In addition, Caco-2 model has been widely used as a predictive tool for the intestine epithelial absorption and permeability

of peptides (Cinq-Mars, Hu, Kitts, and Li-Chan, 2007; Guang, Shang, and Jiang, 2012). However, modifications of protein under thermal treatment may lead to limited absorption and bioavailability. Oxidized forms of sulfur-containing amino acids have been found to display reduced or limited availability regardless of the true ileal digestibility of proteins (Rutherfurd and Moughan, 2012). In addition, formation of Maillard compounds and cross-linked peptide chains limited protein bioavailability (Boye, Wijesinha-Bettoni, and Burlingame, 2012; Millward, Layman, Tomé, and Schaafsma, 2008).

Amino sequencing and characterization of individual peptides are important for an understanding of mode of action of bioactive peptides. It also could shed some lights on the peptide structure-bioactivity relationship. In addition, identification of amino acid sequences of the bioactive peptide will be useful for the synthesis of such peptides for either food or pharmaceutical purposes. Tandem mass spectrometry, where the peptide sequencing is achieved by software using iterative calculations, is now a more widely used approach in peptide sequencing (Bo, Feng, Xi, Baoping, and Yonnie, 2007). In addition to peptide identification, it is also important to understand how a bioactive peptide illustrates its bioactivity. In recent years, molecular docking has been used to predict the interaction between an enzyme and an inhibitor. This is a computational simulation approach that can be used as an assistant tool to explain the structure-activity relationship and the mechanism of peptide binding with the enzyme. To understand how the most potent ACE inhibitory peptide binds to ACE, molecular docking simulation is usually performed based on kinetics data. The docking simulation of TPTQQS binding with ACE showed that T1, T3, and Q4 are the key amino acids for the non-competitive inhibition. These amino acids oriented the peptide

onto the lid structure, keeping the peptide out of the active site of ACE (Ni, Li, Liu, and Hu, 2012) . Mode of inhibition and molecular docking would reveal inhibition mechanisms of an interested peptide.

1.2 Research objectives

The objectives of this study were:

1. To investigate the effect of thermal processes on protein digestibility of chicken breasts and antioxidant activities of digests. To investigate and identify ACE inhibitory peptides presenting in digests of simulated *in vitro* GI digestion of cooked chicken breasts from Kc and Br.
2. To investigate permeability through Caco-2 cell monolayer of digest derived from cooked Kc breast exhibiting the most potent ACE inhibitory activity and identify the transported ACE inhibitor peptides as well as the kinetics of their inhibition and interactions with ACE based on molecular docking simulation.
3. To investigate permeability through Caco-2 cell monolayer of the most potent ACE inhibitory peptides, and identify the transported peptides exhibiting ACE inhibitory activity as well as the kinetics of their inhibition and interactions with ACE based on molecular docking simulation.

1.3 Research hypotheses

Different chicken breeds and muscle types, which are subjected to varied thermal treatments, exhibit different digestibility. This would lead to different peptides upon digestion. Such peptides would process different bioactive properties, especially ACE inhibitory and antioxidant activities. Permeability through Caco-2 cell monolayer of chicken meat cooked at varied thermal treatment would be different. Transportation of peptides across epithelial Caco-2 cells could lead to structural modification of bioactive peptides and changes of ACE inhibitory activity of transported peptides.

1.4 Scope of the study

Chicken muscles of Kc and Br were cooked under various thermal treatments and subjected to GI digestion. Protein digestibility, antioxidant activity and ACE inhibitory activity of digests were determined. The most potent bioactive peptides with ACE inhibitory activity were purified and characterized. Amino acid sequences of ACE inhibitory peptides were identified using LC-MS/MS. Permeability through Caco-2 cell monolayer of digest and peptides that showed the highest ACE inhibitory were investigated using Caco-2 cell monolayers. Transported peptides were purified and identified by LC-MS/MS. Identified transported peptides exhibiting the highest ACE inhibitory activity were investigated for their inhibition kinetics using molecular docking simulation.

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CHAPTER II

LITERATURE REVIEWS

2.1 Chicken meat proteins

2.1.1 Muscle proteins

Myosin and actin are two muscle proteins responsible for contraction. Myosin is the primary structural component of thick filaments, while thin filaments are composed of actin, troponin, and tropomyosin (Vercruysse, Van Camp, and Smagghe, 2005). The functional units of the muscle are thick and thin filaments with arranged into sarcomeres, these sarcomeres are longitudinally repeated to form myofibrils. Myofibrils are arranged in parallel and form myofibers, numerous parallel organized myofibers form muscle fibers (Vercruysse et al., 2005).

2.1.1.1 Myofibrillar proteins

Myofibrillar proteins consist of 65-75% of total protein in muscle and they are major proteins in animal muscle (Venugopal, 2009). These proteins are related to the water holding capacity and other functional properties of proteins such as gelation, emulsification, and foaming (Foegeding, Lanier, and Hultin, 1996; Vareltzis, 2000). Extraction of these proteins can be achieved using neutral salt solutions of ionic strength ranging of 0.30-0.70. The contractile proteins in muscles are myofibrils which are different in size and location in the muscle, as listed in **Table 2.1.**

Table 2.1 Contractile proteins in food myosystems.

Protein	**Relative abundance** (% of contractile proteins)	**Molecular weight** (kDa)	**Location**
Myosin	50-60	470	Thick filaments
Actin	15-30	43-48	Thin filaments
Tropomyosin	8-10	65-70	Thin filaments
Troponins	8-10		Thin filaments
Troponin-C		17-18	
Troponin-I		20-24	
Troponin-T		37-40	
C-protein	-	140	Thick filaments
α-Actin	-	180-206	Z-disk
Z-nin	-	300-400	Z-disk
Titin	5	700-1,000	Gap filaments
Nebulin	5	~600	N_2-line

Source: Ashie and Simpson (1997).

Myofibrillar proteins can be divided into 3 subgroups as follows:

Myosin constitutes approximately 50- 60 % of myofibrillar proteins with molecular weight of 500 kDa which forms thick filament (Foegeding et al., 1996). In addition, it contains a total of six polypeptide chains, two heavy chains, and four light chains. Six polypeptide chains of myosin are assembled in the quaternary structure that resembles a stick (tail) with two pear-shaped heads **(Figure 2.1a).** The tail regions consists of two α-helical heavy chains coiled together into a coiled-coiled α-helical supersecondary structure (Foegeding et al., 1996). This structure terminates at the head region. The main secondary structure in the head is α- helix, accounting for approximately 48% of amino acids. Myosin head includes ATP binding site, actin binding site, DTNB [(5,5-dithiobis)-2-(nitrobenzoic acid)] light chain site, and alkaline light chains site. The tail portion of the heavy chain molecule is responsible for its association with the thick filament and the light chains bind to the α-helical regions of the heavy chain (Foegeding et al., 1996).

The major protein of the thin filaments is actin, which accounts for 20% of myofibrillar proteins of muscle. Actin contains a myosin binding site, which allows myosin to form temporary complex during muscle contractile or the permanent myosin-actin complex of rigor mortis. Actin is also associated with troponin and tropomyosin complex (Xiong, 1997). Actin consists of two peanut shaped domains of equal size. Actin monomers (globular actin or G-actin) are assembled in a double-helical structure called fibrous actin, or F-actin (**Figure 2.1b**). G-actin binds with ATP in the presence of $Mg^{2+,}$ to form F-actin. G-actin has a molecular mass of 42 – 48 kDa. Filaments of F-actin interact with the head portion of myosin. In addition, F-actin can polymerize in the presence of neutral salts at 0.15 M (Foegeding et al., 1996).

Troponin accounts for about 8-10% of myofibrillar proteins, and consists of three subunits namely troponin I, troponin C and troponin T. Troponin C is a calcium binding protein and confers calcium regulation to the contractile process via the thin filament. Troponin I strongly inhibits ATPase activity of actomyosin and troponin T provides a strong association site for binding of tropomyosin (Foegeding et al., 1996).

Tropomyosin is about 8-10% of myofibrillar protein and resembles the tail or rod portion of the myosin molecule. It is composed of two α-helical polypeptides wound together into a two-stranded coiled-coiled supersecondary structure. α- and β-tropomyosin polypeptides have molecular masses of 37 and 33 kDa, respectively. Tropomyosin binds to actin filaments along each groove of the actin double helix such that each molecule interacts with seven G-actin monomers and aggregates end-to-end (Foegeding et al., 1996).

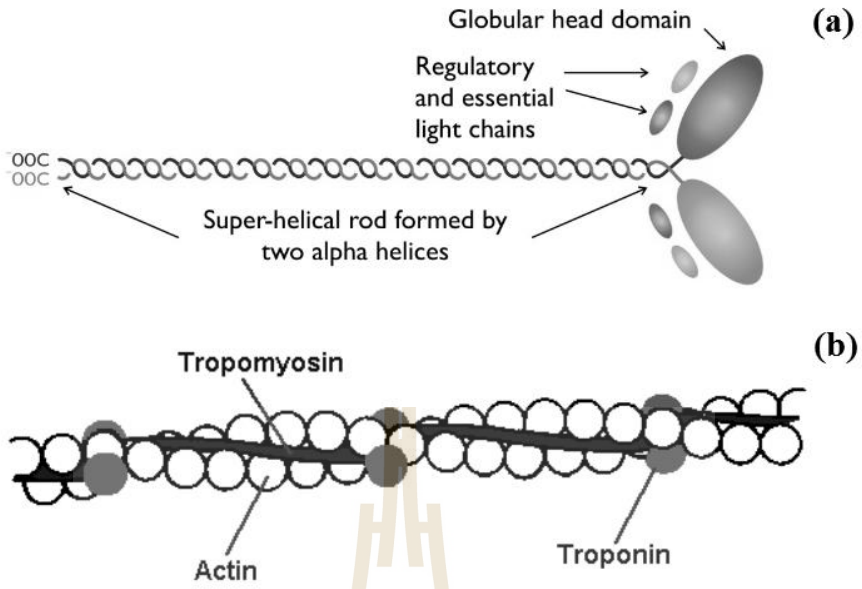


Figure 2.1 Structure of myosin thick filament (a) and arrangement of actin, troponin, and tropomyosin (b). (Mcnally, Lapidos, and Wheeler, 2006; Mu and Plummer, 1988)

Source: Modified from Mcnally, Lapidos, and Wheeler, 2006

2.1.2 Broiler and Korat chickens

Broilers (Br, *Gallus gallus domesticus*) are gallinaceous domesticated fowl that are raised specifically for meat production (Benson and Witzig, 1977). They are a subspecies of the red jungle fowl (*Gallus gallus*). Typical broilers have white feathers and yellowish skin. Commercial broilers are very fast growing with high meat yields. Slaughter weight of broilers ranges from 1.8-3.0 kg, which takes around 32-62 days of rearing. Broilers constitute the largest proportion of the commercially available chickens (Stewart and Amerine, 2012).

Korat chicken (Kc) is one of the Thai indigenous crossbred chicken strains that are derived through a crossbreeding program (Maliwan, Khempaka, and Molee, 2017). Kc is a cross between a male Thai indigenous chicken and a female broiler. Muscle

composition between Kc and Br is different due to differences in age (45 days for broiler and 70 days for Kc) and breed (pure line and hybrid). Consumable live weight of Kc is around 1.2-2.0 kg at age of 40-90 days. Kc meat has been more acceptable than commercial broiler because of a unique taste and texture. It also contains lower fat and higher collagen levels (Maliwan et al., 2017). Overall meat qualities are similar to those of indigenous chickens with better growth performance (Puttaraksa, Molee, and Khempaka, 2012).

2.1.3 Chemical composition of chicken meats

Chickens are economically important poultry, they contain several nutrients including essential fatty acids, proteins, amino acids, vitamins, and minerals such as iron and zinc (Lombardi-Boccia, Lanzi, and Aguzzi, 2005; Van Heerden, Schönfeldt, Smith, and van Rensburg, 2002; Wattanachant, Benjakul, and Ledward, 2004). Proximate composition of meats from various species are shown in **Table 2.2**. Chicken muscles contain higher protein and lower fat than pork, beef, and some fish.

Proximate composition of breast and thigh is different as shown in **Table 2.3**. Breast contains higher protein and lower fat content than thigh. Collagen influences textural characteristics of meat (Jeon et al., 2010). Differences in collagen content among breeds could be attributed to genotypes and/or age differences at slaughter (Dawson, Sheldon, and Miles, 1991). Collagen is the main structural protein (Di Lullo, Sweeney, Körkkö, Ala-Kokko, and San Antonio, 2002). Thigh contains higher level of collagen than breast (Jaturasitha, Srikanchai, Kreuzer, and Wicke, 2008).

Amino acid composition of chicken breast and thigh is presented in **Table 2.4**. They are a good sources of aspartic acid/asparagine, glutamic acid/glutamine, leucine, arginine and lysine. In addition, they also contained essential amino acids including

valine, isoleucine, leucine, phenylalanine, methionine, tyrosine, and histidine. Differences in amino acid content among breeds could be also attributed to genotypes.

Table 2.2 Proximate composition of chicken muscle from different breeds of chicken.

Species	Moisture (%)	Protein (%)	Fat (%)	Ash (%)	Reference
Kaidang, Thai indigenous	74.88	22.05	0.37	1.03	Wattanachant et al., 2004
Broiler	74.87	20.59	0.68	1.10	Wattanachant et al., 2004
Spent Leghorn chickens	74.25	22.65	1.75	1.10	Al-Najdawi and Abdullah, 2002
Pork (*Sus scrofa domesticus*)	73.60	18.30	4.50	1.10	Jensen et al., 2014
Beef cattle (*Bos taurus*)	73.90	18.90	3.21	1.00	Jensen et al., 2014
Beef tenderloin	76.10	18.91	1.81	1.20	Pereira et al., 2017
Silver barb	73.40	17.90	7.40	1.10	Puwastien et al., 1999
Nile tilapia	78.10	19.80	1.80	1.10	Puwastien et al., 1999
Lake trout fish	71.30	17.60	10.91	1.00	Di Lena et al., 2016

(Wattanachant et al., 2004) (Al-Najdawi and Abdullah, 2002; Di Lena et al., 2016; Jensen, Dort, and Eilertsen, 2014; Pereira, López-Alonso, Miranda, Benedito, and García-Vaquero, 2017; Puwastien et al., 1999)

Table 2.3 Chemical composition of breast and thigh meats from different breeds.

Breeds	Moisture (%)	Protein (%)	Fat (%)	Ash (%)	Total collagen (mg/g muscle)	References
Breast meat						
Kaidang, Thai native chicken	74.39	23.05	2.88	0.60	7.27	Chuaynukool et al., 2007
Spent hen, Thai native chicken	74.83	20.34	1.64	0.19	7.47	Chuaynukool et al., 2007
Hanhyup, Korean native chicken	72.50	23.11	1.84	ND	2.14	Jayasena et al., 2013
North Korean native chickens	74.89	24.13	1.30	0.86	1.89	Jeon et al., 2010
Broiler, Thailand	76.62	21.02	1.33	1.44	3.93	Chuaynukool et al., 2007
Broiler, Korean	74.51	22.32	1.97	ND	1.54	Jayasena et al., 2013
Broiler chickens (Ross strain)	74.71	23.04	1.48	0.86	0.65	Jeon et al., 2010
Thigh meat						
Kaidang, Thai native chicken	80.82	20.34	1.14	0.75	10.33	Chuaynukool et al., 2007
Spent hen, Thai native chicken	79.42	16.44	1.28	1.29	13.11	Chuaynukool et al., 2007
Hanhyup, Korean native chicken	76.39	20.10	2.97	ND	4.26	Jayasena et al., 2013
North Korean native chickens	74.37	20.57	3.98	0.81	2.97	Jeon et al., 2010
Broiler, Thailand	77.30	16.98	0.51	1.43	9.59	Chuaynukool et al., 2007
Broiler, Korean	77.35	18.63	3.41	ND	4.38	Jayasena et al., 2013
Broiler chickens (Ross strain)	74.50	19.02	3.58	0.76	2.03	Jeon et al., 2010

(Chuaynukool, Wattanachant, and Siripongvutikorn, 2007; Jayasena et al., 2013; Jeon et al., 2010)

Table 2.4. Amino acid composition (g/ 100g of total amino acids) of breast and thigh meats from various breeds.

Amino acid	Breast			Thigh		
	Broiler, Korean	**Broiler, Thailand**	**Broiler, North Carolina**	**Broiler, Korean**	**Broiler, Thailand**	**Broiler, North Carolina**
Aspartic acid/asparagine	10.44	7.19	10.07	10.28	6.99	9.92
Threonine	5.05	5.97	5.12	5.02	5.94	5.53
Serine	4.46	4.7	5.24	4.61	4.9	5.56
Glutamic acid/glutamine	16.57	12.55	15.58	17.49	12.71	15.69
Glycine	4.61	5.33	4.54	4.49	5.92	5.49
Alanine	6.32	5.53	5.89	6.15	5.34	6.19
Valine	4.31	4.27	4.64	4.14	4.18	4.52
Isoleucine	4.07	4.76	4.37	3.96	4.6	4.31
Leucine	8.97	8.48	8.43	8.87	8.42	8.12
Phenylalanine	4.02	5.95	4.23	4.2	5.9	4.12
Methionine	2.84	3.71	3.45	2.96	3.68	3.32
Arginine	6.67	8.67	6.74	6.62	9.54	6.19
Proline	3.97	3.81	4.51	4.2	4.08	4.68
Cysteine	1.27	0.61	0.61	1.36	0.62	0.68
Tyrosine	3.19	5.99	4.03	3.25	5.82	3.92
Lysine	9.36	6.74	8.73	9.4	6.35	7.94
Histidine	3.87	5.73	3.82	3.01	5	3.82
Reference	Choe et al., 2010	Wattanachant et al., 2004	Hamm, 1981	Choe et al., 2010	Wattanachant et al., 2004	Hamm, 1981

2.2 Thermal treatments of muscle foods

2.2.1 Thermal processing

Thermal processing is mandatory for food preparation, especially for muscle foods, which are usually cooked before being eaten. Heat destroys certainly microorganisms and can, be used to extend shelf-life and safety. Cooked meat products are prepared in many forms by thermal processing such as boiling, steaming, grilling, microwaving, and superheated steaming. Mild thermal processing is the heating temperature at 70 to 100˚C, which can destroy vegetative cells of microorganisms, but has almost no effect on spores. According to the USDA guideline, chicken meat should be heated to 68.3-72˚C to ensure that it is free from pathogen and viruses (Owusu-Apenten, 2002). While high thermal processing (over 100˚C) is aimed at destroying both vegetative cells and spores for long-term shelf-life.

2.2.2 Protein modifications under thermal treatments

Oxidation of meat may cause changes in protein hydrophobicity, conformation, solubility and altered susceptibility to proteolytic enzymes. Degree of denaturation of food proteins is significantly correlated with time and temperature applied (Torreggiani et al., 2008). Mild heating (60 to 80 °C) induces denaturation of myofibrillar proteins and collagen, resulting in enhanced accessibility of gastrointestinal proteases (Bax et al., 2012). There is a loss of almost all secondary and tertiary structure at temperatures above 70˚C (Davis and Williams, 1998), causing random coil conformation. Cooking meat for the extended period for up to 24 h at a mild heating condition significantly increased tenderness due to solubilizing of collagen to gelatin and proteolysis of myofibrillar proteins (Bouton and Harris, 1981). The slow heating regime is an underlying process of various meat cooking methods, including braising, stewing, and

sous vide processing (Baldwin, 2012). The extent of protein denaturation of prolonged heating would be different from that of meat cooked at shorter time. Disulfide bonds were formed with the heating temperature above 90˚C, and extreme thermal treatment over 100 ˚C applied in the canning process induces degradation of peptide bonds, protein aggregation via hydrophobic interactions, and covalent cross-linkings of side chains. The formation of protein carbonyls and modification of aromatic amino acids can also take place (Soladoye, Juárez, Aalhus, Shand, and Estévez, 2015).

Proteins responsible for meat texture include stromal (mostly collagen) and myofibrillar proteins (Califano, Bertola, Bevilacqua, and Zaritzky, 1997; Dawson et al., 1991). It has been indicated that toughness increased during cooking of meat between 40 – 90 ˚C (Bailey and Light, 1989). Extremely high thermal treatment at 121˚C leads to loss of shape and unacceptable textural characteristics (Dawson et al., 1991).

2.2.3 Amino acid modifications under thermal treatments

Thermal processes have triggered the generation of reactive oxygen species (ROS) during meat cooking (Lund, Heinonen, Baron, and Estevez, 2011; Traore et al., 2012). Free radical can attack on protein molecules, resulting in protein cross-linking, protein fragmentation, and/or modification of amino acid side chains, (Lund et al., 2011; Stadtman and Levine, 2003).

A modification of amino acids by ROS attack leads to formation of carbonyl groups and hydroperoxides, while cross-linking has mostly been described as the formation of disulfide and dityrosine through the loss of cysteine and tyrosine residues **(Table 2.5)** (Soladoye et al., 2015). Further, oxidation of glutamyl and aspartyl residues in proteins can lead to peptide bond cleavage forming N-pyruvyl derivative. Uchida et

al., (1990) showed that oxidation of proline residue in proteins can lead to peptide bond cleavage, leading to a detectable 4- aminobutyric acid, which is derived from 2-pyrrolidone, an immediate derivative of the oxidation process.

Table 2.5 Oxidation of amino acid residues and their oxidation products.

Amino acid residue	Process of modification	Product of modification
Arginine	Carbonylation/ metal ion-catalyzed oxidation	γ-Glutamic semialdehyde
Lysine	Carbonylation/ metal ion-catalyzed oxidation	α-Aminoadipic semialdehyde
Proline	Carbonylation/ metal ion-catalyzed oxidation	Glutamic semialdehyde, 2- pyrolidone, 4,5- hydroxyproline, pyroglutamic acid
Cysteine	Glutathiolation/ cross-linking/ metal ion-catalyzed oxidation	Disulfide, cysteic acid, sulfenic acid, sulfinic acid
Threonine	Carbonylation/ metal ion-catalyzed oxidation	2-Amino-3-ketobutyric acid
Leucine	Hydroxylation	3,4,5-Hydroxyleucine
Histidine	Metal ion- catalyzed oxidation	Asparagine, aspartic acid, 2-oxohistidine, 3,4,5- hydroxyleucine, 4- hydroxyl glutamate
Glutamic acid	–	Pyruvate adducts, oxalic acid
Methionine	Sulfoxidation	Methionine sulfoxide, methionine sulfone
Phenylalanine	Hydroxylation	2- , 3- , and 4- Hyrophenylalanine, 2,3-dihydroxyphenylalanine
Tryptophan	Hydroxylation/nitration	2- ,4- ,5- ,6- , and 7- Hydroxykynurenine, kynurenine, and nitrotryptophan
Tyrosine	Metal ion- catalyzed oxidation/nitrosylation	3,4- Dihydroxyphenylalanine, Tyr- Tyr cross- links, 3- nitrotyrosine, and Tyr-oxygen-Tyr
Valine	Hydroxylation	3-Hydroxylvaline

Source: Soladoye et al., 2015 (Soladoye et al., 2015)

Carbonylation of amino acid at site chain of peptide and protein is one of the most remarkable chemical modifications in oxidized proteins (Estévez, 2011). It is an irreversible and non- enzymatic modification of proteins that involves the formation of carbonyl moieties induced by oxidative stress (Berlett and Stadtman, 1997). Carbonyls

can be formed through four different pathways, namely, 1) direct oxidation of the side chains from lysine, threonine, arginine and proline, 2) non-enzymatic glycation in the presence of reducing sugars, 3) oxidative cleavage of the peptide backbone via the α-amidation pathway or via oxidation of glutamyl side chains, and 4) covalent binding to non-protein carbonyl compounds such as 4-hydroxy-2-nonenal (HNE) or malondialdehyde (MDA). Carbonyl content was increased with heat treatment at 90 ˚C and higher amounts of carbonyls were produced at higher temperatures of 123 to 207 ˚C in myofibril (Gatellier, Kondjoyan, Portanguen, and Santé-Lhoutellier, 2010; Promeyrat et al., 2011). In addition, amino acids can be modified by thermal treatment.Cys, Ala, His, and Lys contents were reduced in beef and pork meats cooked at 70˚C for 10 min (Jensen et al., 2014). Thus, these modifications would lead to alteration of the protease recognition sites. For example, activity of trypsin, a serine endopeptidase that hydrolyzes proteins specifically at the carboxyl side of arginine and lysine, would reduce if these amino acids are carbonylated and modified.

2.2.4 Effect of thermal treatment on protein digestibility and bioavailability

Time and temperature at which meat is cooked greatly affect the extent of protein denaturation that occurs and the accessibility of GI enzymes to break down meat proteins. Mild thermal treatment (60 - 80˚C) induces protein unfolding, thus enhancing protease susceptibility (Bax et al., 2012; Soladoye et al., 2015). In contrast, modification of protein by high temperature might have an effect on protein digestibility (Morzel, Gatellier, Sayd, Renerre, and Laville, 2006). These ultimately affect nutritional quality of muscle foods (Morzel et al., 2006; Véronique Santé-Lhoutellier, Engel, Aubry, and Gatellier, 2008). Extreme thermal heating (>100 ˚C) leads to irreversible unfolding, aggregation, disulfide interchanges, protein

fragmentation and chemical modifications of side-chains, including sulfoxidation, carbonylation, hydroxylation, nitrosylation, and glutathiolation (Davis and Williams, 1998; Soladoye et al., 2015). These intermolecular cross-links and formation of aggregates with oxidative modifications also reduced protein susceptibility to enzymatic proteolysis (Dizdaroglu, Gajewski, and Simic, 1984).

Modifications of protein under thermal treatment may reduce bioavilability of amino acids. (Soladoye et al., 2015). Oxidized sulfur-containing amino acids have been found to display reduced or limited nutritional availability regardless of the true ileal digestibility of proteins (Rutherfurd and Moughan, 2012). In addition, formation of Maillard compounds and cross-linked peptide chains also limit protein bioavailability (Boye, Wijesinha-Bettoni, and Burlingame, 2012; Millward, Layman, Tomé, and Schaafsma, 2008)

2.3 GI digestion of muscle protein

Dietary proteins and peptides are susceptible to hydrolysis during different stages of GI digestion, namely ingestion, digestion and absorption (Vermeirssen, Van Camp, and Verstraete, 2004). Proteases are responsible for breaking down proteins and peptides into smaller peptides and amino acids (Hur, Lim, Decker, and McClements, 2011). Pepsin and pancreatic enzymes are mainly present in the stomach and small intestine, respectively. Daily pepsin secretion is about 20–30 kU of enzyme activity at 37 °C in adults, and dietary intake of protein comprises around 75 g/day (Bublin et al., 2008; Mills, Jenkins, Alcocer, and Shewry, 2004). Proteins are firstly digested in the stomach by the action of pepsin at acidic pH 2-3, then the polypeptides are further cleaved by intestinal endo- and exo-peptidases namely trypsin, α-chymotrypsin,

elastase, and carboxypeptidase A and B at more alkaline pH 7-8 (Vermeirssen et al., 2004). Increasing in dietary protein uptake is induced more secretion of pancreatic proteolytic enzymes (Boisen and Eggum, 1991). These enzymes exhibit different specificity as shown in **Table 2.6**.

Table 2.6. Bond specificities of secreted peptidases.

Enzyme		Specificity (*)
Pepsin	H_2N—○○○○*○○—COOH	Pro, Leu
Trypsin	H_2N—○○○○*○○—COOH	Arg, Lys
Chymotrypsin	H_2N—○○○○*○○—COOH	Phe, Tyr
Elastase	H_2N—○○○○*○○—COOH	Ala, Gly, Ile, Leu, Val, Ser
Carboxypeptidase A	H_2N—○○○○○*○—COOH	Tyr, Phe, Ile, Thr, Glu, His, Ala
Carboxypeptidase B	H_2N—○○○○○*○—COOH	Lys, Arg

* Means specificity indicated by filled circle

Source: Modified from Mahato et al., (2003)[Mahato, Narang, Thoma, and Miller, 2003]

An ideal *in vitro* digestion model would provide highly accurate results in a short time and could serve as a tool to study the digestibility of various foods (Hur et al., 2011). *In vitro* digestion models for foods were shown in **Table 2.7**. There are various experimental parameters of digestion model. These include the type of enzyme pepsin, pancreatin, trypsin, and α-chymotrypsin. The most frequently used enzymes within *in vitro* digestion models of food proteins were pepsin to simulate the stomach and then pancreatin to simulate the small intestine with digestion time 1-3 h and 0.5-3 h, respectively. Digestion temperature is usually performed at 37 ˚C, pH 2.0-2.5 for pepsin, and pH 7.0-8.0 for pancreatin. Different enzyme levels have been used, which would lead to variations. Concentration and composition of enzymes are also very important factors to consider when designing *in vitro* digestion model. Several studies utilized enzymes collected from human subjects, whereas others have used enzymes

Table 2.7 *In vitro* digestion systems used in various food proteins.

Subject of study	Sample and buffer	Enzymes	Digestion times	Stop reaction	References
Digestion of raw pork meat	10% (w/v) of raw pork, pH 2 with 6 M HCl	Pepsin (1% enzyme/substrate)	2 h	Boiled 95˚C for 10 min	Escudero et al., 2010
	pH 7.2 with 1 M $NaHCO_3$	Pancreatin (2% enzyme/substrate)	3 h		
ACE inhibitory activity of soy protein digests	5% (w/v) of sample, pH 2 with 1 M HCl	Pepsin (2% enzyme/protein)	1 h	Boiled for 10 min	Lo and Li-Chan, 2005
	pH 5.3 with 1 M $NaHCO_3$, then pH 7.5 with 1 N NaOH	Pancreatin (4% enzyme/protein)	2 h		
Availability of various iron fortificants in bread and milk	2% (v/v) of milk, pH 2 with 1 M HCl	Pepsin 0.2 g/mL	1 h		Yeung et al., 2002
	pH 6 with 1 M $NaHCO_3$, then pH 7 with 1 M $NaHCO_3$	Pancreatin 0.05/mL	2 h	-	
Digestible iron and zinc content of Okra sauce	10% (w/v) of raw pork, pH 2 with 1 M HCl	Pepsin solution (14900 U/mL)	1 h	-	Avallone et al., 2007
	pH 4.0 with 0.15 mM PIPES buffer, then pH 7, add bile extract solution	Pancreatin (1.85 mg/mL)	2 h		

(Avallone, Bohuon, Hemery, and Treche, 2007; Escudero, Sentandreu, Arihara, and Toldrá, 2010; Lo and Li-Chan, 2005; Yeung, Glahn, and Miller, 2002)

Table 2.7 *In vitro* digestion systems used in various food proteins. (continued)

Subject of study	Samples and buffer	Enzymes	Digestion times	Stop reaction	References
Digestion of cooked eggs	5% (w/v)of cooked egg, pH 2 with 1 M HCl,	Pepsin (4% enzyme/protein)	3 h	Boiled 95 °C for 10 min	Majumder et al., 2009
	pH 7.5 with 0.1 M NaOH	Pancreatin (2% enzyme/protein)	3 h		
InfoGest consensus method of solid samples	Minced sample was mix 1:1 with simulated gastric gluid pH3	Pepsin (2,000U/mL)	2 h	Add inhibitor, freeze -80C, then freeze dry	Minekus et al., 2014
	Mix 1:1 with simulated intestinal fluid pH 7.0	Pancreatin (based on 100U/mL of trypsin)	2 h		
Digestion of sarcoplasmic and myofibrillar protein	1% of samples (dry weight), pH 2.5 with 6 M HCl	pepsin (2%, w/w of protein)	2 h	deep-freezing(–25 °C)	Storcksdieck et al., 2007
	pH 6.0 with 0.1 M NaHCO3	pancreatin (2%, w/w of protein)	2 h		
Digestion of myofibrillar protein in lamb meat	0.8 mg/ml in 33 mM glycine buffer at pH 1.8	pepsin (20 U/mg proteins)	1 h	15% of trichloroacetic acid	Santé-Lhoutellier et al., 2008
	pH 8 with 33 mM glycine buffer at pH 1.8	Trypsin (6.6 U) and α-chymotrypsin (0.33) U/mg of proteins	0.5 h		

(Majumder and Wu, 2009; Minekus et al., 2014; Veronique Santé-Lhoutellier, Astruc, Marinova, Greve, and Gatellier, 2008; Storcksdieck, Bonsmann, and Hurrell, 2007)

extracted from animal or plant sources. Then, digestion was terminated by boiling, acid, and freezing.

The standard method for GI digestion has been developed such as *in vitro* digestion systems for screening the digestibility of food materials (Lee, Lee, Chung, and Hur, 2016) and the COST Action InFoGest (the static protocol for simulating digestion in the upper GI tract) (Mackie and Rigby, 2015). However, the standard method for *in vitro* GI digestion of muscle meat has not been reported.

2.4 Bioactive peptides derived from GI digestion

Most of physiological activities of proteins are caused by peptide sequences encrypted in the parent protein which becomes active when cleaved. Bioactive peptides have been defined as "food derived components that exert a physiological effect in the body" (Bouglé and Bouhallab, 2017; Ryan, Ross, Bolton, Fitzgerald, and Stanton, 2011). Bioactive peptides released by enzymatic proteolysis of food proteins may act as potential physiological modulators of metabolism during GI digestion. Bioactive peptides usually contain 2–20 amino acid residues and their activities are based on their amino acid composition and sequence (Himaya, Ngo, Ryu, and Kim, 2012; Pihlanto, Akkanen, and Korhonen, 2008). Peptides generated in the GI tract may have different biological properties, such as antioxidant and antihypertensive activity. Antioxidant and ACE inhibitory peptides derived from GI enzymes digestion of food proteins are shown in **Table 2.8**.

Table 2.8 ACE inhibitory and antioxidant peptides derived from GI enzymes digestion of food proteins.

Protein source	Examples of identified sequence	IC50 (μM)	Enzymes	References
1) ACE inhibitory activity				
Cooked eggs	VRF	6.59	Pepsin (4%, w/w of protein), and pancreatin (2%, w/w of protein),	Majumder and Wu, 2009
Pork	KAPVA PTPVP	45.6 382	Pepsin (1%, w/w of substrate), and pancreatin (2%, w/w of substrate)	Escudero et al., 2012
Common Bean	YAGGS	ND	Pepsin (5%, w/w of substrate), and pancreatin (5%, w/w of substrate)	Mojica et al., 2015
Camel milk	IPP	5.0	Pepsin (1115 U/L) and pancreatin (1115 U/L	Tagliazucchi et al., 2016
Cooked eggs	VRF	6.59	Pepsin (4%, w/w of protein), and pancreatin (2%, w/w of protein),	Majumder and Wu, 2009
Sardine muscle	KW	1.63	Pepsin (1%, w/w of substrate), and trypsin and α-chymotrypsin (1%, w/w of substrate)	Matsufuji et al., 1994
Sake Lees	VW VWY WY	1.4 9.4 10.5	Pepsin (0.1 mg/mL) and pancreatin (4.47 mg/mL)	Yoshiyuki et al., 1994

ND means not determine

(Escudero, Toldrá, Sentandreu, Nishimura, and Arihara, 2012; Majumder and Wu, 2009; Matsufuji et al., 1994; Mojica, Chen, and Mejía, 2015; Tagliazucchi, Shamsia, and Conte, 2016; Yoshiyuki, Keiko, Akitsugu, and Satoshi, 1994)

Table 2.8 Antioxidant and ACE inhibitory peptides derived from GI enzymes digestion of food proteins. (continued)

Protein source	Bioactive activity	Examples of identified sequence	Enzymes used	References
2) Antioxidant activities				
Sunflower protein	Copper-chelating activity	Fractionated GI digest	Pepsin (5%, w/w of substrate), and pancreatin (5%, w/w of substrate),	Megías et al., 2008
Horse mackerel visceral protein	DPPH and hydroxyl radicals scavenging	ACFL	Pepsin (1%, w/w of substrate), trypsin and α-chymotrypsin (1%, w/w of substrate)	Sampath et al., 2011
Croaker muscle protein	DPPH and hydroxyl radical scavenging, inhibits the lipid peroxidation and DNA damage	HNGCFTK	Pepsin (1%, w/w of substrate), trypsin and α-chymotrypsin (1%, w/w of substrate)	Nazeer et al., 2012
Cooked chicken egg	Antioxidant with ORAC, ABTS and DPPH assays	63 identified peptides	Pepsin (2%, w/w of protein), and pancreatin (2%, w/w of protein)	Remanan et al., 2014
3) Antioxidant and ACE inhibitory activities				
Okara protein	ACE inhibitor, power reduction and radical scavenging activity	TIIPLPV	Pepsin (5%, w/w of substrate), and pancreatin (5%, w/w of substrate)	Jiménez-Escrig et al., 2010

(Megías et al., 2008) (Jiménez-Escrig, Alaiz, Vioque, and Rupérez, 2010; Nazeer, Kumar, and Ganesh, 2012; Remanan and Wu, 2014; Sampath Kumar, Nazeer, and Jaiganesh, 2011)

2.4.1 Antioxidant peptides

Peptides have also been shown to possess antioxidant activity, which can protect cellular damage caused by reactive oxygen species (ROS) or free radicals and reduces the risk of several diseases, including diabetes, cancer, cardiovascular diseases, and Alzheimer's Disease (Quirós, Dávalos, Lasunción, Ramos, and Recio, 2008). Protein hydrolysates and their isolated peptides have shown promising *in vitro* antioxidant activity (Sarmadi and Ismail, 2010). Most studies have been focused on the production of antioxidant peptides using commercial or new sources of proteases (Udenigwe and Aluko, 2012). For examples, peptides derived from porcine myofibrillar proteins hydrolysate using papain and Actinase E inhibited peroxidation of linoleic acid, DPPH scavenging and metal chelating activities (Saiga *et al.*, 2003). Five identified from this hydrolysate including: DSGVT (actin), IEAEGE (unknown), DAQEKLE (tropomyosin), EELDNALN (tropomyosin) and VPSIDDQEELM (myosin heavy chain) showed the highest antioxidant activity (Ryan et al., 2011). In addition, chicken essence, a traditional product consumed in China, possessed various antioxidant activities including the inhibition of linoleic acid autoxidation, DPPH radical scavenging activity, reducing power and the ability to chelate metal ions (Hui-Chun, Bonnie Sun, Che-Lang, and Chyuan-Yuan, 2005). Two peptides, HVTEE and PVPVEGV, displayed inhibition of the autoxidation of linoleic acid in a model system (Hui-Chun et al., 2005). Antioxidant peptides derived from GI digestive system of food proteins have also been reported as shown in **Table 2.8.** These studies revealed that food proteins could provide an excellent source of antioxidant peptides upon GI digestion. Antioxidant peptides released upon GI digestion could also play an important role in human health. Thus, consumption of food proteins not only provides nutritional

value in the form of amino acids, but also yields bioactive peptides. However, antioxidant peptides generated from the GI digestion of cooked meat have not been systematically elucidated.

It has not been fully understood the exact mechanism underlying the antioxidant activity of peptides. Various studies have displayed that they are inhibitors of lipid peroxidation, scavengers of free radicals, chelators of transition metal ions (Sarmadi and Ismail, 2010). The antioxidative properties of peptides are more related to their composition, structure, and hydrophobicity. Tyr, Trp, Met, Lys, Cys, and His are examples of amino acids exhibiting antioxidant activity (Chen, Muramoto, Yamauchi, Fujimoto, and Nokihara, 1998; W. Wang, Mejia, and Gonzalez, 2005). Amino acids with aromatic residues can donate protons to electron deficient radicals (Rajapakse, Mendis, Byun, and Kim, 2005). It is proposed that the antioxidative activity of His-containing peptides is in relation to the hydrogen-donating ability, lipid peroxyl radical trapping and/or the metal ion chelating ability of the imidazole group (Chan and Decker, 1994; Rajapakse et al., 2005). On the other hand, SH group in cysteine has an independently crucial antioxidant action due to its direct interaction with radicals (Qian, Jung, and Kim, 2008). In addition, fractions enriched with di-, tri- and tetrameric peptides containing tryptophan and proline exhibited the strongest radical scavenging activity (Ma, Xiong, Zhai, Zhu, and Dziubla, 2010). In addition, sequence of amino acids in a peptide chain also plays an important role in the antioxidant activity (Saito et al., 2003). **Table 2.9** provides information regarding the effect of amino acid compositions and their required position for antioxidant activity.

Table 2.9 Amino acid compositions and their positioning in relation with antioxidant activity.

Amino acids	Mechanism of action	Detail
Aromatic AAs (Tyr, His, Trp, Phe)	Converting radicals to stable molecules by donating electron, while keeping their own stability via resonance structure Improving the radical-scavenging properties of the amino acids residues	- His at N-termini as an effective metal ion cheater - His at C-termini as an effective scavenger against various radicals - Tripeptides with Trp or Tyr at C-terminus as strong radical scavengers but weak peroxynitrite scavengers
Hydrophobic	Enhancing the solubility of peptide in lipid which facilitates accessibility to hydrophobic radical species, Gly as hydrogen donor	- Val or Leu, at the N-terminus and Pro, His, or Tyr in the sequences - Ala or Leu at the C or N terminus, Gln and a Pro residue in the sequences of peptide from gluten
Acidic and basic	Carboxyl and amino groups in the side chains as chelator of metal ions, as hydrogen donor	- Asp (acidic amino acid) and His (basic amino acid) residues in peptide purified from fermented mussel sauce
Cysteine	SH group as radical scavenger, protecting tissue from oxidative stress, improving the glutathione activity	- Tripeptides with Cys as strong scavengers against peroxynitrite radicals - In curry leave protein SH group together with other functional groups involved in its antioxidant activity

Source: Modified from Sarmadi and Ismail, 2010 (Sarmadi and Ismail, 2010)

2.4.2 ACE inhibitory peptides

The chronic diseases such as cardiovascular diseases (CVDs) are increasing rapidly every year. High blood pressure is an independent risk factor for CVDs and is responsible for most preventable deaths worldwide (Kearney et al., 2005; Mohamed, 2014). In addition, hypertension can lead to many serious diseases including disorders of the heart and blood vessels, kidney disease, arteriosclerosis, and stroke (World

Health Organization, 2014). ACE is a multifunctional, zinc-containing enzyme, located in lungs endothelial and kidney epithelial cells (Kierszenbaum and Tres, 2015). It is one of crucial enzymes in the renin- angiotensin system (RAS) that controls hypertension and electrolyte homeostasis. ACE converts angiotensin I to a potent vasoconstrictor, angiotensin II, which also induces the release of aldosterone as shown in **Figure 2.2**. Therefore, it increases sodium concentration and blood pressure. ACE also takes part of the kinin–kallilrein system by hydrolyzing bradykinin. Kinin–kallilrein is a system of blood proteins that plays a role in inflammation, blood pressure control, coagulation, and pain.

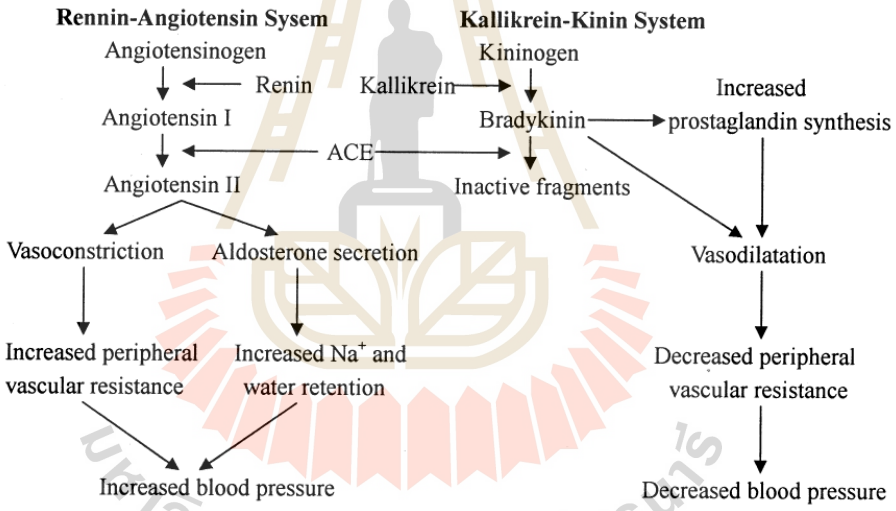


Figure 2.2 Role of angiotensin converting enzyme in blood pressure regulation.

Source: Li et al., (2004)G.-H. Li, Le, Shi, and Shrestha, .

ACE inhibitory peptide was firstly discovered in snake venom (Ferreira, Bartelt, and Greene, 1970). ACE inhibitory peptides from food proteins have been reported in various protein sources, such as milk, egg, meat, fish, and plant proteins (Hernández-Ledesma, del Mar Contreras, and Recio, 2011). ACE inhibitory peptides have been identified in the protein hydrolysates of muscle proteins, their sequences were released

from actin, myosin, and troponin (Muguruma et al., 2009). The most active ACE inhibitory peptide, RMLGQTPTK (IC_{50} of 34 μM), derived from porcine skeletal muscle hydrolyzed by pepsin was released from the regulatory protein troponin C and categorized as being a non-competitive inhibitor (Katayama et al., 2003; Katayama et al., 2004). Crude myosin light chain was hydrolyzed by pepsin, generating VKKVLGNP (IC_{50} of 28.5 μM). This purified peptide reduced systolic blood pressure (SBP) of spontaneously hypertensive rat (SHR) fed at 10 mg per kilogram of animal weight up to 3 h post-administration (Katayama et al., 2007). When crude porcine myosin B was hydrolyzed with pepsin, peptide M6 (KRVITY) corresponding to positions 191–196 on the myosin heavy chain, was obtained (Muguruma et al., 2009). Systolic blood pressure of SHR was immediately reduced following oral administration of M6 peptide with a maximum decrease of 23 mmHg after 6 h. (Muguruma et al., 2009).

ACE inhibitory peptides have also been reported to be released during GI digestion as shown in **Table 2.8**. Escudero et al., (2010) studied ACE inhibitory peptides after GI digestion of pork meat and found that the strongest ACE inhibition was observed in peptide KAPVA (IC_{50} of 46.56 μM) followed by PTPVP (IC_{50} = 256.41 μM). ACE inhibitory peptides derived from GI digestion of cooked eggs was also identified, seven peptides including VDF (IC_{50} of 6.59 μM), LPF (IC_{50} of 10.59 μM), MPF (IC_{50} of 17.98 μM), YTAGV (IC_{50} of 23.38 μM), ERYP (IC_{50} of 8.76 μM), IPF (IC_{50} of 8.78 μM), and TTI (IC_{50} of 24.94 μM) exhibited potent ACE-inhibitory activity, while a tripeptide VRF showed strong activity with IC_{50} of 6.59 μM (Majumder and Wu, 2009). In addition, ACE inhibitory peptides released from GI digestion of raw pork were derived from titin (KAPVA and PTPVP), nebulin (RPR),

troponin T (PKIPEGEKY), and actin (DSGDGVT) (Escudero, Sentandreu, Arihara, et al., 2010; Escudero, Sentandreu, and Toldra, 2010; You, Zhao, Regenstein, and Ren, 2010).

ACE inhibitory peptides are the most studied bioactive peptides, whose sequences have been complied in BIOPEP and antihypertensive peptide database (AHTPDB). The BIOPEP database currently displays the sequences of peptides with 44 various bioactivities (Iwaniak and Minkiewicz, 2008). BIOPEP contains 3,389 peptide sequences (assessed May 1, 2017) and 740 of them are ACE inhibitors (http://www.uwm.edu.pl/biochemia/index.php/ pl/biopep). AHTPDB contains 5,978 sequences of ACE inhibitor peptides, and most ACE inhibitors are di- and tri-peptides (assessed May 1, 2017). This database is a manually curated database of experimentally validated antihypertensive peptides (Kumar et al., 2015). Information pertaining to peptides with antihypertensive activity was collected from research articles and from various peptide repositories.

ACE inhibitory peptides and their structural requirement have been studied. Quantitative structure activity relationship (QSAR) is based on statistical models that predict the relationship between structure (e.g. amino acid sequence) and activity (e.g. ACE-inhibition) (Pripp, Isaksson, Stepaniak, Sørhaug, and Ardö, 2005). This approach provides a tool to predict the amino acid sequence that would give a potent inhibitory potential. QSAR has been successfully applied in the structure-activity relationship of ACE inhibitory peptide (Martinez-Mayorga and Medina-Franco, 2009; Nakai, Li-Chan, and Dou, 2005; Pripp, Isaksson, Stepaniak, and Sørhaug, 2004). The majority of ACE inhibitory peptides are relatively short sequences containing 2 - 12 amino acids. The activity of ACE is to cleave the C-terminal dipeptide

of oligopeptide substrates with a wide specificity. Inhibitory activity of ACE inhibitory peptides is strongly influenced by their C-terminal tripeptide sequence as well as hydrophobic amino acids (Gly, Ile, Leu, and Val) at the N-terminus. Amino acids with cyclic or aromatic rings (Pro, Tyr, Trp) are found at the C-end of the ACE inhibitors (FitzGerald, Murray, and Walsh, 2004; Vermeirssen et al., 2004). Most di- and tripeptides contain Pro at the C terminal end as well as N-terminal branched side aliphatic amino acids resulted in high ACE inhibitory activity (Iwaniak and Dziuba, 2009).Pripp et al., (2005) reported that the QSAR model of dipeptide ACE inhibitors, contain hydrophobic and aromatic amino acids in the C-terminus. Iwaniak (2011) applied principal component analysis to analyze 98 dipeptide ACE inhibitors collected in the BIOPEP database, the results showed that bulkiness of the C-terminal amino acids showed high correlation with inhibitory activity. For tripeptides, the 1st residue was usually aromatic, the 2nd was positively charged, and the 3rd residue (C-terminus) was hydrophobic (J. Wu, Aluko, and Nakai, 2006). Peptides with a long chain and contain positively charged guanidine (Arg) and ε-amine groups (Lys) contributed to an increased inhibitory activity. Replacement of Arg caused a total loss of activity (FitzGerald et al., 2004). Some sequences of ACE inhibitors contain a glutamic acid at the C-terminus, which can chelate zinc of ACE active center (FitzGerald et al., 2004).

2.4.3 Peptides possessing dual bioactive activities

The combination of ACE inhibitory and antioxidant activities could be very helpful for the control of cardiovascular diseases by synergistically acting on different regulatory mechanisms. Antonio et al., (2010) reported that digest with molecular weight <1 kDa derived from GI digestion of okara protein (the by-product of the soymilk) exhibited high potency on both ACE inhibitory and antioxidant activities.

Identified peptide, TIIPLPV, showed the highest ACE inhibitory activity. This study suggested that digest derived from GI digestion exhibit multifunctional health benefits. However, most reports showing dual bioactivity are derived from enzymatic hydrolysates of food proteins, but those derived from GI digestion has not been extensively elucidated.

2.5 Permeability of peptides

2.5.1 *In vitro* transport model of peptide

The human intestinal colorectal adenocarcinoma (Caco-2) cell line is typically available *in vitro* model of absorptive enterocytes for small intestinal transport of drugs and food compounds (Cinq-Mars, Hu, Kitts, and Li-Chan, 2007). In the model, cultured Caco-2 cells grow attached to a substrate, forming a monolayer. When they attain confluence, they spontaneously differentiate and acquire the morphofunctional traits of mature enterocytes (Herold, Rogler, Rogler, and Stange, 1994). The differentiated cells express characteristics of mature epithelium, such as microvillus structure, the tight junction at the apical side between cells to serve as a model of the paracellular movement of compounds across the monolayer, numerous brush border enzymes.

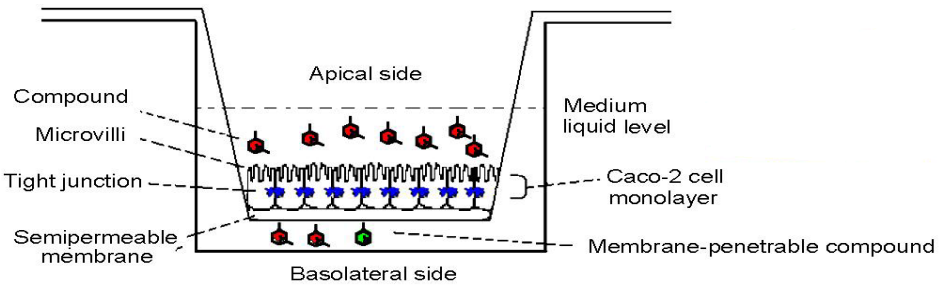


Figure 2.3 Schematic representation of a Caco-2 permeability assay.

Source: Yang et al. (2007) (Yang et al., 2007)

Permeability study is performed using Caco- 2 cell monolayers grown on semipermeable plastic supports in multi-well insert systems (**Figure 2.3**).

In the experiment for peptide transport, the test sample can be added to either the apical or basolateral chamber to measure permeability in the absorptive (apical to basolateral) or secretive (basolateral to apical) directions. Samples are then taken from the opposite chamber at various time intervals to measure the amount of test compound passing through the cell monolayer. The permeability of peptides passing through the monolayers can be monitored using TNBS method (Samaranayaka, Kitts, and Li-Chan, 2010; Toopcham, Mes, Wichers, Roytrakul, and Yongsawatdigul, 2017) and HPLC analysis with absorbance at 214 nm (Guang, Shang, and Jiang, 2012; Lacroix, Chen, Kitts, and Li- Chan, 2017). To ensure the integrity of Caco- 2 cell monolayers, a function of tight junctions can be determined and monitored by the measurement of transepithelial electrical resistance (TEER) (Cinq-Mars et al., 2007; Samaranayaka et al., 2010). The integrity of cultured monolayers is also detectable by carrying out transport studies using water-soluble reference compounds that can be absorbed by tight junctions (TJ) channels between the cells such as Lucifer Yellow, and fluorescein (Isabelle ME, Xiu Min, David, and Eunice CY, 2017; Konishi, 2003)

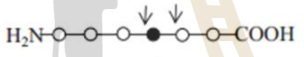
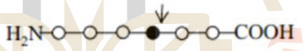
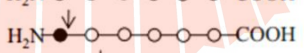
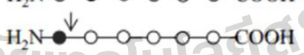
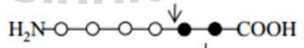
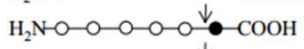
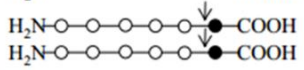
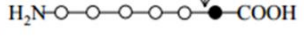
2.5.2 Intestinal absorption of peptides

Free amino acids are absorbed into the enterocytes across the brush border membrane via distinct amino acid transport systems. Dietary proteins were hydrolyzed by GI enzymes, while oligopeptides derived from GI digestion undergo further hydrolysis by the action of brush border peptidases, resulting in a mixture largely consisting of free amino acids, di- and tripeptides, and oligopeptides (Howell, Kenny, and Turner, 1992; Mahato et al., 2003). Protein digestion generates many peptides in

the gut lumen and some of these short peptides are absorbed intact from the GI tract (Roberts, Burney, Black, and Zaloga, 1999). These newly formed peptides might or might not exert bioactivity. Then, the transported peptides were further hydrolyzed by blood protease before reaching to target tissues and organs.

Brush border membrane contains various types of enzymes such as alkaline phosphatase, sucrase, and a large number of peptidases (Bernkop- Schnurch and Walker, 2001; Woodley, 1993). Intestinal brush border membrane peptidases and their different substrate specificity are shown in **Table 2.10**.

Table 2.10 Intestinal brush border membrane peptidases and their substrate specificity.

Enzyme		Specificity
Endopeptidases		
Endopeptidase-24.11*		Hydrophobic amino acids
Endopeptidase-24.18		Aromatic amino acids
Endopeptidase-3		Arg-Arg?
Enteropeptidase		$(Asp)_4$-Lys
Exopeptidases : Amino-terminus		
Aminopeptidase N*		Many
Aminopeptidase A		Asp. Glu
Aminopeptidase P *		Pro
Aminopeptidase W *		Trp, Tyr, Phe
Dipeptidyl peptidase IV*		Pro, Ala
γ-Glutamyl transpeptidase*		γ-Glutamic acid
Exopeptidases : Carboxy-terminus		
Peptidyldipeptidase A*		His, Leu
Carboxypeptidase P		Pro, Gly, Ala
Carboxypeptidase M		Lys, Arg
γ-Glutamyl carboxypeptidase		γ-Glutamic acid

***** Enzyme that express on surface and inside membrane. **(Howell et al., 1992; Mahato et al., 2003)**
Source: modified from Mahato et al, (2003) and et al, (1992).

Intact peptides with 2–6 amino acids can be absorbed through intestinal cells, but the absorption rate decreases with peptide chain length (Bouglé and Bouhallab, 2017; Grimble, 1994). Brush border enzyme activity is generally greater in the

duodenum and the jejunum than in the ileum, and membrane enzymes are very low in the colon. Many peptides are quickly degraded by brush border peptidases (Kreil, Umbach, Brantl, and Teschemacher, 1983). For example, β-casein peptide LHLPLP was hydrolyzed by brush border peptidases to HLPLP prior to transport across the intestinal epithelium (Quirós et al., 2008), dry-cured ham peptide AAATP was hydrolyzed to AAAT, AAA and ATP (Gallego et al., 2016), and tripeptides LAP and LSP were also quickly digested by brush border peptidases. Although peptides are thought to be rapidly metabolized to amino acids, but several peptides such as tripeptides IPP, VPP, IKP and LQP are resistant to these physiological processes and can reach the circulation (Foltz et al., 2008; Hernández-Ledesma et al., 2011; Ohsawa et al., 2008). The presence of proline and hydroxyl proline results in peptide resistance to digestive enzymes, especially tripeptides with Pro-Pro at the C-terminus, which are resistant to proline-specific peptidases (FitzGerald and Meisel, 2000). Modification of bioactive peptides during transport through Caco-2 cell monolayer are shown in **Table 2.11.**

Table 2.11 Modification of bioactive peptides during transport through Caco-2 cell monolayer.

Parent protein	Bioactivity of peptide	Peptide	Parent and degraded peptide products		Reference
			Apical	Basal	
β-casein protein	ACE inhibitor	LHLPLP	LHLPLP, HLPLP	HLPLP* (paracellular passive diffusion**)	Quirós et al., 2008
Egg ovotransferrin	ACE inhibitor	IRW	IRW	IRW (PepT1**, 0.4%***)	Bejjani and Wu, 2013
Fish gelatin	ACE inhibitor	GPHyp	GPHyp	PHyp (PepT1**)	Aito-Inoue et al., 2007
			PHyp		
Egg proteins	ACE inhibitor	YPI	YPI	YPI* (PepT1**, 0.4%***)	Miguel et al., 2008
		RPPGFSPFR	RPPGFSPFR	RPPGFSPFR	
		FRADHPFL	FRADHPFL, RADHPFL, ADHPFL, DHPFL	FRADHPFL, ADHPFL, DHPFL	
		RADHP	RADHP	-	
		YAEERYPIL	YAEERYPIL, EERYPIL	YAE, YPI*	
		YAEER	YAEER	YAEER	
DNA recombinant	ACE inhibitor	VLPVP	VLPVP	VLPVP* (paracellular diffusion and transport**, 0.25%***)	Lei et al., 2008

* Peptide showing ACE inhibitory activity, ** Peptide transport route, and *** Percentage of peptide transported.
(Aito-Inoue, Lackeyram, Fan, Sato, and Mine, 2007; Bejjani and Wu, 2013; Lei, Sun, Liu, Liu, and Li, 2008; Miguel et al., 2008; Quirós et al., 2008)

Table 2.11 Modification of bioactive peptides during transport through Caco-2 cell monolayer. (continued)

Parent protein	Bioactivity of peptide	Peptide	Parent and degraded peptide products		Reference
			Apical	Basal	
β-casomorphin	Opioid peptide	VLPVPQK	VLPVPQK	VLPVPQK (PepT1mediated/SOPT2**, 1.07%***)	Vij et al., 2016
Egg white ovalbumin	ACE inhibitor	QIGLF	QIGLF	QIGLF* (tight junctions**)	Ding et al., 2014
Ovotransferrin	ACE inhibitor	RVPSL	-	RVPSL (tight junctions**)	Ding et al., 2015
Fermented milk	ACE inhibitor	VPP	VPP,	VPP (1.93%***),	Makoto Satake et al., 2002
			PP	PP	
Lactoferricin	ACE inhibitor	RRWQWR	RRWQ, WR	RRWQ,	Fernández-Musoles et al., 2013
			RRW, QWR,	WQ	
		RWQ	RWQ, WQ	RWQ, WQ	
		WQ	WQ	WQ	
Rapeseed protein	Antioxidant Activities	YWDHNNPQI-R	WDHNNPQIR, DHNNPQIR, YWDHNNPQ	WDHNNPQIR*, DHNNPQIR*	Xu et al., 2017

* Peptide showing ACE inhibitory activity, ** Peptide transport route, and *** Percentage of peptide transported.
(Ding, Wang, Zhang, and Liu, 2015; Ding et al., 2014; Fernández-Musoles et al., 2013; Satake et al., 2002; Vij, Reddi, Kapila, and Kapila, 2016; Xu et al., 2017)

Table 2.11 Modification of bioactive peptides during transport through Caco-2 cell monolayer. (continued)

Parent protein	Bioactivity of peptide	Peptide	Parent and degraded peptide products		Reference
			Apical	Basal	
Dry-cured ham protein	ACE inhibitor	AAATP	AAATP, AATP, ATP,	AAAT, AAA, ATP	Gallego et al., 2016
		AAPLAP	AAPLAP PLAP	APLA, AAP PL, LA	
		KPVAAP	KPVAAP VAAP, KPV VA, AP, KP	KPVAAP VAAP, KPV VA, AP	
Milk protein	DPP-IV inhibitor	LPYPY	LPYPY, PYP, YPY, LP, PY, YP	LPYPY*, YPY*, LP*, PY*,YP*	Lacroix et al., 2017
		IPI	IPI, IP, PI	IPI*, IP*, PI*	
Tilapia muscle proteins	ACE inhibitor	GI digest		LLP*, AHL*, PQP*, MCS*, ALSC* (Overall peptide transported 14.8%***)	Toopcham et al., 2017
Pacific Hake protein	Antioxidative (ABTS radicals scavenging)	GI digest		2.8%*** Overall peptides transported	Samaranayaka et al., 2010
Chinese pimple milk	ACE inhibitor	GI digest		7.78%*** Overall peptides transported	Guang et al., 2012

* Peptide showed ACE inhibitory activity, ** Peptide transport route, and *** Percentage of peptide transport.

(Gallego et al., 2016; Guang et al., 2012; Isabelle ME et al., 2017; Samaranayaka et al., 2010; Toopcham et al., 2017)

The rate absorption of peptides in the small intestine is very low (Garcia-Redondo, Roque, Miguel, López-Fandiño, and Salaices, 2010; Regazzo, 2010). It has been indicated that a small portion of bioactive peptides can pass the intestine barrier and although it is usually too small to be considered nutritionally important (Gardner, 1988). The mechanism of transepithelial transport of oligopeptides includes passive and active transport processes as shown in **Figure 2.4** (Renukuntla, Vadlapudi, Patel, Boddu, and Mitra, 2013). Passive transport involves the diffusion of peptide molecules in the direction of concentration gradient (Gibaldi, 1977). Passive diffusion of peptides can be described by a combination of two processes: 1) paracellular transport (this route is diffusion of low molecular weight hydrophilic through the tight junctions between cells by energy independent passive diffusion process), and 2) transcellular transport (this route is ideal for lipophilic drugs which express relatively high affinity for the lipid bilayer of cell membrane). While, active transport involves the movement of peptide molecules against concentration gradient (i.e. from low to high concentration) by transmembrane proteins with expenditure of ATP molecules. Active transport includes carrier mediated transport (this mechanism involves the movement of small molecules, or macromolecules via membrane proteins transporters), and transporter (exit of some peptides from the enterocyte into the portal circulation via a peptide transporter (PepT1) located in intestinal basolateral membrane). Oligopeptides with 2–51 amino acids can cross the intestinal barrier intact with diferrent transport route and exhibit their biological functions at the tissue level (Roberts et al. 1999). Size of peptide also affects transport route., all 400 di- and 8,000 tripeptides can be actively transported by PepT1, irrespective of the amino acid sequence. Main pathway for peptides with molecular weight 0.5-1.6 kDa is paracellular route and their permeability were 9.54-

10.66%. In addition, brush border peptidases were responsible for peptide degradation in the paracellular and transcellular routes (Wang and Li, 2017). However, as the molecular weight of peptides increases, their chance to pass the intestinal barrier decreases (Roberts et al., 1999).

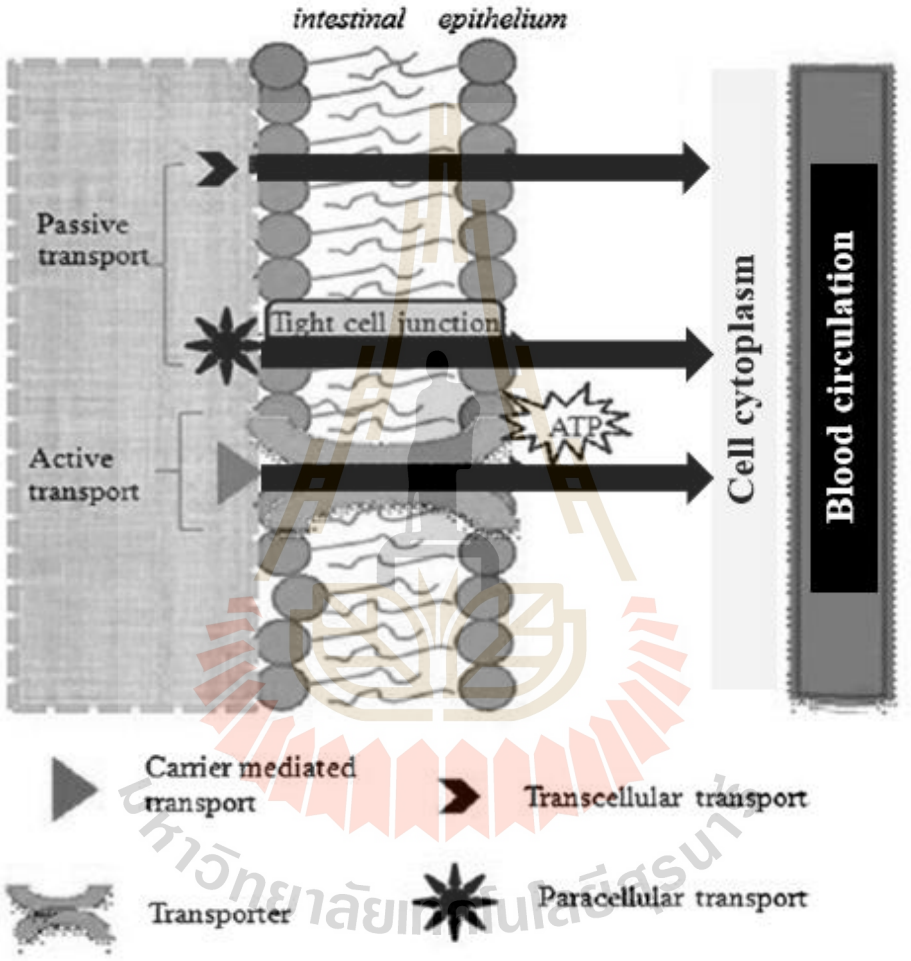


Figure 2.4. Mechanisms for intestinal transport of peptides.

Source: Modified from Renukuntla et al., 2013

Permeability of several intact ACE inhibitory peptides based on Caco-2 model has been reported to be less than 2%, however, it still shows an effective antihypertensive effect *in vivo* such as YPI and VPP (Miguel et al., 2008; Satake et al., 2002). Caco-2 cell monolayers are known to be tighter than mammalian intestinal

tissues (Boisset, Botham, Haegele, Lenfant, and Pachot, 2000), while brush border membrane- associated enzyme activities are generally lower (Bolte, Wolburg, Beuermann, Stocker, and Stern, 1998). Peptides that can transport across the intestinal wall into blood circulation are further hydrolyzed by blood peptidases, which reduce stability of some peptides in the blood to as little as one minute (Walsh et al., 2004). However, HLPLP was partially hydrolyzed and retained in human blood plasma for 2 h, indicating that peptide was resistant to blood proteases hydrolysis (Quirós et al., 2008).

2.6 Overview of inhibition mode and molecular docking of peptide

ACE inhibitory peptides from different food proteins display various inhibition mode are shown in **Table 2. 12**. Competitive and non- competitive inhibitors are commonly reported. The competitive inhibitor binds competitively with the substrate to the active site of ACE, while the non-competitive inhibitor can bind with an enzyme molecule to produce a dead- end complex, regardless of the bonding of a substrate (Laidler and Bunting, 1973). In the uncompetitive inhibitor, peptide binds only to a substrate-enzyme complex and decreases the maximum enzyme activity, so that it takes longer for the substrate or product to leave the active site. The mixed- mode inhibitor binds to ACE at either active or nonactive sites, in case of binding to the active site, it could hamper substrate binding while binding at nonactive site would lead to enzyme conformational changes that reduce substrate affinity (Laidler and Bunting, 1973).

The molecular docking simulation has been used to elucidate the molecular mechanisms of interactions between enzyme and inhibitors (Goodsell, 2009). This is a computational simulation that would provide insights on peptide- ACE interactions,

gaining a deeper understanding of the mechanism of ACE inhibition by peptides (Goodsell, 2009). To understand on how the most potent ACE inhibitory peptide binds to ACE, molecular docking simulation was performed based on kinetics results (mode of inhibition). More studies have been focused on the structure-activity relationship and the mechanism of peptide binding with ACE using molecular docking simulation such as YR and IR (Ko et al., 2017), WG and PRY (Fu, Alashi, Young, Therkildsen, and Aluko, 2017). Generally, the binding site of ACE-HHL complex involves 7 amino acid residues of Tyr523, His513, His353, Try520, Lys511, Gln281 and Ala354, while the catalytic site is composed of Glu384 and a zinc ion bonded with H383, H387 and E411(tri-coordination) (X. Wang, Wu, Xu, Xie, and Guo, 2011). It has been shown that the inhibitor combined with ACE through the interaction of hydrogen bonds, hydrophobic, van der Waals, electrostatic force and metal acceptor interaction (P. Li et al., 2014). Among these forces, hydrogen bonds plays the most important role for stabilizing the docking complex and enzyme catalytic reactions (Chaudhary, Vats, Chopra, Biswas, and Pasha, 2009). ACE inhibitors including; captopril, enalaprilat, and lisinopril are bound to zinc ion, rendering significant conformational changes in ACE. Thus, displacement of zinc ion from the active site resulted in the inhibition of ACE (Ghassem, Arihara, and Babji, 2012; Masuyer, Schwager, Sturrock, Isaac, and Acharya, 2012). The docking simulation of TPTQQS binding with ACE showed that T1, T3, and Q4 are key amino acids for the non-competitive inhibition, these amino acids oriented the peptide onto the lid structure, keeping the peptide out of the active site of ACE (Ni, Li, Liu, and Hu, 2012). The docking results of VGPV and SSGP revealed that they interacted outside the active site of ACE (Fu et al., 2016; Q. Wu, Du, Jia, and Kuang, 2016). Many ACE peptide inhibitors have shown to contain

hydrophobic amino acids at third position (C-terminal end), which allowed interaction with the subsites S1, S1′, and S2′ of ACE. Generally, the S1’ and S2’ subsites are conserved and important subsites which are occupied by competitive inhibitors (Ni et al., 2012; Pina and Roque, 2009). S1 pocket includes A354, E384 and Y523 residues and S2 pocket includes Q281, H353, K511, H513 and Y520 residues, while S1′ contains E162 residue (Rohit, Sathisha, and Aparna, 2012). Thus, mode of inhibition and molecular docking would reveal interactions between ACE and a peptide inhibitor at the molecular level.

Table 2.12. Protein-derived ACE inhibitory peptides categorized by inhibition modes.

Source	Preparation	Peptide	K_i value (μM)	Reference
1. Competitive inhibitors				
- Cupuassu seed protein	Alcalase	LDNK	30.84	da Cruz et al., 2016
		MVVDKLF	13.83	
- Hen egg white lysozyme	Pepsin, α-chymotrypsin and trypsin	MKR	16.30	Rao et al., 2012
		RGY	52.10	
		VAW	2.40	
2. Non-competitive inhibitors				
- Salmon protein byproducts	Alcalase	VWDPPKFD	19.52	Ahn et al., 2012
- Cupuassu seed protein	Alcalase	FLEK	18.50	da Cruz et al., 2016
		MEKHS	13.31	
- Sea cucumber	Alcalase	CRQNTLGHNTQTSIAQ	210	Forghani et al., 2016
3. Un-competitive inhibitor				
- Hen egg white lysozyme	Papain and trypsin	FESNFNTQATNR	57.40	Asoodeh et al., 2012
4. Mixed mode inhibitor				
- Salmon protein byproducts	Alcalase	FEDYVPLSCF	10.48	Ahn et al., 2012
		FNVPLYE	77.84	
- Cupuassu seed protein	Alcalase	GSGKHVSP	0.74	da Cruz et al., 2016

(Ahn, Jeon, Kim, and Je, 2012; Asoodeh, Memarpoor Yazdi, and Chamani, 2012; da Cruz, Pimenta, de Melo, and Nascimento, 2016; Forghani et al., 2016; Rao et al., 2012)

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CHAPTER III

CHEMICAL AND CELLULAR ANTIOXIDANT ACTIVITYIES OF CHICKEN BREAST MUSCLE SUBJECTED TO VARIOUS THERMAL TRETMENTS FOLLOWED BY SIMULATED GASTROINTESTINAL DIGESTION

3.1 Abstract

The effect of thermal treatments on chemical and cellular antioxidant activities of chicken breasts subjected to *in vitro* gastrointestinal digestion was investigated. Breast of Korat crossbred chicken (Kc) and commercial broiler (Br) were cooked under various conditions, namely heating at 70 °C for 30 min (H-0.5) and 24 h (H-24), autoclaving (AC) at 121°C for 15 min (AC-15) and 60 min (AC-60). Protein digestibility decreased upon the extreme thermal treatment of AC-60. The H-0.5 improved metal chelating activity of Kc digest, FRAP, and anti-liposome oxidation of Br digest. Digest of Br/H-0.5 and Kc/AC-15 at 50 μg/mL exhibited the highest cytoprotective effect against tert-butyl hydroperoxide (TBHP)-induced oxidative damage of HepG2 cells. In addition, the Kc/AC-15 digest at a concentration as low as 12.5 μg/mL inhibited intracellular TBHP-induced reactive oxygen species (ROS)

production ($P < 0.05$). Thus, the digest of Kc breasts subjected to AC-15 provides not only nutritional value but also antioxidant activity at the cellular level.

Keywords: antioxidant activity, chicken, cooking, gastrointestinal digestion, HepG2 cell line

3.2 Introduction

The antioxidant properties of protein hydrolysates from various sources have been reported (Kong and Xiong, 2006; Nimalaratne, Bandara, and Wu, 2015; Wiriyaphan, Chitsomboon, Roytrakul, and Yongsawadigul, 2013). Most studies have focused on the production of antioxidant peptides using commercial or new sources of proteases (Udenigwe and Aluko, 2012). Antioxidant peptides released upon human gastrointestinal (GI) digestion could also play an important role in human health. Consumption of muscle foods not only provides nutritional value in the form of amino acids but also yields bioactive peptides. However, encrypted bioactive peptides released upon digestion have not been as well studied as their hydrolysate counterparts.

Consumption of broiler (Br) meat has been increasing annually and is estimated to reach 90.8 pounds per capital worldwide in 2016 (The National Chicken Council 2016). Chicken breast is a great source of nutrition with a high protein content and low fat content. Korat crossbred chickens (Kc) are a new crossbreed between the male line of Thai indigenous chickens and the female line of Br chickens. The new breed offer a firmer and chewier texture and contains less fat and higher quantities of collagen. It is thought that the muscle protein composition of Kcs and Brs may be different. It is not known if such differences would result in a different production of peptides upon GI digestion, leading to varied bioactive properties.

Measuring chemical antioxidant activity using radical scavenging assays may not always correlate with biological responses (Alamed, Chaiyasit, McClements, and Decker, 2009). Therefore, a cellular model might provide more relevant information on *in vivo* systems. The liver is particularly susceptible to toxic and oxidative stimulants because the portal vein brings blood to this organ after intestinal absorption. Thus far, only human HepG2 cells have been used for biochemical, pharmacological, and nutritional studies because they retain a hepatocytic morphology and most of the functional properties of hepatocytes in culture (Alia et al., 2006). Any compound with the ability to protect against oxidation-induced cytotoxicity of HepG2 cells would be of significant value in various biological systems.

Thermal processing is mandatory for food preparation, especially for muscle foods. The degree of denaturation of these foods is significantly correlated with time and temperature applied (Torreggiani et al., 2008). Mild heating (60 to 80 °C) induces denaturation of myofibrillar proteins and collagen, resulting in enhanced accessibility of gastrointestinal proteases (Bax et al., 2012). Cooking meat for extended period for up to 24 h at a mild heating condition significantly increased tenderness due to solubilizing of collagen to gelatin and proteolysis of myofibrillar proteins (Bouton and Harris, 1981). The slow heating regime is an underlying process of various meat cooking methods, including braising, stewing, and sous vide processing (Baldwin 2012). The extent of protein denaturation of prolonged heating would be different from that of meat cooked at shorter time. In addition, extreme thermal treatment over 100 °C applied in the canning process induces degradation of peptide bonds, protein aggregation via hydrophobic interactions, and covalent cross-linkings. The formation of protein carbonyls and the modification of aromatic amino acids can also take place

(Soladoye, Juárez, Aalhus, Shand, and Estévez, 2015). Such changes of muscle proteins would inevitably affect the extent of GI digestion, resulting in varied degrees of digestibility and types of peptides formed. Bioactive properties, in particular antioxidant activity, of released peptides can also vary with the degree of thermal treatment applied. The objective of this study was to investigate the effect of thermal processes on protein digestibility of chicken breasts from 2 different breeds, Kc and Br. In addition, antioxidant activities based on applied thermal treatments and chemical and biological assays of digest from both breeds were elucidated.

3.3 Materials and methods

3.3.1 Materials

Whole breasts of Br were purchased from a local supermarket at Nakhon Ratchasima and Kcs were obtained from the University Farm of Suranaree Univ. of Technology, Thailand. The age of the commercial Br was approximately 42 d with live weight of 1.8 to 2.0 kg. The Kcs were slaughtered at 70 d with live weight of 1.15 to 1.25 kg. Breast muscles used in the study were 24 h postmortem. Samples were cut into pieces of 2 × 2 × 2 cm, packed in nylon/polyethylene laminated bags, and cooked under various treatments including heating at 70 °C for 0.5 h (H-0.5) and 24 h (H-24) using a water bath (SAP12; Grant Instrument Ltd., Cambridgeshire, U.K.) and autoclaving (MLS-3020; Panasonic Corp., Tokyo, Japan) at 15 psi and 121 °C for 15 min (AC-15) and 60 min (AC-60). The heating regime of H-24 reflected a long cooking time used in stewing or long sous vide process (Baldwin, 2012). It should be noted that the AC-60 treatment was not commercially practiced, but it was intended to demonstrate the effect of extremely severe thermal treatment. After each thermal treatment, samples were

immediately cooled on ice for 10 min. All samples (both raw and cooked forms) were stored at −20 °C and analyzed within 3 d.

2,2′-Azinobis (3-ethyl-benzothiazoline-6-sulfonate [ABTS]) and 2,4,6-tripyridyl-s-triazine (TPTZ) were purchased from Biochemika (Buchs, Switzerland). Pepsin from porcine stomach mucosa, pancreatic enzyme, trinitrobenzenesulfonic acid (TNBS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), L-α-phosphatidylcholine from soybean, 2-deoxy d-ribose, amino acid standards, dichloro-dihydro-fluorescein diacetate (DCFH-DA), and tert-butyl hydroperoxide (TBHP) were purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). The HepG2 human hepatoma cell line was obtained from the American Type Culture Collection (Manassas, Va., U.S.A.). Dulbecco's Modified Eagle Medium (DMEM) was purchased from Gibco BRL, Life Technologies (Gaithersburg, Md., U.S.A.). Other chemicals and reagents were of analytical grade.

3.3.2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970). Raw and cooked samples (1 g wet weight) were homogenized using an IKA homogenizer (IKA Works Asia, Bhd, Malaysia) in 19 mL of a 5% SDS solution for 5 min and heated at 95 °C for 10 min. Samples were then centrifuged at 10000 x g for 10 min. Protein (15 μg) was loaded onto 4% stacking and 10% running acrylamide gels. Gels were run at a constant voltage of 120 V, stained with 0.125% Coomassie brilliant blue R-250, and destained in a solution containing 25% ethanol and 10% acetic acid.

3.3.3 Preparation of *in vitro* GI digestion

In vitro digestion was carried out according to Garrett et al. (1999) with slight modifications. Briefly, raw or cooked samples (5 g dry weight) were homogenized in distilled water (250 mL) using an IKA homogenizer (IKA Works Asia) for 1 min and adjusted to pH 2.0 with 5 M HCl. Pepsin was added (2.86% [w/w], based on dry substrate), and the samples were incubated in a shaking incubator at 37 °C for 1 h. The pH was adjusted to 5.3 with a 0.9 M $NaHCO_3$ solution and then further to pH 7.5 with 5 M NaOH. Pancreatin was then added (4.00% [w/w], based on dry substrate), and the samples were further incubated under the same conditions for 2 h. Subsequently, all digested samples were submerged in a 95 °C water bath for 10 min, cooled on ice to room temperature, and centrifuged at 10000 x g for 10 min. The peptide content of the supernatant was determined using the Lowry method with tyrosine as a standard (Wiriyaphan et al., 2013). The degree of digestibility was determined based on TNBS according to Adler-Nissen (1979) using leucine as a standard and calculated using the following equation:

$$\textbf{Digestibility}\ (\%) = \left(\frac{LA_3 - LA_0}{LA_t}\right) X\ 100\%$$

where LA_3 is the α-amino content of the digest, LA_o is the α-amino content of the sample at time 0 and LA_t is the total α-amino content of raw or cooked samples obtained from hydrolyzing the samples with 6 M HCl at 120°C for 24 h.

3.3.4 Antioxidant activities

3.3.4.1 ABTS radical scavenging activity assay

ABTS radical scavenging activity assays were performed according to Arnao et al. (2001) with some modifications. Twenty microliters of diluted digest (0.1 mg/mL) was added to 1980 µL of fresh ABTS solution. The mixture was then incubated in the

dark for 5 min, and absorbance was measured at 734 nm. Trolox was used as a standard, and the antioxidant activity is expressed as mM Trolox equivalents (TE; Kong and Xiong 2006).

3.3.4.2 Ferric-reducing antioxidant power assay (FRAP assay)

FRAP assays were performed according to Benzie and Strain (1996) with some modifications. One hundred microliters of diluted digest (0.1 mg/mL) was added to 1 mL of fresh FRAP reagent. The mixture was then incubated in a water bath at 37 °C for 15 min, and absorbance was measured at 593 nm. Trolox was used as a standard. The reducing ability of the samples is expressed as mM TE.

3.3.4.3 Metal chelating activity

The ability of digest to chelate ferrous ions (Fe^{2+}) was assessed according to Decker and Welch (1990). One hundred microliters of digest (0.1 mg/mL) was mixed with 2.4 mL of distilled water. The mixture was then reacted with 50 μL of 2 mM $FeCl_2$ and 100 μL of 5 mM 3-(2-pyridyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4-triazine (ferrozine) for 20 min at room temperature in the dark, and absorbance was measured at 562 nm. The results are expressed as mM ethylenediaminetetraacetic acid (EDTA) equivalents.

3.3.4.4 Inhibition of β-carotene bleaching

A β-carotene emulsion was prepared according to Koleva et al. (2002) with some modifications. β-Carotene (0.5 mg) was mixed with 1 mL of chloroform, 25 μL of linoleic acid, and 200 μL of Tween-40. Chloroform was evaporated under vacuum at 45 °C, and the β-carotene mixture was resolved with 100 mL of distilled water. One hundred microliters of digest (0.1 mg/mL) was mixed with 2 mL of the fresh β-carotene emulsion and incubated at 50 °C for 2 h in a water bath; absorbance was measured at

470 nm. The oxidation of β-carotene was monitored by measuring a reduction of absorbance at 470 nm. Butylated hydroxyanisole at 0.01% was used as a positive control.

3.3.4.5 Hydroxyl radical-scavenging activity

Hydroxyl radicals generated by a Fenton reaction were assayed using the 2-deoxyribose oxidation method according to Sakanaka and Tachibana (2006) with slight modifications. One hundred microliters of digest (0.1 mg/mL) was mixed with 0.45 mL of 0.2 M sodium phosphate buffer (pH 7.4), 0.15 mL of 10 mM 2-deoxyribose, 0.15 mL of 10 mM $FeSO_4$–EDTA, 0.15 mL of 10 mM hydrogen peroxide (H_2O_2), and 0.5 mL of deionized water (DI). The reaction was incubated in a 37 °C water bath for 4 h. Subsequently, 0.75 mL of 2.8% trichloroacetic acid (TCA) and 0.75 mL of 1.0% thiobarbituric acid (TBA) were added. The mixture was boiled for 10 min and cooled on ice. The absorbance of the reaction was measured at 532 nm. The control was prepared as described above except that DI water was used instead of the peptide solution. The degree of scavenging activity was calculated as follows:

$$\textbf{Scavenging activity}\ (\%) = \frac{(\text{Absorbance of control} - \text{Absorbance of sample})}{\text{Absorbance of control}} \text{ x } 100$$

3.3.4.6 Anti-liposome oxidation

Lecithin liposome was prepared according to the method of Yi et al. (1997). Lecithin was suspended in DI water at a concentration of 8 mg/mL, and the suspension was sonicated (Elma, Model D-78224, Singen, Germany) for 30 min. Digest (15 μg) was dissolved in 15 mL of liposome solution and sonicated for 2 min. The reaction was initiated by adding 20 mL of 0.15 M cupric acetate and incubating at 37 °C in the dark with a shaking speed of 120 rpm (Julabo, Model SW22, Seelbach, Germany). Oxidation

of the lecithin liposome system was monitored at 0, 2, 4, 6, 8, 10, 20, 40, and 60 h by measuring the formation of thiobarbituric acid reactive substances (TBARS) and conjugated dienes (CD). Trolox (1 mg/mL) was used as a positive control and DI water was used as a negative control.

TBARS measurements were performed as described by Buege and Aust (1978) with slight modifications. The liposome sample (0.5 mL) was mixed with 2.5 mL of TBARS solution (0.375% TBA and 15% TCA in 0.25 M HCl). The mixture was homogenized, boiled for 10 min and cooled on ice for 1 min. After centrifugation at 5000 × g for 10 min (5415C, Eppendorf, Hamburg, Germany), the optical density of the supernatant was measured at 532 nm. TBARS values are expressed as mg MDA equivalent/mL of liposome oxidation. The CD values of liposome oxidation were measured at 234 nm as detailed by Frankel et al. (1997).

3.3.5 Cellular antioxidation

3.3.5.1 Cytotoxicity effect of digest on HepG2 cells

HepG2 cells were grown in DMEM containing 10% fetal bovine serum (FBS), N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES) buffer pH 7.4 (15 mM), penicillin (100 U/mL), and streptomycin (100 µg/ mL). Cells were incubated at 37 °C in 5% CO_2 with 95% relative humidity and subcultured by trypsinization when they reached confluence. Cells were incubated with Kc/AC-15 digest at concentrations ranging from 12.5 to 1,000 µg/mL at 37 °C in 5% CO_2 for 24 h, followed by addition of 100 µL of 0.5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and incubation for another 2.5 h. Subsequently, the cells were centrifuged at 2000 rpm and 4 °C for 5 min. The culture medium was discarded and dimethyl sulfoxide (DMSO) was added at 100 µL/well. The absorbance was measured at 540

nm. The results are expressed as percentage of viable cells compared to the control sample (without digest).

3.3.5.2 Determination of TBHP-induced cytotoxicity on HepG2 cells

Cytotoxicity of TBHP (0 to 1200 μM) on HepG2 cells was determined. Cells were grown in a 96-well plate (3.0×10^4 cells/well) as described above for 24 h. Various concentrations of TBHP were then added, and the cells were incubated for 2 h. The culture medium was removed and replaced with fresh medium containing 100 μL of 0.5 mg/mL MTT and then further incubated for 2.5 h. Subsequently, the cells were centrifuged at 2000 rpm and 4 °C for 5 min, the culture medium was discarded and DMSO was added at 100 μL/well. Absorbance was monitored at 540 nm by a Benchmark Plus Microplate Spectrophotometer System (Bio-Rad Laboratories, Inc., Hercules, Calif., U.S.A.). The results are expressed as percentage of viable cells compared to the control sample (without TBHP).

3.3.5.3 Protective effect of digest samples on TBHP-induced cytotoxicity of HepG2 cells

HepG2 cells grown in a 96-well plate as described above were incubated with digest samples at various concentrations (0, 250, 500, 750, and 1000 μg/mL) dissolved in DMEM supplemented with 10% FBS for 24 h. The fresh culture medium containing 500 μM TBHP was replaced, and cells were further incubated for 2 h. Cell viability was determined using MTT assays as described above. The results are expressed as a percentage of viable cells compared to the control (without TBHP).

3.3.5.4 Cellular ROS by DCFH-DA

Detection of intracellular formation of ROS was performed using the oxidation-sensitive dye DCFH-DA as a substrate according to Qian et al. (2008) with slight

modifications. The diffused DCFH-DA was deacetylated by cellular esterases to DCFH, which was oxidized by ROS to form the highly fluorescent 2′,7′-dichlorofluorescin (DCF). HepG2 cells grown in a 96-well plate for 24 h were preincubated with digest at varying concentrations of 0, 12.5, 50, 100, 250, and 500 μg/mL in DMEM with 10% FBS for 6 h. The culture medium was replaced by 10 μM DCFH-DA in Hank's balanced salt solution (HBSS) and incubated in the dark for 30 min. The cells were then washed 3 times in PBS and further incubated with 500 μM TBHP in HBSS for 1 h. The fluorescence intensity proportional to the ROS levels within the cell cytosol was monitored at an excitation wavelength (Ex) of 485 nm and an emission wavelength (Em) of 530 nm using a Spectramax Gemini EM fluorescence microplate reader (Molecular Devices, Inc., Sunnyvale, Calif., U.S.A.).

3.3.6 Statistical analyses

All tests were performed in triplicate and the results were analyzed by one-way analysis of variance. Duncan's new multiple range tests were carried out to determine the significance of differences within a 95% confidence interval using SPSS 17.0 software (SPSS Inc., Chicago, Ill., U.S.A.).

3.4 Results and Discussion

3.4.1 SDS-PAGE pattern

Raw chicken breasts from both breeds showed typical SDS-PAGE patterns of muscle protein separation with major bands representing myosin heavy chain (MHC) and actin at 220 and 42 kDa, respectively (**Figure 3.1a and b**). Comparable protein patterns to raw were also observed in the sample subjected to H-0.5. However, degradation of MHC was evident in samples treated with H-24. Prolonged cooking time (H-24) resulted in severe degradation of MHC similar to the AC-15 treatment.

Degradation of actin was also obvious with some retentions for both treatments. Prolonged heating time does not only destroy integrity of muscle protein, but also meat palatability. Chicken *Pectoralis profundus* heated at 58 °C for 17 h showed less juiciness than that heated for 6 h, although tenderness of samples was comparable (Christensen et al., 2012). Disintegration of muscle proteins would result in less protein binding capacity, which, in turn, reduced juiciness. Complete loss of MHC, actin, and other muscle proteins occurred when a temperature of 121 °C was applied for 60 min (AC-60). Disintegration of muscle proteins subjected to mild temperature (70 °C) for long time (H-24) or high thermal treatments (AC-15, AC-60) observed in chicken of both breeds (**Figure 3.1a and b**) was in agreement with the study of Wen et al. (2015) who reported almost loss of various muscle proteins of pork heated at 100 °C for 3 h. Our study also indicated that thermal aggregation of degraded muscle proteins was observed as seen by the retention of higher molecular weight proteins at the top of the stacking gel. This finding indicates that extreme thermal treatments induced muscle protein degradation and modification.

Thermal treatments can lead to modifications of amino acid side chains, polymerization, and aggregation of the protein. Under high thermal treatment, free radicals can react with the protein at a specific site, resulting in fragmentation. Oxidation of glutamyl and aspartyl residues in proteins can lead to peptide bond cleavages, forming N-pyruvyl derivatives, and oxidation of proline residues leads to detectable 4-aminobutyric acid, which is derived from 2-pyrrolidone, an immediate derivative of the oxidation process (Uchida, Kato, and Kawakishi, 1990). Cross-linking modifications, including dityrosine bonds, and Schiff base formation between side chains of lysine with histidine, commonly occur as a result of thermal treatment. In

addition, disulfide bond formation was found to increase in chicken breasts cooked at 60 to 100 °C for 50 min (Cui, Zhou, Zhao, and Yang, 2009). Such modifications would explain aggregation and degradation observed by SDS-PAGE analysis with samples subjected to 121 °C treatment (AC-15 and AC-60 min).

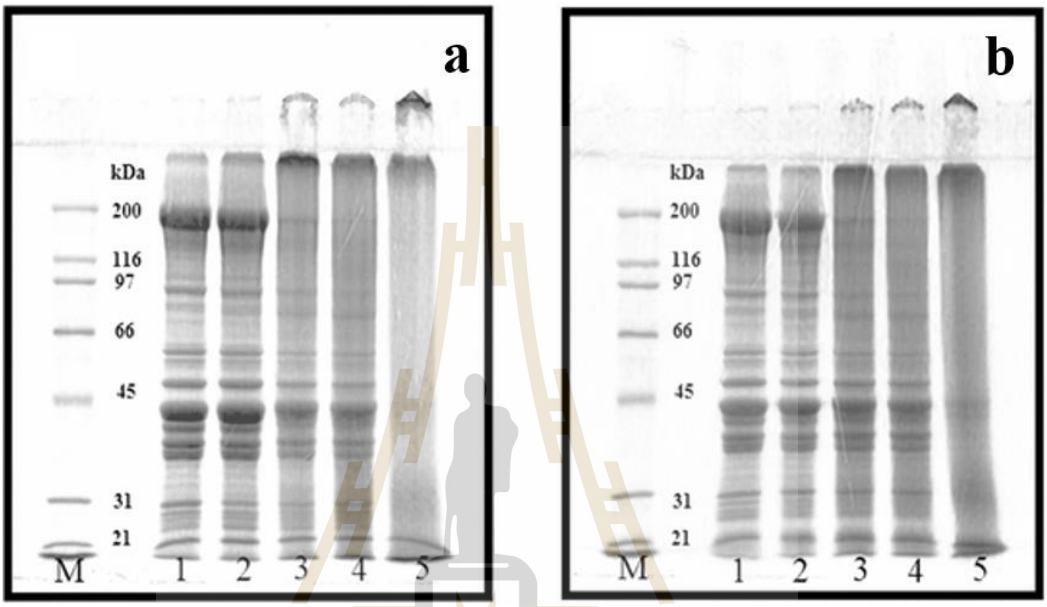

Figure 3.1 SDS-PAGE (10% acrylamide) patterns of Korat crossbred chicken (a) and commercial broiler (b) breasts under various thermal treatments. (M) protein standard markers, (1) raw, (2) 70 °C for 30 min, (3) 70 °C for 24 h, (4) 121 °C for 15 min, and (5) 121 °C for 60 min.

3.4.2 Degree of *in vitro* digestion (DH)

Digestibility in both chicken breeds was in the range of 39% to 55% (**Figure 3.2**). In both breeds, raw and samples heated by H-0.5, H-24 and AC-15 resulted in comparable digestibility ($P > 0.05$), although the extreme thermal treatment (AC-60) of Br led to the lowest digestion ($P < 0.05$; **Figure 3.2**). Protein aggregation and disintegration induced by H-0.5, H-24 and AC-15 as observed on SDS-PAGE (**Figure 3.1**) did not

appear to affect accessibility and hydrolysis of GI proteases. However, larger aggregates of the AC-60 sample that appeared on the top of 4% stacking gel limited the digestibility by GI proteases (**Figure 3.1 and 3.2**). A reduction of *in vitro* digestibility was also observed when pork was heated at 100 °C for 3 h as compared to that heated at 60 to 70 °C for up to 30 min (Wen et al., 2015). High thermal treatments over 100 °C have been found to generate ROS, leading to several amino acid side chain modifications including carbonylation, caboxylation, nitrosylation, chlorination, and hydroxylation (Traore et al., 2012). Carbonylation of arginine and lysine due to

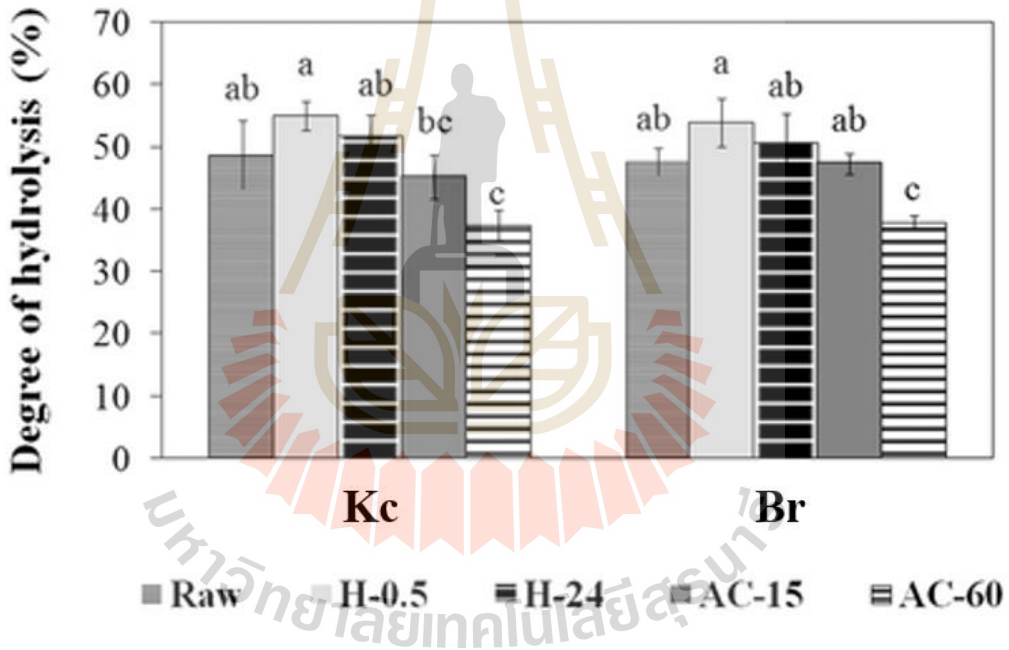

Figure 3.2 Protein digestibility of chicken breasts subjected to various thermal treatments. H; heating at 70 °C. AC; autoclaving at 121 °C. Kc, Korat crossbred chicken; Br, commercial broiler; H-0.5, heated at 70 °C for 30 min; H-24, heated 70 °C for 24 h; AC-15, autoclaved at 121 °C for 15 min; AC-60, autoclaved at 121 °C for 60 min. Bars with different letters within the breed indicate mean with significant differences ($P < 0.05$).

oxidation resulted in a resistance to trypsin digestion because trypsin shows specificity toward arginine and lysine (Morzel, Gatellier, Sayd, Renerre, and Laville, 2006). An increase in protein carbonyl content has been reported in myofibrillar protein subjected to heat treatment over 90 °C (Gatellier, Kondjoyan, Portanguen, and Santé-Lhoutellier, 2010). Therefore, the extreme thermal treatment (AC-60) of chicken breast would likely limit the protein digestibility.

3.4.3 Antioxidant activity of digest samples

The antioxidant activities of cooked digest samples varied with thermal treatments and tested methods. Digest samples of cooked chicken breast were able to scavenge ABTS radicals (**Figure 3.3a**). The AC-60 resulted in GI-digested peptides with less scavenging activity in Kc samples ($P < 0.05$). These results imply that peptides released by cooked chicken breast still exhibit a hydrogen-donating ability upon GI digestion and mild heating (70 °C) appeared to be more favorable in yielding ABTS radical scavenging peptides. Amino acids including Trp, Tyr, Cys, and His, which contain indole, phenol, thiol, and imidazole groups, respectively, have been reported to be hydrogen donors (Aliaga and Lissi, 2000).These 4 amino acids constitute 7% to 13% of the total protein in chicken breasts (Wattanachant, Benjakul, and Ledward, 2004). Thus, these amino acids could contribute to ABTS activity of chicken breast digest. You and Wu (2011) also found that egg white and egg yolk hydrolyzed by pepsin/pancreatin showed higher antioxidant activities than those hydrolyzed by non-gastrointestinal enzymes. Peptides with molecular weight less than 500 Da showed higher antioxidant activity than larger peptides (You and Wu, 2011). Gastrointestinal digestion of chicken breast likely results in small peptides with specific amino acids exerting hydrogen-donating ability.

The H-0.5 improved the chelation ability of Kc digest ($P < 0.05$). In contrast, the metal chelating ability of digest drastically decreased when extreme thermal treatment was applied (**Figure 3.3b**). Acidic (Asp and Glu) and basic (His, Lys, and Arg) amino acids with carboxyl and amino groups in the side chain positions, respectively, are thought to play an important role in chelating metal ions (Saiga, Tanabe, and Nishimura, 2003). The extent of digestibility did not correlate with metal chelating activity, implying that size of peptide is not the main factor governing its chelating ability.

All thermal treatments increased the FRAP values of both Kc and Br digest samples. Notably, extreme thermal treatment of AC-15 improved the reducing power of Kc digest, but the opposite trend was observed in Br counterparts (**Figure 3.3c**).This implies that the extent of thermal denaturation and aggregation between the 2 breeds was different, resulting in varied peptides upon GI digestion. In raw digests, Kc samples exhibited a higher reducing power than Br samples, but after thermal treatment of H-0.5, Br digest showed the highest activity at twice that of raw digest ($P < 0.05$). The FRAP assay is based on an electron transfer mechanism that donates one electron to Fe^{3+}, reducing it to Fe^{2+} in acidic conditions, whereas the ABTS radical-scavenging assay involves both electron and hydrogen atom transfers (Huang, Ou, and Prior, 2005). Thus, the mild thermal treatment (H-0.5) applied to Br samples led to the release of peptides with a high electron donating ability upon GI digestion.

The inhibition rate of β-carotene bleaching depends on the antioxidant activity of the tested samples. In general, digest of Br samples exhibited a higher β-carotene bleaching activity than that of Kc samples (**Figure 3.3d**). The extreme thermal treatment (AC-60) of Kc samples enhanced the β-carotene bleaching ability of its

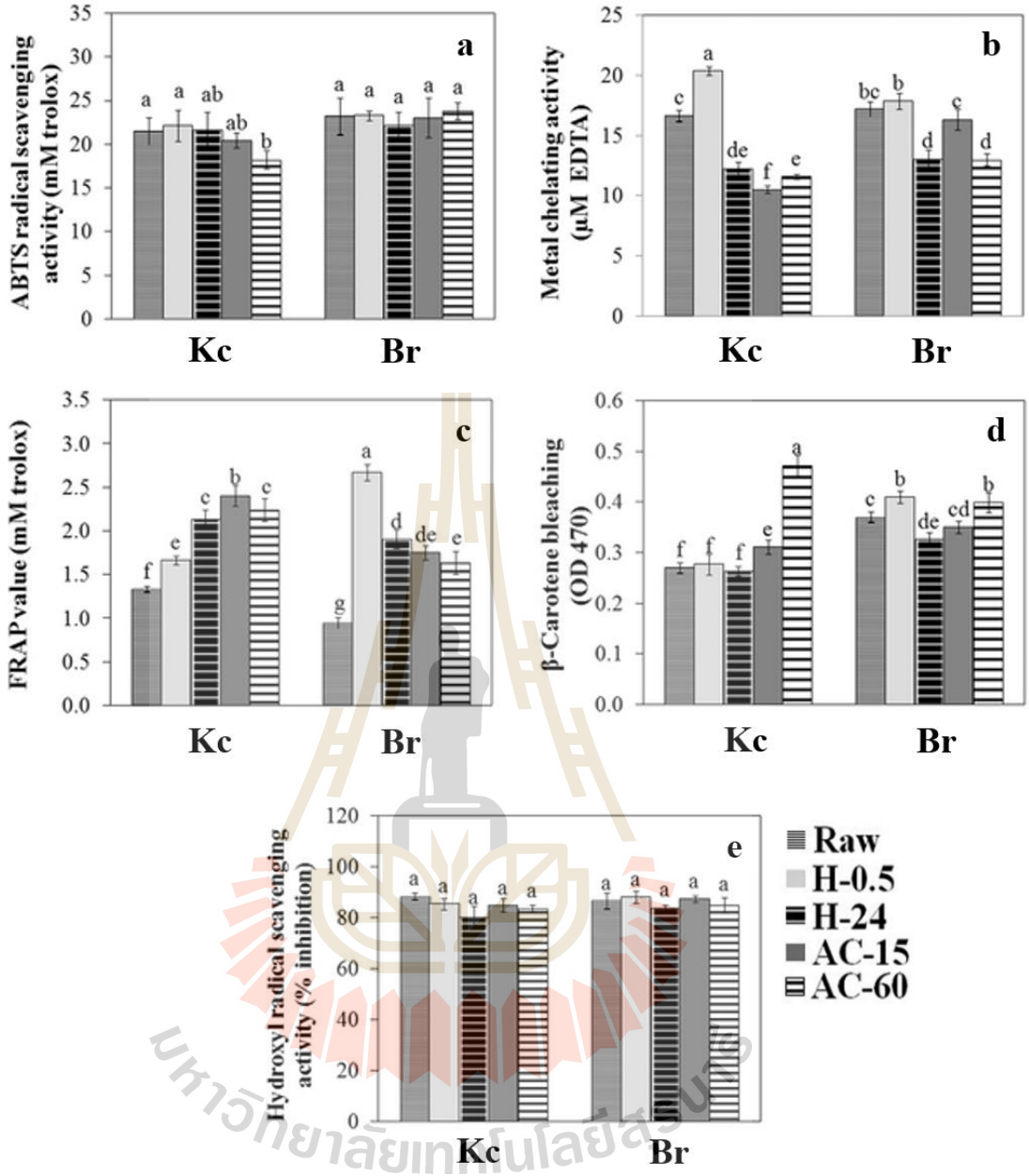

Figure 3.3 Antioxidant activities of digest samples (0.1 mg/mL) of chicken breasts subjected to various thermal treatments. (a) ABTS radical scavenging activity, (b) metal chelating activity, (c) ferric-reducing antioxidant power (FRAP), (d) hydroxyl radical scavenging activity, and (e) β-carotene bleaching ability. Values are expressed as the mean ± SD (n = 4). Abbreviations are the same as Figure 3.2. Bars with different letters indicate mean with significant differences ($P < 0.05$).

digest. Hydrophobic antioxidants typically exhibit a higher capacity in preventing oxidation in oil-in-water emulsion systems compared to hydrophilic counterparts (Wijeratne, Amarowicz, and Shahidi, 2006). Thus, it could be speculated that digest of Br samples may contain higher amounts of hydrophobic peptides as higher activity was observed compared to Kc counterparts. The AC-60 treatment of Kc could also result in a greater release of hydrophobic peptides upon GI digestion, rendering the highest β-carotene bleaching activity.

Hydroxyl radicals are the most reactive among the oxygen radicals that are capable of inducing severe damage to adjacent biomolecules; this damage includes aging, cancer, and other diseases (Jeong, Park, Lam, and de Lumen, 2003). The hydroxyl radical scavenging activity of the digest of all samples was comparable regardless of thermal treatment applied or breed ($P > 0.05$; **Figure 3.3e**). The most reactive amino acids interacting with hydroxyl radicals include the nucleophilic sulfur-containing amino acids (Cys and Met), the aromatic amino acids (Trp, Tyr, and Phe), and the imidazole-containing amino acid (His; Elias et al. 2008).

The ability of hydrolysate or peptides to protect against liposome oxidation has been previously demonstrated (Kansci, Genot, Meynier, Gaucheron, and Chobert, 2004). Liposomes are a great model in which to evaluate how oxidation of the phospholipid bilayer in cell membranes is counteracted (Frankel et al., 1997). In control samples, oxidation of liposomes increased with time and reached a maximum as denoted by CD and TBARS values taken at 10 and 40 h, respectively ($P < 0.05$; **Figure 3.4a to d**). The formation of dienes typically occurs at the early stages of lipid oxidation, although hydroperoxides are further decomposed to secondary products, malonaldehydes, at a later stage (Frankel et al., 1997). All digests delayed liposome

oxidation. In digest obtained from raw samples, Kc samples had greater inhibition of liposome oxidation than Br samples as measured by TBARS values. Thermal treatment appears to reduce the ability of peptides to protect against liposome oxidation. Digest of Br/H-0.5 samples had the highest inhibitory activity against liposome oxidation, although that obtained from Kc/AC-15 samples was the most effective based on TBARS values (**Figure 3.4c to d**). Our results indicate that thermal treatments and

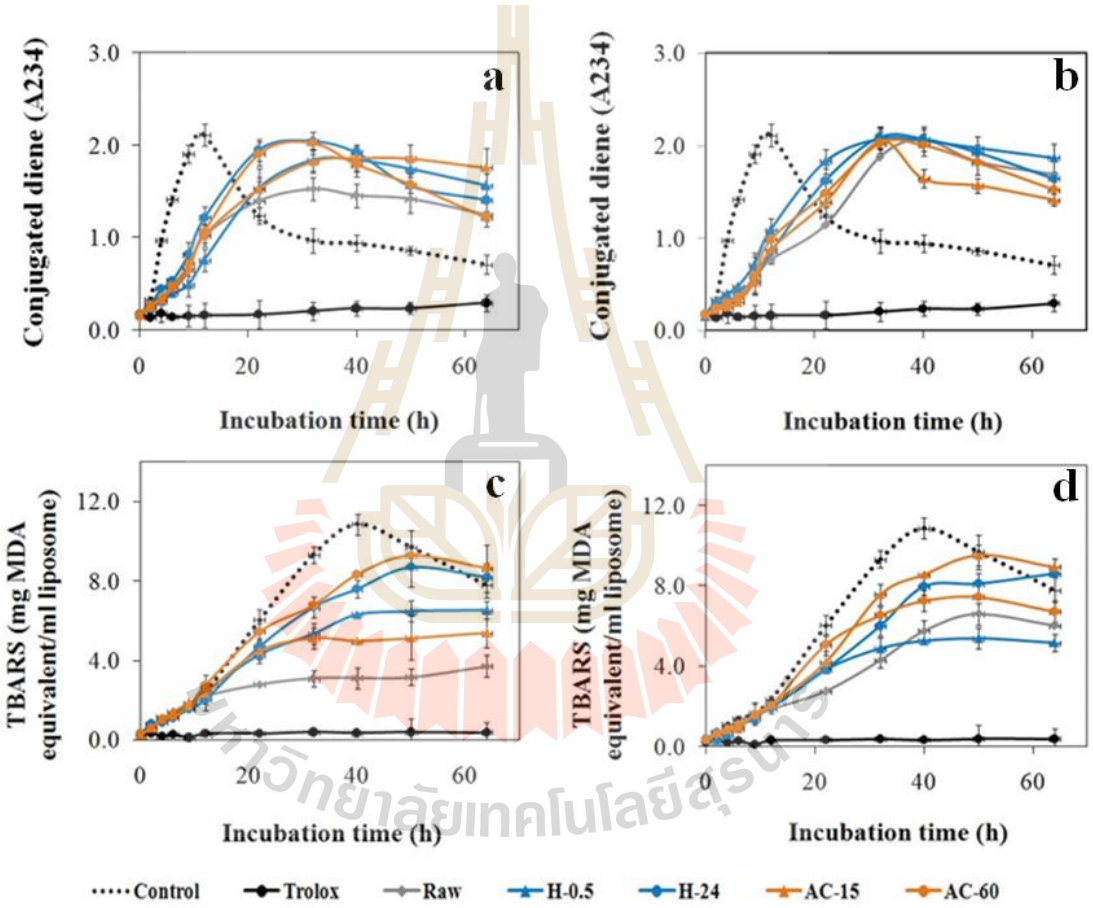

Figure 3.4 Antioxidant activity of digest samples of chicken breasts subjected to various thermal treatments in the lecithin–liposome system as measured by the formation of conjugated dienes (a: Kc, b: Br) and TBARS values (c: Kc, d: Br). Values are expressed as the mean ± SD (n = 4).

breed greatly affect the release of antioxidant peptides upon GI digestion. It should be noted that although the extreme thermal treatments (AC-15, AC-60) reduced the degree of digestibility, the peptides generated from *in vitro* GI digestion of such treatments possessed antioxidant activity in various modes.

3.4.4 Cytoprotective effect of digest samples

The effect of digest of Kc/AC-15 samples on HepG2 cells was determined at various peptide concentrations up to 1000 μg/mL. Cell viability of samples treated with 12.5 to 50.0 μg/mL was comparable to that of control (without digest) and higher viability was observed at digest concentrations of 100 to 1000 μg/mL (**Figure 3.5a**).These results indicate a mitogenic effect of chicken breast digest at concentrations greater than 100 μg/mL. Similar findings have been reported with whey protein hydrolysate (Cross and Gill, 1999) and rice bran extract (Suzuki et al., 2013). In addition, growth factor–like molecules that induce a mitogenic effect were generated in protein hydrolysates from sardine waste (Ravallec-Plé et al., 2001).

All digests at 50 μg/mL had no cytotoxic or mitogenic effect on HepG2 cells. Thus, this concentration of Kc and Br digest was selected for further investigation of their cytoprotective effects on TBHP-induced ROS formation in HepG2 cells. At 500 μM TBHP, approximately 42.6% of cells underwent cell death in the control sample (**Figure 3.5b**). All digest samples at 50 μg/mL had a protective effect against TBHP-induced cytotoxicity. Our results demonstrate that chicken breast digest not only provides nutritional value by supplying amino acids but also displays antioxidant activity at the cellular level. It should be noted that cellular cytoprotective effects correlated well with FRAP assay results. TBHP-induced cytotoxicity of hepatocytes generated free radical intermediates through cytochrome P-450 activity, resulting in

cell injury, leakage of lactate dehydrogenase, and alanine amino transferase and breakage of DNA strands (Tseng, Wang, and Kao, 1996). Thus, the reducing ability of digest by electron transfers observed during FRAP assays may help to protect cells from TBHP-induced oxidative damages.

Although the raw digest of Br samples exhibited a greater cytoprotective effect than their Kc counterparts, thermal treatments boosted the cytoprotective effect of Kc samples, particularly Kc/AC-15 digest, which exhibited the greatest protective effect when compared to 200 μg/mL of α-tocopherol (**Figure 3.5b**). Digest of Br samples subjected to high thermal treatments (H-24, AC-15, and AC-60) had less of a cytoprotective effect than their respective Kc digests. High thermal treatments, particularly AC-15, appeared to be favorable for Kc breast to possess cellular antioxidant activity upon GI digestion. This confirmed that muscle structure and composition of these 2 breeds were varied.

As digest of Kc/AC-15 exhibited the highest efficacy, it was, thus, chosen for investigation of the intracellular ROS scavenging activity in HepG2 cells using a fluorescent DCFH-DA probe. The DCFH-DA fluorescent probe can be oxidized to form fluorescent DCF by intracellular ROS generated by TBHP. A decrease in fluorescence intensity indicated that cytoprotection of digest takes place intracellularly. The fluorescence intensity was reduced in a dose-dependent manner when cells were pretreated with Kc/AC-15 digest (**Figure 3.5c**). These findings suggest that Kc/AC-15 samples at concentrations as low as 12.5 μg/mL are able to inhibit intracellular TBHP-induced ROS production ($P < 0.05$). It should be noted that the efficacy of cytoprotection of Kc/AC-15 digest at 500 μg/mL was comparable to that of 200 μg/mL α-tocopherol ($P > 0.05$).

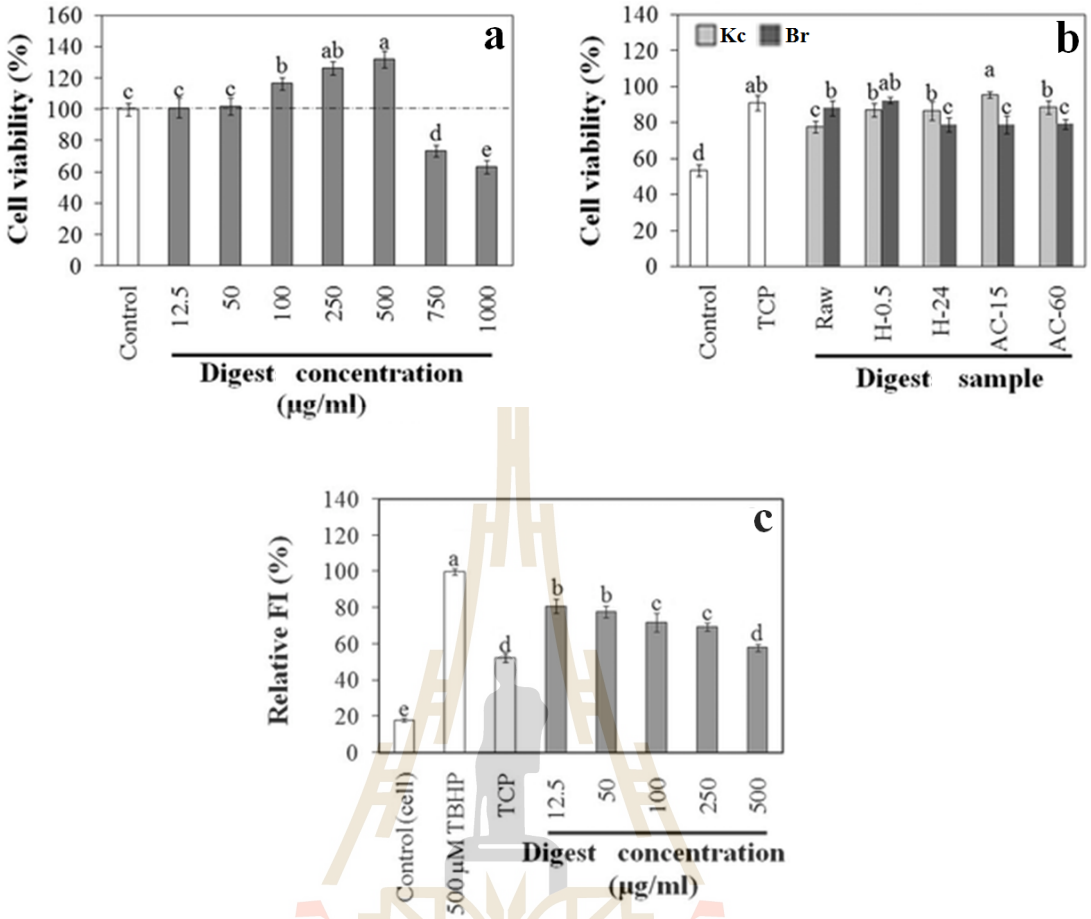

Figure 3.5 Cytoprotective effect and cellular radical scavenging activities of chicken breast digest samples on HepG2 cells. (a) cytotoxic effect of Kc/AC-15, (b) cytoprotective effect of digest against 500 µM TBHP-induced cytotoxicity, and (c) cellular radical scavenging activities of digest of Kc/AC-15 against 500 µM TBHP-induced cytotoxicity. TCP, α-tocopherol. Values are expressed as the mean ± SD (n = 4). Bars with different letters indicate significant differences ($P < 0.05$).

Aside from peptides generated from *in vitro* GI digestion, natural antioxidant peptides, namely carnosine, anserine, and homocarnosine, could contribute to observed antioxidant activity. Chan and Decker (1994) reported that anserine and carnosine

inhibited lipid oxidation through a combination of free radical-scavenging and metal chelation. However, heating at 70 or 100 °C caused significant loss of these dipeptides (Peiretti, Medana, Visentin, Dal Bello, and Meineri, 2012). This might partly explain a decrease in metal chelating activity observed in digests of cooked samples (**Figure 3.3b**). Peptides released upon *in vitro* GI digestion could play a more vital role in cytoprotection compared to these natural antioxidant peptides. Identification and characterization of antioxidant peptides released from cooked Kc breast by GI proteases deserve further investigation.

3.5 Conclusions

Upon *in vitro* GI digestion, chicken breast muscles release peptides, which exhibit antioxidant activity toward chemical radicals and cellular systems. Mild thermal treatment (H-0.5) promoted antioxidant activities of Br breast digest, although extreme thermal treatment of AC-60 yielded Kc digest with higher β-carotene bleaching and cytoprotective effect. The Br/H-0.5 and Kc/AC-15 digests, both at concentrations of 50 μg/mL, exhibited the greatest protective effect against TBHP-induced oxidative damage in HepG2 cells. The cytoprotection of Kc/AC-15 was mediated partly by intracellular ROS scavenging activity in a dose-related manner. Therefore, the Kc subjected to AC-15 could provide an excellent source of antioxidant peptides upon GI digestion, which could exert some health benefits in addition to its nutritional value.

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CHAPTER IV

ANGIOTENSIN CONVERTING ENZYME (ACE) INHIBITORY PEPTIDE DERIVED FROM THE SIMULATED *IN VITRO* GASTROINTESTINAL DIGESSTION OF COOKED CHICKEN BREAST

4.1 Abstract

The release of encrypted angiotensin converting enzyme (ACE) inhibitory peptides upon simulated *in vitro* gastrointestinal digestion of Korat crossbred (Kc) and commercial broiler (Br) breast meat varied with cooking methods. Digests of Kc breast meat heated at 70 °C for 0.5 h (H-0.5) showed the highest ACE inhibitory activity, while digests of Kc chicken heated at 121 °C for 1 h showed the lowest inhibitory activity. ACE inhibitory activity varied with the protein digestibility. After a series of chromatographic techniques, we identified KPLLCS, ELFTT, and KPLL, novel potent ACE inhibitor peptides whose sequences were homologous to that of myosin and whose IC_{50} values were 0.37, 6.35 and 11.98 μM, respectively. ELFTT and KPLL were resistant to plasmin hydrolysis for up to 60 min. Therefore, Kc chicken meat heated at H-0.5 could serve as a source of ACE inhibitory peptides upon gastrointestinal digestion.

Keywords: Chicken breast, *in vitro* digestion, bioactive peptide, angiotensin converting enzyme

4.2 Introduction

Muscle foods are one of the important sources of protein in the diet. Beyond their nutritional properties, muscle proteins provide bioactive peptides that are released upon digestion. The angiotensin converting enzyme (ACE) inhibitory activity of protein hydrolysates derived from muscle foods has been extensively reported based on the use of various proteases, including alcalase, thermolysin, Aspergillus protease, neutrase, pronase E and gastric proteases (trypsin and chymotrypsin) (Bhat, Kumar, and Bhat, 2015; Ryan, Ross, Bolton, Fitzgerald, and Stanton, 2011). The release of ACE inhibitory peptides upon GI digestion has been reported to a lesser extent and only focuses on raw meat (Escudero, Sentandreu, Arihara, and Toldrá, 2010). This has no relevance to health benefits since raw meat is not commonly consumed due to food safety and palatability concerns. ACE inhibitory peptides generated from the GI digestion of cooked meat have not been systematically elucidated.

The time and temperature at which meat is cooked greatly affect the extent of protein denaturation that occurs and the accessibility of GI enzymes to break down the meat into varied bioactive peptides. Mild thermal treatment (60–80 °C) induces protein unfolding, thus enhancing protease susceptibility ((Bax et al., 2012; Soladoye, Juárez, Aalhus, Shand, and Estévez, 2015). In contrast, extreme thermal heating (>100 °C) leads to irreversible unfolding, aggregation, disulfide interchanges, protein fragmentation and chemical modifications of side-chains, including sulfoxidation, carbonylation, hydroxylation, nitrosylation, and glutathiolation (Davis and Williams,

1998; Soladoye et al., 2015). Such modification would lead to alteration of the enzyme recognition sites. For example, trypsin, a serine endopeptidase that hydrolyzes proteins specifically at the carboxyl side of arginine and lysine, will have a reduced activity if these amino acids are carbonylated (Morzel, Gatellier, Sayd, Renerre, and Laville, 2006). The peptide profiles of pork samples cooked at 60–100 °C and GI digested were different (Wen et al., 2015). We assume that the ACE inhibitory activity varies with the type of heating regime used to cook the meat.

Chicken breast is a great source of dietary protein, and consumption of chicken meat has been increasing every year (The National Chicken Council 2016). The Korat crossbred (Kc) chicken is a new breed that is a cross between a male Thai indigenous chicken and a female broiler. The muscle composition between a Br and a Kc chicken is expected to be different due to differences in age (45 days for Br vs. 70 days for Kc) and breed (pure line vs. hybrid). It is reasonable to assume that the digest from both types of chicken would contain different peptides which could lead to varied ACE inhibitory activity. ACE inhibitory peptides generated from the GI digestion of cooked chicken meat has never been identified and characterized. The identification of bioactive peptides could shed some light on the protein structure-function relationship. The objectives of this study were to investigate and identify the ACE inhibitory activity of digest obtained upon simulated gastrointestinal digestion of cooked chicken breasts and identify and characterize the potent peptides.

4.3 Materials and methods

4.3.1 Materials

A mix of 20 male and female Kc and Br chickens were used for the experiment. Seventy-day-old Kc chickens were obtained from the University Farm of Suranaree University of Technology, Thailand with a mean carcass weight of 1.2±0.5 kg. Br chickens aged 45-day-old were purchased from Charoen Pokphand Foods Co. (Nakhon Ratchasima, Thailand) with a mean carcass weight of 1.5±0.2 kg. A breast was removed from each carcass 3 h postmortem and stored at 4°C for 24 h. The breast muscle was cut into pieces of 2x2x2 cm pieces, and 6 pieces were packed into each nylon vacuum bag. Samples were heated at 70 °C for 0.5 h (H-0.5) or 24 h (H-24) or autoclaved at 15 psi and 121 °C for 15 min (AC-15) or 60 min (AC-60) using an autoclave (Sanyo Model MLS-3020, Japan). Then, samples were immediately cooled on ice for 10 min and kept at −20 °C. All analyses were performed within 3 days.

Angiotensin I-converting enzyme (ACE) from rabbit lungs, hippuryl-histidyl-leucine (HHL), hippuric acid (HA), pepsin from porcine stomach mucosa, pancreatin from porcine pancreas, plasmin from human plasma, acetonitrile (ACN) and trifluoroacetic acid (TFA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Other chemicals were of analytical grade.

4.3.2 *In vitro* gastrointestinal (GI) digestion

In vitro digestion was performed according to Garrett, Failla and Sarama (1999) with slight modifications. Raw or cooked samples (5 g dry solid) was homogenized (IKA Works Asia, Bhd, Malaysia) in 250 mL of distilled water (DI) for 1 min. The pH was adjusted to 2.0±0.02, pepsin (2.86% of substrate, dry basis) was added, and digestion was performed at 37 °C for 1 h. Subsequently, the pH was increased to 7.5±0.02 by adding 5 M NaOH. Pancreatin (4.00% of substrate, dry basis) was added

and the reaction was carried out at 37 °C for 2 h. The enzymatic digestion was terminated by submerging the sample in a 95 °C water bath for 10 min and cooling on ice to room temperature. The digested mixtures were centrifuged at 10,000 x g for 10 min. The peptide content of the supernatant was determined using the Lowry method with tyrosine as a standard (Wiriyaphan, Chitsomboon, Roytrakul, and Yongsawadigul, 2013). The degree of digestibility was also determined based on TNBS according to Adler-Nissen (1979) using leucine as a standard and calculated using the following equation:

$$\textbf{Digestibility}\ (\%) = \left(\frac{\alpha A_1 - \alpha A_0}{\alpha A_t}\right) \text{X } 100\%$$

where αA_1 is the α-amino content of the digest, αA_0 is the α-amino content of the sample at time 0 and αA_t is the total α-amino content of the sample obtained from hydrolyzing the samples with 6 M HCl at 120°C for 24 h.

4.3.3 Distribution of the molecular weight of digests

The distribution of the molecular weights of digests was determined using a Superdex 30 prep grade column (1.6 x 100 cm, GE Healthcare, Piscataway, NJ, USA). One hundred microliters of digest (0.1 mg) was loaded onto a column, and the elution was performed using 30% acetonitrile containing 0.1% trifluoroacetic acid (TFA) at a flow rate of 0.5 mL/min. Fractions (1 mL) were collected, and their absorbance was monitored at 214 nm. The molecular weight of the fractionated peptides was determined using a set of molecular weight standards: cytochrome C (12,000 Da), aprotinin (6,512 Da), AGNQVLNLQADLPK (1480.6 Da), and MILLLFR (1,095 Da). Molecular weight distribution was divided into 3 ranges, namely >5, 1-5, and <1 kDa. Relative

proportion of each range was calculated by dividing peak area of respective range to total peak area of the chromatogram (Cudennec, et al., 2016).

4.3.4 ACE inhibitory activity assay

In vitro ACE inhibitory activity was assayed according to Cushman and Cheung (1971) with slight modifications. The reaction mixture contained 50 µL of peptide and 50 µL of ACE (25 mU/mL) and was pre-incubated for 5 min at 37°C. Then, 150 µL of substrate (8.3 mM HHL in 0.1 M Tris-HCl buffer containing 500 mM NaCl at pH 8.3) was added, and the sample was further incubated at 37°C for 60 min. The reaction was stopped by adding 250 µL of 1 M HCl. The released hippuric acid (HA) was extracted using 1.5 mL of ethyl acetate. The upper layer of the ethyl acetate phase (1 mL) was dried in an 80°C sand bath. The dried sample was dissolved in 1 mL of deionized (DI) water and filtered through a 0.45-µm membrane filter. HA was separated using an Agilent 1260 HPLC system equipped with a Zorbax Eclipse XDB C18 column (4.6 mm i.d. x 150 mm, Agilent, Palo Alto, CA, USA). Elution was carried out using mobile phase A (0.05% TFA in water) and mobile phase B (0.05% TFA) in acetonitrile at a flow rate of 0.5 mL/min. The gradient of elution started at 5% of mobile phase B, linearly increased to 60% in 10 min, was maintained for 2 min at 60%, and then returned to 5% for 3 min. HA was monitored at 228 nm and quantitated using an external standard. Positive controls were prepared using DI water instead of peptide, while negative controls were prepared by adding HCl before enzyme and peptide. ACE inhibition (%) was calculated as follows:

$$\text{ACE inhibitory activity (\%)} = \left[\frac{(HC_0 - HC_{CB}) - (HS_0 - HS_{RB})}{(HC_0 - HC_{RB})}\right] \text{x } 100$$

where HC_0 is the HA content of the control (without a peptide sample), HC_{CB} is the HA content of the control blank, HS_0 is the HA content of the reaction with a peptide sample, and HS_{RB} is the HA content of the reaction blank.

4.3.5 Purification of ACE inhibitory peptides

4.3.5.1 Ion exchange chromatography (IEC)

Kc/H-0.5 digest exhibiting the highest ACE inhibitory activity was used for peptide purification. A lyophilized sample (200 mg peptide) was dissolved in DI water (10 mL), and 1 mL of peptide solution was loaded onto a DEAE–Sephacel ion exchange column (2.6 x 6.5 cm, GE Healthcare, Piscataway, NJ, USA) equilibrated with buffer A (50 mM Tris-HCl, pH 8.0). The unbound fraction was washed with two column volumes of buffer A, and the bound fraction was eluted with a linear gradient of NaCl (0–1 M) in buffer A with one column volume at a flow rate of 1 mL/min using an AKTA Purifier (GE Healthcare, Piscataway, NJ, USA). The eluate was monitored at 214 nm and collected in 8 mL volume fractions. Ion exchange chromatography was repeated 10 times. Two peaks were obtained from each injection. Three fractions at each peak were separately collected, determined for ACE inhibitory activity at the final concentration in the reaction mixture of 0.43 mg/mL. The fraction showing the highest ACE inhibitory activity was lyophilized. The concentration of inhibitor required to inhibit 50% of the ACE activity (IC_{50}) of the fraction exhibiting the highest ACE inhibitory activity was determined.

4.3.5.2 Size exclusion chromatography (SEC)

The lyophilized fraction exhibiting the highest ACE inhibitory activity obtained from IEC (64 mg peptide) was dissolved in DI water (2 mL). The reconstituted peptide (100 µL) was applied to a Superdex Peptide 10/300 GL column (10 × 300 mm, GE Healthcare, Piscataway, NJ, USA) equilibrated with DI water. The elution was performed using an AKTA Purifier (GE Healthcare, Piscataway, NJ, USA) with an isocratic mode of DI water at a flow rate of 0.5 mL/min. The elution was monitored at 214 nm and 0.5 mL fractions were collected. The purification was repeated 20 times. Peptide peak fractions were individually collected, lyophilized and analyzed for ACE inhibitory activity at the final concentration in the reaction mixture of 0.32 mg peptide/mL. The IC_{50} of the fraction exhibiting the highest ACE inhibitory activity was determined. The molecular weight of the SEC fractionated peptides was also determined using cytochrome C (12,000 Da), aprotinin (6,512 Da), AGNQVLNLQADLPK (1480.6 Da), and MILLLFR (1,095 Da) as molecular weight standards.

4.3.5.3 Reverse phase chromatography (RPC)

The lyophilized powder obtained from the Superdex peptide column (13.3 mg peptide) was dissolved in DI water (0.5 mL). The reconstituted peptide (50 µL) was applied to a SOURCE™ 5RPC ST 4.6/150 column (GE Healthcare, Piscataway, NJ, USA) equilibrated with DI water containing 0.05% (v/v) trifluoroacetic acid (TFA). The elution was carried out using mobile phase A (DI water containing 0.05% (v/v) TFA) and mobile phase B (acetonitrile (ACN) containing 0.05% (v/v) TFA). The elution gradient was started with 0-20% of mobile phase B for 20 min and 20-100% for 10 min, followed by 100% over 5 min at a flow rate of 0.5 mL/min. The elution was

monitored at 214 nm, and 0.5-mL fractions were collected. Purification was repeated 10 times. Peaks of mobile phase typically appeared in the first 5 min. Peptides were eluted after 10 min and were separately collected, lyophilized and analyzed for ACE inhibitory activity at 0.19 mg/mL of peptide (the final concentration in the reaction). The IC_{50} of the fraction exhibiting the highest ACE inhibitory activity was determined.

4.3.6 LC-MS/MS for peptide sequencing

The lyophilized powder of the fraction exhibiting the highest ACE inhibitory activity obtained from the SOURCE™ 5RPC ST column was subjected to amino acid sequencing 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. The peptides were separated on a nanocolumn (Acclaim PepMap 100 C18, 3 mm, 100A, 75 mm i.d. × 150 mm) with 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) for 13 min at a flow rate of 300 nL/min. Peptide mass was operated in a positive ion MS/MS mode. A full-scan MS/MS was performed for each sample with an acquisition range m/z 50–1990 Da. The de novo peptide sequencing was carried out using PepNovo, which was accessed at http://proteomics.ucsd.edu/LiveSearch/ (Frank and Pevzner, 2005). The input parameters included: enzyme, none; parent mass tolerance, ±2.0 Da; ion tolerance, ±0.5 Da. Sequences showing high PepNovo score (>80%) were chosen. Identification of protein precursors was carried out using NCBI Protein Blast (chicken, Gallus gallus, taxid:9031) for peptides containing >4 amino acid residues, and using BIOPEP (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep) for shorter peptides (A.

Iwaniak, Dziuba, and Niklewicz, 2005). All selected peptides were also searched within chicken proteins (Gallus gallus, 9031, http://www.uniprot.org/peptidesearch/) using UniProt Peptide Match (Boutet, Lieberherr, Tognolli, Schneider, and Bairoch, 2007). A search of ACE inhibitory peptides was carried out through BIOPEP and Anti Hypertensive Inhibiting Peptide database (AHTPDB, Kumar et al., 2015).

4.3.7 Peptide syntheses

Peptide sequences based on high Pepnovo scores and matched with chicken peptides were synthesized using a solid-phase peptide synthesis method (GL Biochem, Shanghai, Ltd.). The synthetic peptides, including VDHV, ELFTT, DDL, KPLLCS, KPLL, EPVQ, TEL and LTE, were purified using an HPLC column to 98% purity. The molecular mass of the synthesized peptides was confirmed by the manufacturer using liquid chromatography coupled to a mass spectrometer (LC-MS/ESI). All peptides were dissolved in DI water and their IC50 values were determined.

4.3.8 Plasmin hydrolysis

The R2 fraction obtained from RPC or synthetic peptides (2 mg) was dissolved in 1 mL of 50 mM Tris–HCl, pH 7.4, 0.15 mM NaCl, and 0.01% Tween 80. Peptide samples were then hydrolyzed with plasmin at 0.05 U/mL at 37 °C. At each time interval of 0, 60, 120 and 180 min, the samples were withdrawn from the reaction vessel and boiled for 5 min to terminate the plasmin activity. The α-amino group content, indicating the extent of plasmin hydrolysis, and IC_{50} of hydrolyzed samples were measured.

4.3.9 Statistical analyses

Data in triplicate were analyzed using one-way analysis of variance (ANOVA), and a comparison of the means was performed using Duncan's multiple range test (DMRT) as the significant difference within the 95% confidence interval using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA).

4.4 Results and discussion

4.4.1 ACE inhibitory activity of digests

ACE inhibitory activity of digest of cooked samples varied with thermal treatments and breeds. In raw and ST under the same condition, Kc showed lower ACE inhibitory activity than did Br, but digests of Kc subjected to 70 °C for either 0.5 h or 24 h showed higher inhibitory activity (p<0.05, **Table 4.1**). Extreme thermal treatment of 121 °C decreased the ACE inhibitory activity of digests from both breeds (p<0.05). In general, the ACE inhibitory activity of digests appeared to vary with the digestibility of the sample (**Figure. 4.1**). The highest digestibility and highest ACE inhibitory activity was found in samples cooked by mild thermal treatment (H-0.5). The H-0.5 treatment also resulted in digest with the highest proportion of small peptides (<1 kDa), while digest of meat cooked by extreme sterilization conditions (AC-60) contained the highest ratio of large peptides (> 5 kDa) (p<0.05, **Table 4.1**). Peptide size is vital for ACE inhibitory activity. Most ACE inhibitory peptides have been reported to be short sequences containing 2-12 amino acids (Darewicz, Borawska, Vegarud, Minkiewicz, and Iwaniak, 2014; Norris and FitzGerald, 2013). Larger peptides have difficulty binding to the ACE active site, resulting in decreased inhibitory activity (Natesh, Schwager, Sturrock, and Acharya, 2003). This might partly explain the low inhibitory

activity of the ST digests. High thermal treatment (>100 °C) induced protein cross-linking, massive aggregation and modifications via oxidation of various amino acid residues including sulfoxidation, carbonylation, and hydroxylation (Davis and Williams, 1998; Estévez, 2011; Soladoye et al., 2015). These protein modifications lead to a lower susceptibility to GI proteases, rendering larger peptide fragments upon digestion. Our study revealed that mild heating for 30 min (H-0.5) is the preferred cooking method for obtaining ACE inhibitory peptides upon GI digestion of both Kc and Br chicken breasts.

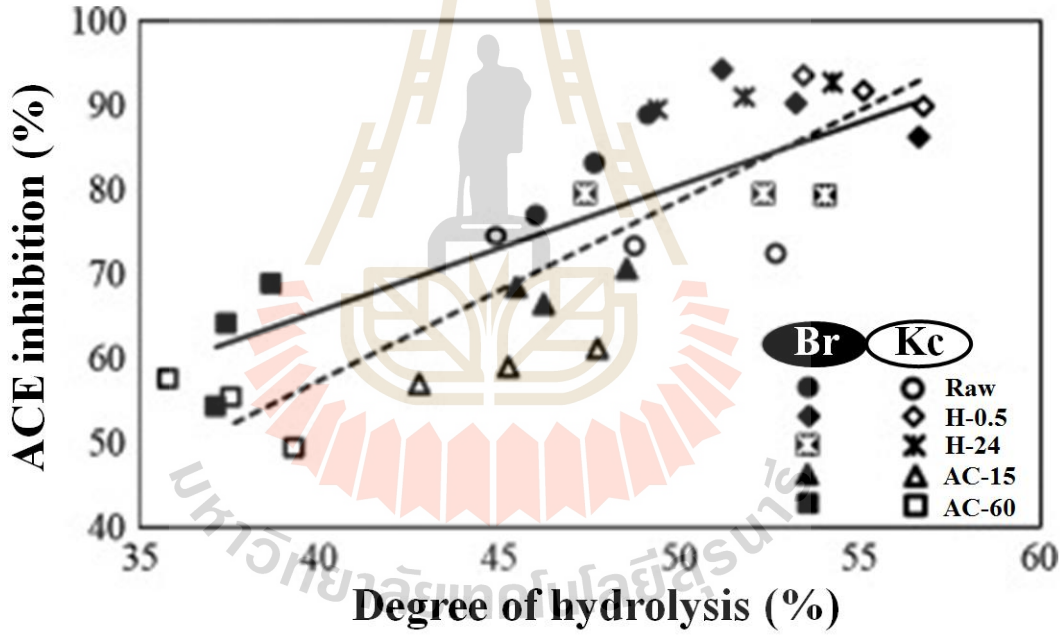

Figure 4.1 The relationship between digestibility and ACE inhibitory activity of Kc and Br chicken digests heated at different temperatures and times. Each value is the average of 3 replicates.

Table 4.1 ACE inhibitory activity, digestibility and apparent molecular weight distribution of the GI digests of raw and cooked chicken breasts.

Breed	Code	ACE inhibitory activity (%)	Apparent molecular weight distribution (% of total peak area)		
			>5 kDa	1-5 kDa	<1 kDa
Korat crossbred (Kc)	Kc/ Raw	73.33±1.08^{d}	9.67±0.01^{dA}	23.94±0.42^{fB}	66.39±0.32^{jC}
	Kc/ H-0.5	91.64±1.89^{h}	3.31±0.12^{aA}	30.66±0.17^{iB}	66.02±0.28^{iC}
	Kc/ H-24	85.12±1.53^{fg}	29.93±0.21^{gB}	19.16±0.37^{eA}	50.91±0.47^{dC}
	Kc/ AC-15	58.90±2.09^{b}	35.91±0.18^{hB}	17.72±0.45^{dA}	46.37±0.32^{cC}
	Kc/ AC-60	55.29±3.12^{a}	44.04±0.23^{iC}	15.19±0.26^{bA}	40.77±0.21^{bB}
Commercial broiler (Br)	Br/ RAW	81.99±1.91^{ef}	7.68±0.24^{cA}	28.32±0.13^{gB}	64.01±0.65^{hC}
	Br/H-0.5	86.76±2.53^{g}	4.22±0.42^{bA}	32.01±0.46^{jB}	63.77±0.23^{gC}
	Br/H-24	79.54±1.04^{e}	18.22±0.32^{eA}	29.25±0.53^{hB}	52.53±0.41^{eC}
	Br/AC-15	68.54±2.07^{c}	27.3±0.14^{fB}	15.92±0.31^{cA}	56.77±0.74^{fC}
	Br/AC-60	62.09±2.83^{b}	49.98±0.15^{jC}	13.01±0.46^{aA}	37.01±0.11^{aB}

Note: The ACE inhibitory activity was assayed at final concentration of 0.43 mg/ml in the reaction mixture. The mean and standard deviation of triplicate experiments are presented for each sample type. Values within a column bearing different letters (a-h) and within a row bearing different letters (A-C) are significantly different at $p < 0.05$.

4.4.2 Purification and identification of ACE inhibitory peptides

Since the digest of Kc/H-0.5 exhibited the most potent ACE inhibitory activity (**Table 4.1**), it was selected for peptide purification. After DEAE-Sephacel chromatography, both bound and unbound fractions exhibited ACE inhibitory activity. The bound fraction B1 showed the highest ACE inhibition (approximately 81%), while the activity of unbound fractions ranged from 48% to 60% **(Figures 4.2a, b)**. When the B1 fraction was subjected to SEC, the S2 fraction, possessing molecular masses ranging from 400 to 600 Da, showed the highest activity **(Figures 4.2c, d)**. After the RPC of S2, 3 major peaks were obtained, with the R2 fraction being the most potent inhibitor **(Figures 4.2e, f)**. Based on their IC_{50} value, the purification scheme applied in this study improved the purity of the ACE inhibitor peptides by 5.3-fold, based on the IC50 value **(Table 4.2).** The yield of the isolated ACE inhibitory peptide was approximately 2.6% of the crude digest. It should be mentioned that the number of ACE peptides generated upon *in vitro* gastrointestinal digestion of Kc/H-0.5 was likely to be greater than 2.6% since some inhibitor peptides, namely, UB1, UB2, UB3 **(Figure 4.2a)**, and S1, S2 **(Figure 4.2c)**, were excluded.

The digests obtained from other food proteins also show ACE inhibitory activity, including boiled and fried whole eggs, egg whites, egg yolks (Majumder and Wu, 2009), soy protein isolate (Lo, Farnworth, and Li-Chan, 2006), and pea and whey proteins (Vermeirssen et al., 2005). Our study demonstrated that chicken breast does not only serve as an important nutrient in the diet but also provides a source of ACE inhibitory peptides.

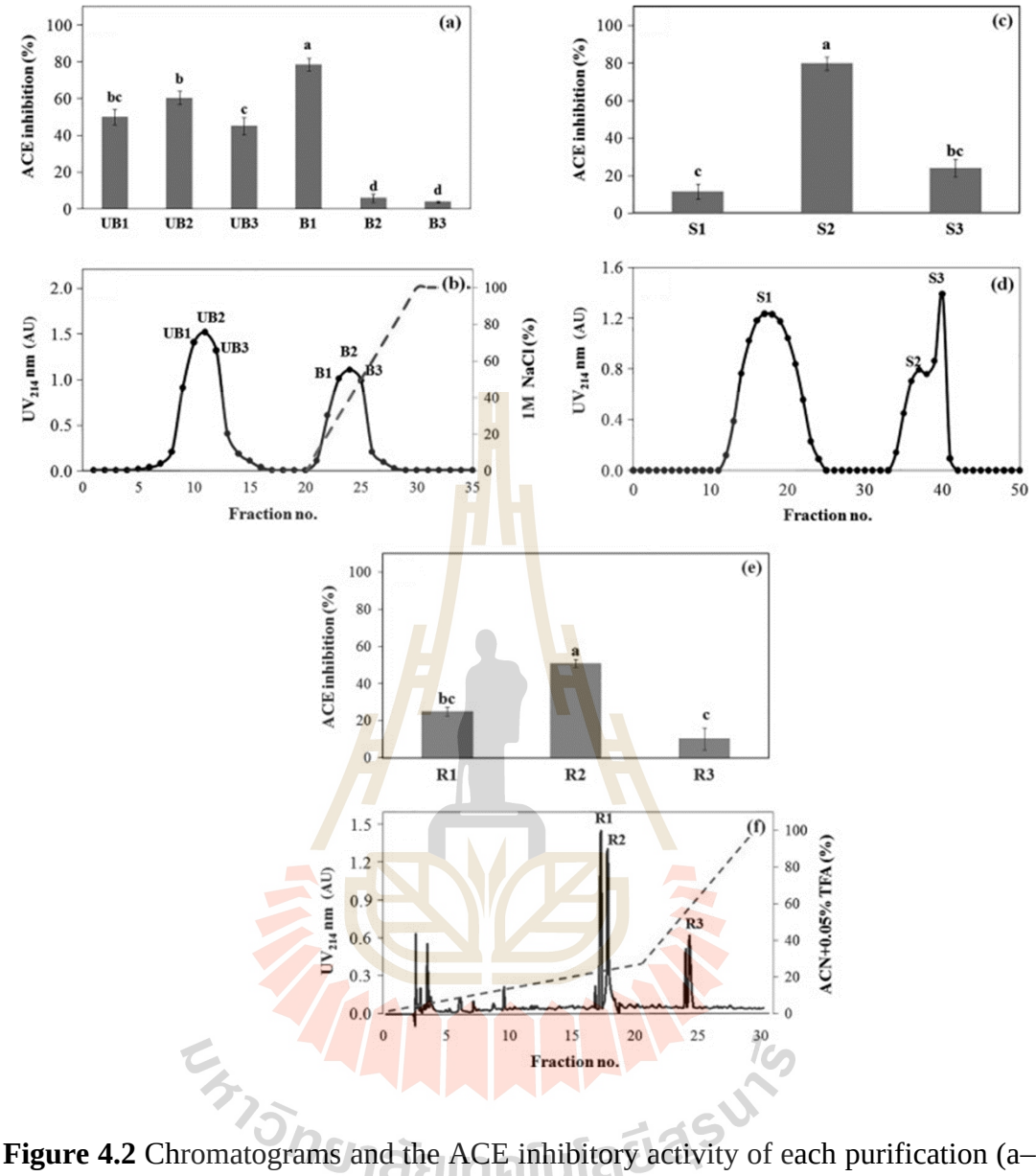

Figure 4.2 Chromatograms and the ACE inhibitory activity of each purification (a–f); (a and b) DEAE-Sephacel ion exchange chromatogram of Kc/H-0.5 digest eluted with NaCl (0–1 M) in 50 mM Tris-HCl buffer at pH 8, (c and d) Superdex peptide 10/300 GL size exclusion chromatogram of the B1 fraction eluted with DI water, and (e and f) RP-HPLC SOURCE™ 5RPC ST 4.6/150 chromatogram of the S2 fraction eluted with ACN containing 0.05% TFA.

Table 4.2 Purification table and IC_{50} values of the Kc/H-0.5 digest.

Step	Fraction	Peptide content (mg)	Recovery rate (%)	IC_{50} [a] (μg/mL of peptide content)
Crude digest	-	200.00	100.00	103.32±1.02
Ion exchange	B1	64.13	32.07	37.54±0.42
Gel filtration	S2	13.30	6.65	29.36±0.27
Reverses phase	R2	5.21	2.61	19.37±0.11

[a] The average and standard deviation of triplicate experiments are presented.

4.4.3 Amino acid sequences of the purified peptides

Eight peptides in the active R2 fraction with high scores were identified from *de novo* sequencing and showed similarities to myofibrillar proteins of chicken based on various databases (**Table 4.3**), indicating that myofibrillar proteins, namely myosin, actin, tropomyosin, and titin, are parent sources of the encrypted ACE inhibitor peptides from cooked chicken meat. The ACE-inhibitory capacity of each peptide was found to be highly dependent on the peptide sequence. The most potent ACE inhibitor identified in this study, KPLLCS, was derived from uncharacterized chicken protein based on UniProt Peptide Match, and showed high similarity (83%) to a myosin peptide, KPLLKS, reported in BIOPEP. It was speculated that this peptide could be encrypted in Korat chicken myosin. The MS/MS spectrum of KPLLCS is shown in **Figure 4.3**. In addition, two peptides derived from myosin, namely ELFTT and KPLL, exhibited high ACE inhibitory potency. These 3 peptide inhibitors have not been previously reported. Other 5 peptides identified also showed ACE inhibition, but with much lower inhibitory activity (**Table 4.3**). Typically, small peptides (up to 6 amino acid residues) are absorbed into the small intestinal epithelial cell, and further digested by brush

border peptidases before being transferred into bloodstream (Bouglé and Bouhallab, 2017). It should be noted that all reported peptides shared common sequences with ACE inhibitor dipeptides previously reported in both BIOPEP and AHTPDB (**Table 4.3**). It could, therefore, be hypothesized that dipeptides released from these chicken peptides by brush border peptidases would still exhibit ACE inhibitory activity at the target organ.

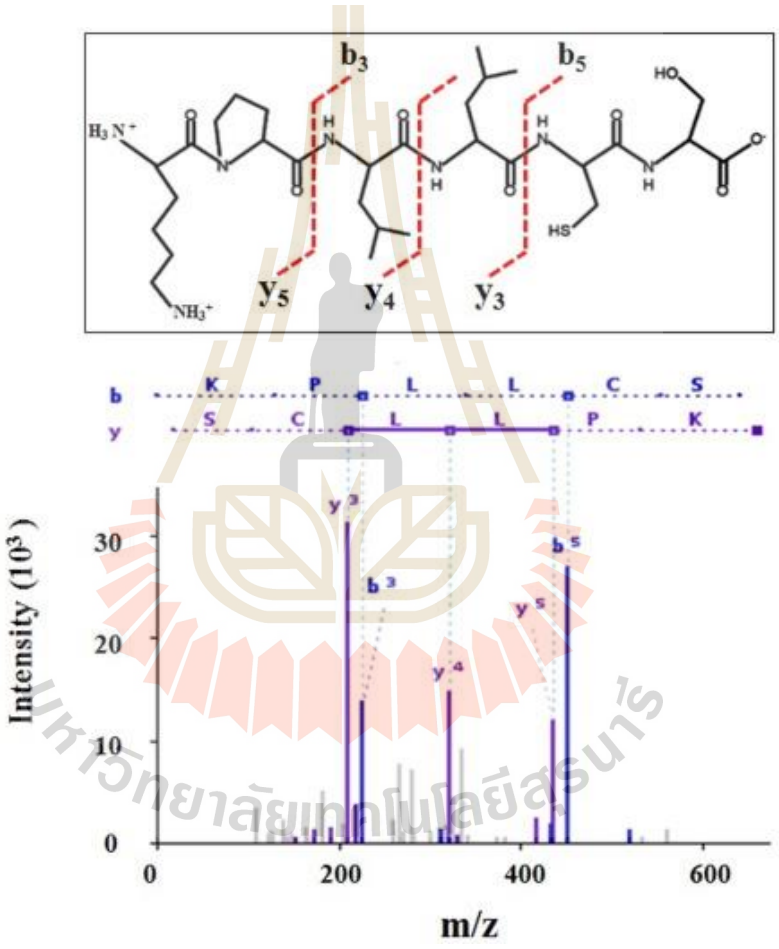

Figure 4.3 Representative spectrum of nano-LC–MS/MS of KPLLCS obtained by *de novo* sequencing.

Table 4.3 IC_{50} values and amino acid sequences of purified peptides based on the *de novo* peptide sequencing algorithm and source of their parent proteins.

Sequence	Mass (Da)	Parent protein	Database[c]	IC_{50} (μM)[d]	Previously reported sequences
KPLLCS	659.84	Heavy meromyosin[b]	BIOPEP id: 1123	0.37 ± 0.12	KP[e,f]
		Uncharacterized protein[a]	UniProtKB id: E1C3T2,		
ELFTT	609.67	Heavy meromyosin[a]	NCBi id: pdb|3J04|B	6.35 ± 1.06	-
		Myosin[a]	NCBi id: pdb|2MYS|B		
KPLL	469.62	Heavy meromyosin[a]	NCBi id: pdb|3J04|A	11.98 ± 1.65	KP[e,f], LL[f]
		Myosin[a]	BIOPEP id: 1123		
TEL	361.39	Myosin[a]	BIOPEP id: 1124	410.26 ± 3.20	TE[e,f]
		Connectin[a] (titin)	BIOPEP id: 1076		
		Actin[a]	UniProtKB id: Q90738		
VDHV	468.5	Myosin[a]	NCBi id: pdb|2DFS|A	461.90 ± 14.29	VD[f], LL[f]
		Myosin[a]	NCBi id: pdb|1OE9|A		
EPVQ	471.5	Connectin[a] (titin)	BIOPEP id: 1076	603.57 ± 14.76	EP[f], VQ[f]
		Unconventional myosin[a]	UniProtKB id: Q5ZLA6		
DDL	361.35	Myosin[a]	BIOPEP id: 1124	926.79 ± 15.25	DL[f]
		Unconventional myosin[a]	UniProtKB id: Q02440		
		Tropomyosin[a]	BIOPEP id: 1128 and 1130		
LTE	361.39	Connectin[a] (titin)	BIOPEP id: 1124	1640.4 ± 82.59	TE[e,f]
		Tropomyosin[a]	BIOPEP id: 1131 and 1133		

[a] 100% matching score with reported parent protein(s).
[b] 83.33% matching score with reported parent protein, KPLLKS.
[c] Databases used for assignment of peptide sequences
[d] Average and standard deviation of 3 triplicate were presented.
[e] BIOPEP database: peptide database, http://www.uwm.edu.pl/biochemia/index.php/pl/biopep .
[f] AHTPDB database: peptide database, http://crdd.osdd.net/raghava/ahtpdb/pep.php.

ACE inhibitory peptides released from pork meat proteins include KAPVA, RPR and PTPVP, which show significant antihypertensive activity in spontaneously hypertensive rats after oral administration (Anna Iwaniak, Minkiewicz, and Darewicz, 2014). Potent ACE inhibitory peptides are generally short peptides whose C-terminal plays an important role in their inhibitory activity. Peptides possessing a Ser (S), Leu (L) or Thr (T) at the C-terminal have been reported to be potent ACE inhibitor peptides, based on the AHTPDB database e.g., VFPS (IC_{50} = 0.46 µM), MIFPGAGGPEL (0.03 µM), PYVRYL (1.90 µM), and IRWCT (1.00 µM). In addition, the presence of a hydrophobic Pro residue at one or more positions in the sequence positively influences ACE inhibitory activity (Norris and FitzGerald, 2013). The presence of Leu residues in the penultimate positions also contributes to ACE inhibition due to the interaction of this residue with the three hydrophobic sub-sites located on the active site of ACE (Ryan et al., 2011). In addition, the inhibition of ACE is achieved by hydrophobic peptides that display a high affinity to the active sub-sites of ACE (Ryan et al., 2011). Thus, the presence of S, L, and T at the C-terminal of novel peptides identified in this study was in agreement with previously reported sequence characteristics of ACE inhibitor peptides.

ACE inhibitory peptides have been derived from skeletal muscle proteins, namely, myosin, tropomyosin, troponin, actin and collagen (Bhat et al., 2015). The potent ACE inhibitory peptides, KPLLCS, KPLL and ELFTT, were encrypted in myosin, while other peptides were derived from titin, the largest known protein with a molecular mass of 3.0-3.7 MDa (Tskhovrebova and Trinick, 2010). It should be noted that the novel peptide KPLLCS (IC_{50} of 0.37 µM) showed considerably higher inhibitory activity than GFHypGTHypGLHypGF and GAHypGLHypGP, which were

derived from chicken breast extract (Saiga et al., 2003) and collagen (Saiga et al., 2008) and whose IC_{50} values were 42 µM and 29 µM, respectively. These novel peptides derived from Kc/H-0.5 could be good candidates for further *in vivo* efficacy studies.

4.4.4 Plasmin hydrolysis

Protein digestion generates many peptides in the gut lumen and some of these short peptides are absorbed intact from the gastrointestinal tract (Roberts, Burney, Black, and Zaloga, 1999). However, transported peptides with an ACE inhibitory activity need to resist blood protease digestion. To explore the behavior of peptides in human plasma *in vitro*, the purified R2 and 3 potential peptides were treated with plasmin. The α-amino group content of the purified R2 increased concomitant with a decrease in the IC_{50} value with hydrolysis time (**Fig. 4.4a**). This result indicated that the purified R2 peptides were hydrolyzed into smaller fragments by plasmin and such smaller peptides exerted a higher ACE inhibitory activity. The IVY derived from wheat germ hydrolysate remains intact in rat or human plasma for up to 4 h (Matsui et al., 2000), while HLPLP from β-casein degrades by one and a half times the initial amount at 2 h of incubation (Quirós, Dávalos, Lasunción, Ramos, and Recio, 2008); therefore, the Kc/H-0.5 digest could still exert its ACE inhibitory activity while in blood circulation.

Of the 3 synthesized and tested peptides, ELFTT and KPLL were resistant to plasmin hydrolysis for up to 60 min (**Fig. 4.4b**), and their IC_{50} values were unchanged. In contrast, KPLLCS was hydrolyzed by plasmin after 15 min, and its IC_{50} value increased from 0.37 µM to 411.42 µM after 60 min, indicating a significant decrease in its ACE inhibitory activity. Gardner (1998) reported that the half-life of peptides in plasma is rather short with an order of magnitude of 1 min. Thus, all 3 peptides would

be likely to be unaffected by plasmin *in vivo*. It would be reasonable to assume that peptides from digest of Kc/H-0.5, particularly those presenting in the purified R2 (i.e., ELFTT, KPLL, KPLLCS), would potentially inhibit ACE *in vivo*, as they were resistant to plasmin hydrolysis.

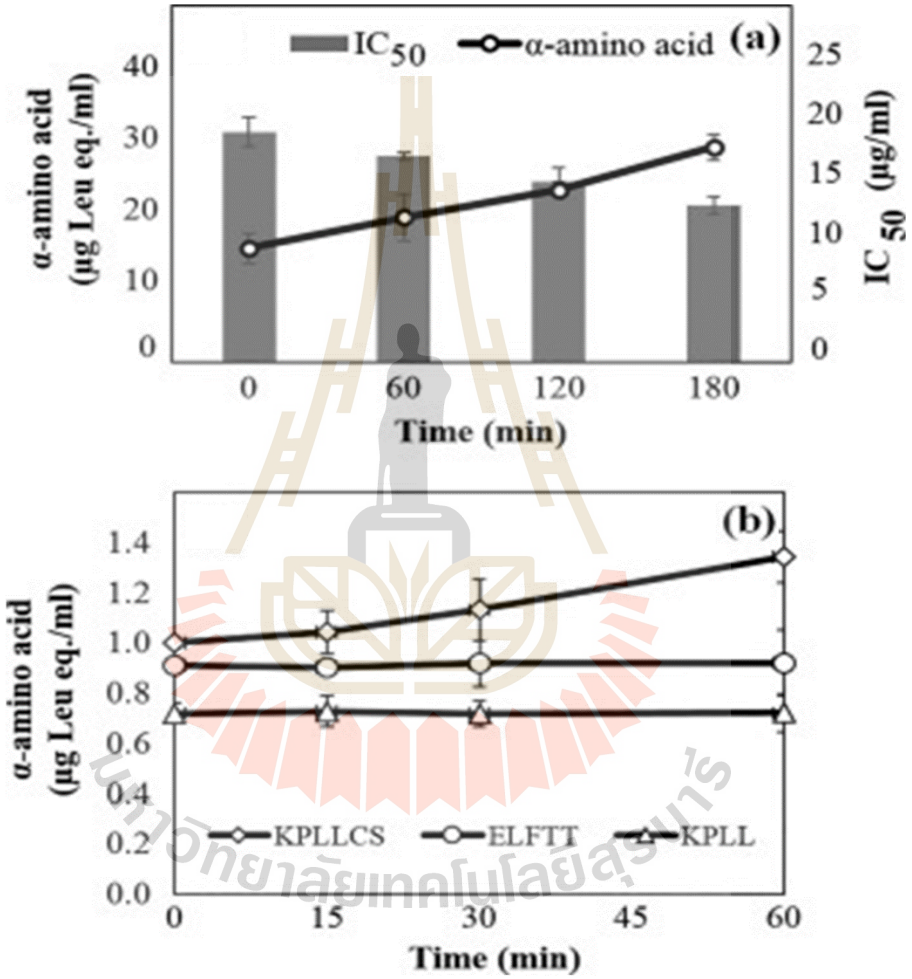

Figure 4.4 The α-amino acid content and IC_{50} values of peptides incubated with human plasmin. (a) The purified R2 fraction incubation up to 180 min. (b) The synthetic peptides KPLLCS, ELFTT and KPLL up to 60 min. Each value is the average of 3 replicates.

4.5 Conclusion

The *in vitro* GI digestion of cooked chicken breast generated a number of potent ACE inhibitory peptides. Mild thermal treatment at 70 °C for 0.5 h resulted in the highest ACE inhibitory activity of Kc digest. The novel potent ACE inhibitory peptides were identified as KPLLCS, ELFTT and KPLL with IC_{50} values of 2.57, 44.46, and 76.96 µM, respectively. ELFTT and KPLL were stable against plasmin digestion for up to 60 min, while KPLLCS was hydrolyzed by plasmin after 15 min, resulting in an increase in its IC_{50} value. This study confirmed that chicken breast heated at Kc/H-0.5 is a source of health-promoting peptides. Further studies on the bioavailability of the peptides using an animal model would be necessary to prove the efficacy of Kc peptides for lowering blood pressure.

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CHAPTER V

ANGIOTENSIN CONVERTING ENZYME (ACE) INHIBITORY PEPTIDES DERIVED FROM SIMULATED *IN VITRO* GASTROINTESTINAL DIGESTION OF COOKED CHICKEN MUSCLE: PERMEABILITY AND IDENTIFICATION OF TRANSPORTED PEPTIDES

5.1 Abstract

Korat-chicken breast and thigh were subjected to heating at 70, 100 or 121˚C for 30 min and simulated *in vitro* gastrointestinal digestion. At 70 or 100˚C heating, digests of breast possessed higher ACE inhibitory activity than those of thigh. The highest ACE inhibitory activity was found in the digest of breast cooked at 70˚C (B/H-70), whereas breast heated at 121˚C (B/H-121) exhibited the lowest. The 1-kDa permeate of the B/H-70 (PM-1-70) digest revealed higher permeability through colorectal adenocarcinoma (Caco-2) monolayers and ACE inhibitory activity than did B/H-121 (PM-1-121). Among transported peptides of PM-1-70, tripeptide APP derived from myosin showed the highest ACE inhibition, with a non-competitive characteristic and K_i of 0.93 μM. Molecular docking showed that APP interacts with ACE via hydrogen bonds, electrostatic and van der Waals interactions. In conclusion, mild thermal treatment of chicken breast resulted in a higher amount of transported peptides, exerting higher ACE inhibitory activity, which could lead to potential health benefits.

Keywords: Muscle protein, poultry, angiotensin converting enzyme, bioactive peptide, permeability

5.2 Introduction

Bioactive peptides with angiotensin converting enzyme (ACE) inhibitory activity and a hypertensive effect *in vivo* have been widely studied in food protein hydrolysates from various sources (Aluko, 2015; Vermeirssen, Van Camp, and Verstraete, 2004). Most of the ACE inhibitory peptides reported in the literature are derived from hydrolysis by commercial proteases (Aluko, 2015; Vermeirssen et al., 2004). *In vitro* gastrointestinal digestion (GI) has been reported to generate ACE inhibitory peptides from muscle foods, including chicken breast, pork and beef (Escudero, Sentandreu, Arihara, and Toldrá, 2010; Sangsawad, Roytrakul, and Yongsawatdigul, 2017). This would imply that a muscle protein diet is likely to provide peptides with ACE inhibitory activity in addition to the nutritional value of amino acids.

Korat chicken is a new crossbreed between the male line of Thai indigenous chickens and the female line of the broiler. This chicken strain has a higher growth rate than indigenous chickens, but its muscles are firmer and chewier in texture than the commercial broiler (Maliwan, Khempaka, and Molee, 2017). Cooking has been noted to trigger the generation of reactive oxygen species, which, in turn, increase the tendency for protein oxidation, especially at high thermal treatment (>100 ˚C). This leads to protein modifications, including protein cross-linking, generation of carbonyl groups, reduction of free thiol groups and extensive protein aggregation (Soladoye, Juárez, Aalhus, Shand, and Estévez, 2015). A high degree of protein modification can

lead to limited enzymatic hydrolysis (Lund, Heinonen, Baron, and Estevez, 2011). The degree of protein modification greatly depends on the muscle type, species and heating conditions (Lund et al., 2011; Soladoye et al., 2015). Chicken breast and thigh have different compositions, with breast containing a higher proportion of muscle type II fiber than thigh and thigh containing a higher level of collagen than breast muscle (Jaturasitha, Srikanchai, Kreuzer, and Wicke, 2008). Thus, it might be reasonable to assume that digests derived from meat that was cooked with various thermal treatments would lead to varied ACE inhibitory activity.

Bioactive peptides must be transported across the intestinal wall into blood circulation and reach the sites of target organs in an active form in sufficient quantities (Foltz et al., 2008). An *in vitro* bioavailability assay based on Caco-2 cell monolayers is typically applied as a model system for small intestinal transport of drugs and food compounds (Cinq-Mars, Hu, Kitts, and Li-Chan, 2007). The amount of transported ACE inhibitory peptides from various food sources including tilapia muscle, casein and Pacific hake was estimated to be 14.80%, 10.25-23.14% and 2.80%, respectively (Samaranayaka, Kitts, and Li-Chan, 2010; Toopcham, Mes, Wichers, Roytrakul, and Yongsawatdigul, 2017; Wang, Wang, and Li, 2016). Apart from the quantitative aspect, the cell model is also useful to monitor structural changes in transported peptides. Oligopeptides are typically transported through epithelial cells by various means, including passive transport, transcytosis, and peptide transporter 1 (PepT1) (Daniel, 2004; Vermeirssen et al., 2004). They can be further digested during this process by intestinal brush border membrane peptidases, including carboxypeptidase P, aminopeptidase N, aminopeptidase A, endopeptidase-24.11, ACE-1, ACE-2, aminopeptidase P, aminopeptidase W, endopeptidase-2 and dipeptidyl-peptidase-IV

(DPP-IV) (Howell, Kenny, and Turner, 1992). Some potent ACE inhibitory peptides can be produced upon hydrolysis of absorbed peptides by surface brush border membrane peptidases. For example, the peptide LHLPLP from fermented milk is hydrolyzed to HLPLP by aminopeptidase as it is transported through a Caco-2 cell monolayer.[19] In addition, AAATP derived from dry-cured ham extract was not found in the apical or basolateral compartments during incubation with a Caco-2 cell monolayer for 60 min; it was hydrolyzed by intestinal brush border peptidases to AAAT (Gallego et al., 2016). Therefore, ACE inhibitor peptides identified in protein hydrolysates, which have been widely published, might not necessarily be "true" inhibitors reaching the target organ (Quirós, Dávalos, Lasunción, Ramos, and Recio, 2008). The use of a cell model would allow one to understand the structural changes of transported peptides and to identify the "true" ACE inhibitors. Currently, the transport of ACE inhibitory peptides derived from muscle digests has not been systematically studied.

ACE inhibitory peptides have been reported as competitive, non-competitive, and mixed-competitive inhibitors. Peptides acting as competitive inhibitors typically contain hydrophobic amino acid residues at their C-terminus, allowing them to bind the active site of ACE (Iwaniak, Minkiewicz, and Darewicz, 2014). They can occupy S1' and S2' subsites of ACE, leading to inhibition of the enzyme (Ni, Li, Liu, and Hu, 2012; Pina and Roque, 2009). However, the mechanism of ACE inhibition by non-competitive inhibitors has not been clearly elucidated. Molecular docking simulations can provide insights on peptide-ACE interactions, providing a deeper understanding on the mechanism of ACE inhibition by peptides.

The objectives of this study were to compare ACE inhibitory activity of *in vitro* digests of Korat chicken breast and thigh subjected to various thermal treatments. Permeability of ACE inhibitory peptides derived from digests was determined based on the Caco-2 cell monolayer model system, and transported peptides exhibiting ACE inhibitory activity were identified and characterized. Molecular docking was conducted to investigate the nature of the interaction of the most potent peptide with ACE. This investigation should shed light on structural changes in ACE inhibitory peptides that occur across the small intestine and provide insight into the peptide structure-ACE inhibition relationship.

5.3. Materials and methods

5.3.1 Materials

Angiotensin-I-converting enzyme (ACE) from rabbit lung, hippuryl-histidyl-leucine tetrahydrate (HHL), hippuric acid (HA), acetonitrile (ACN), trifluoroacetic acid (TFA), Hank's balanced salt solution (HBSS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Lucifer yellow and sodium dodecyl sulfate (SDS) were purchased from Sigma-Aldrich Canada Ltd. (Oakville, Canada). Synthetic peptides were purchased from GL Biochem, Shanghai, Ltd (Shanghai, China). Eagle's minimum essential medium (EMEM), fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Invitrogen (Burlington, Canada). Caco-2 (HTB-37th) cells passage 18 were obtained from the American Type Culture Collection (Manassas, USA). Cell culture inserts were purchased from Corning (VWR, Mississauga, Canada). Other chemicals and reagents were of analytical grade.

5.3.2 Sample preparation

Korat chickens (70 days old, with live weights of 1.2-1.3 kg) were obtained from University Farm of Suranaree University of Technology, Thailand. Breast and thigh were removed from the carcasses 24 h postmortem, cut into 2x2x2 cm pieces, and packed in nylon/polyethylene laminated bags. Thermal treatments were conducted as described by Sangsawad et al. (2016). Briefly, samples were cooked under various treatments, including heating for 30 min at 70°C (H-70) and 100°C (H-100) using a water bath (SAP12; Grant Instrument Ltd., Cambridge shire, UK) and autoclaving at 15 psi, 121°C (H-121; MLS-3020; Panasonic Corp., Tokyo, Japan). The latter treatment was the most extreme thermal condition applied. After each thermal treatment, samples were immediately cooled on ice for 10 min. All samples (raw and cooked) were stored at − 20 °C prior to analysis, and all analyses were performed within 3 days.

5.3.3 *In vitro* gastrointestinal (GI) digestion

In vitro pepsin–pancreatin hydrolysis was carried out according to Garrett et al. (1999) with slight modifications. The homogenized raw or cooked muscles (5 g dry solid in 100 mL of distilled water) were adjusted to pH 2.0 with 5 M HCl. Pepsin (E/S 1:35 w/w) was added with continuous shaking at 37 °C. After one hour of incubation, the pH was adjusted to 5.3 with 0.9 M $NaHCO_3$ and further increased to pH 7.5 using 5 M NaOH. Subsequently, pancreatin (E/S 1:25 w/w) was added, followed by continuous shaking at 37 °C for 2 h, after which the mixture was submerged in a 95 °C water bath for 10 min to inactivate the enzymes. These samples were cooled to room temperature and centrifuged at 10,000×g for 10 min, and referred to as “digest.” The ACE inhibitory activity of digests was assayed at a final concentration of 0.28 mg/mL in the reaction mixture. The samples that showed maximal ACE inhibitory activity were then lyophilized.

Lyophilized digests (10 g) were dissolved in DI water (100 mL) and subjected to sequential ultrafiltration using an Ultracel Amicon ultrafiltration unit (model 8400, Millipore Corporation; Bedford, USA). Nitrogen gas at 50 psi was applied during sequential ultrafiltration using membranes with molecular weight cutoffs (MWCOs) of 10, 3 and 1 kDa. The four fractions obtained after sequential ultrafiltration (UF) include three retentates with MWCOs of 10 kDa, 10-3 kDa and 3-1 kDa, and a permeate with a MW ≤1 kDa, which were referred to as RT-10, RT-10-3, RT-3-1 and PM-1, respectively. PM-1 was desalted to reach a NaCl content lower than 2% using an electro-dialyzer (Micro Acilyzer S1, Tokyo, Japan). Residual sodium content was determined using an Accumet® sodium ion selective electrode (Fisher Scientific, Fairlawn, USA). The electro-dialyzed samples were lyophilized (Labconco Corporation, Kansas City, USA). All recovered fractions were lyophilized and stored in sealed vials at −80 °C. Peptide yield was calculated based on the dried weight of each lyophilized ultrafiltration fraction. They were also tested for their ACE inhibitory activity based on a final concentration of 0.28 mg/mL.

5.3.4 ACE inhibitory activity assay

ACE inhibitory activity of digests was assayed according to Cheung and Li-Chan (2010) with some modifications. ACE and HHL were solubilized in assay buffer (0.05 M Tris-HCl buffer at pH 8.3 containing 0.3 M NaCl). The reaction mixture contained 30 µL of sample and 30 µL of ACE (0.083 U/mL) and was preincubated at 37 °C for 10 min. Then, 150 µL of 6.5 mM HHL was added and the samples were incubated at 37 °C for an additional 60 min. Finally, 250 µL of 0.1 M HCl was added to inactivate the enzyme. The released HA was extracted using 1 mL of ethyl acetate.

The upper ethyl acetate phase (0.7 mL) was dried in a heating block at 120°C for 30 min. The dried sample was dissolved in 1.3 mL of DI water, and the HA concentration was estimated by measuring absorbance at 228 nm. Assays for the ACE inhibitory activity of samples were carried out in triplicate. Positive controls were prepared using DI water instead of peptide, and negative controls (blank of control and samples) were prepared by adding HCl before the enzyme. ACE inhibition (%) was calculated as follows:

$$\text{ACE inhibitory activity (\%)} = \left[\frac{(HC_P - HC_N) - (HS_p - HS_N)}{(HC_P - HC_N)}\right] \text{x } 100$$

where HC_P is the HA content of the positive control, HC_N is the HA content of the blank control, HS_P is the HA content of a reaction with peptide sample, and HS_N is the HA content of the blank sample.

5.3.5 Total and free amino acids analysis

The peptide contents of the desalted and lyophilized PM-1 fractions of samples cooked at 70˚C (PM-1-70) and at 121˚C (PM-1-121) were estimated from differences between their respective total amino acids and free amino acids content, as described by Cheung and Li-Chan (2010). For total amino acid analysis, samples were hydrolyzed in 6 M HCl with 1% phenol at 110 °C for 24 h. Eighteen amino acids were separated on a Waters Pico-Tag high-performance liquid chromatography (HPLC) system after precolumn derivatization with phenylisothiocarbamate. Free amino acid profiles were also determined using the same precolumn derivatization in the samples without acid hydrolysis. Both the total and free amino acid contents were analyzed at the Advanced

Protein Technology Center at The Hospital for Sick Children (Toronto, Canada) (http://www.sickkids.ca/Research/SPARC/Amino-Acid-Analysis/index.html).

5.3.6 Viability of Caco-2 cells

Caco-2 cells of passages 22-29 were cultured and maintained in tissue culture flasks with EMEM medium supplemented with 10% FBS and antibiotics (100 µg/mL penicillin and 100 U/mL streptomycin) in a fully humidified atmosphere with 5% CO_2 at 37 °C. Cells were sub-cultured weekly by trypsin-EDTA treatment. Medium was replaced every 2-3 days, and cells were grown for 21 days. To investigate the cytotoxicity of peptide samples, cells were seeded in 96-well culture plates (density of 1 x 10^5 cells/cm^2). The media was replaced with PM-1-70 and PM-1-121, which were dissolved in EMEM at concentrations of 8, 10, 12 and 15 mg peptide/mL, and incubated at 37 °C in 5% CO_2 for 24 h, followed by the addition of 100 µL of MTT (0.05 mg in complete EMEM) and further incubation in the dark for another 4 h. After the addition of 100 µL of SDS-HCl solution (10% SDS in 0.01 M HCL) and overnight incubation, the release of formazan was measured at 570 nm using a Multiskan Spectrum microplate spectrophotometer (Thermo Labsystems, Chantilly, USA).

5.3.7 Caco-2 cell permeability

For cell permeability studies of PM-1-70 and PM-1-121, cells were seeded onto 24-well cell culture inserts (high pore density polyethylene terephthalate, 0.4-µm pore size, 100±10 x 10^6/cm^2 pore density, 0.3 cm^2 grown surface) at a density of 2.5 x 10^5 cells/cm^2; the basal side contained 0.7 mL EMEM. Culture medium was carefully replaced every other day for at least 21 days as described above. Then, cells were washed twice with HBSS and equilibrated at 37˚C for 1 h. Monolayer integrity was

measured by transepithelial electrical resistance (TEER) using a Millicell ERS-2 voltohmmeter (EMD Millipore, Darmstadt, Germany), and only monolayers with TEER exceeding 250 ΩU/cm^2 were chosen for the transport study. To further validate monolayer integrity, Lucifer yellow was added to the apical side of the insert and incubated with the cells for 1 h either before or after transport experiments.(Chen, Elisia, and Kitts, 2010) TEER values and the permeability of Lucifer yellow (<10% of the initial concentration) were measured before and after the experiment. The amount of Lucifer yellow transported to the basolateral compartment was quantified using a luminometer (Fluoroskan Ascent, Fisher Scientific UK Ltd., Loughborough, England, UK) at Ex_{425} nm and Em_{530} nm. For the peptide transport study, 0.3 mL of test samples (15 mg peptide/mL) dissolved in HBSS was added to the apical side, and 0.7 mL of HBSS was added to the basal side. The plates were incubated under an atmosphere containing 5% CO_2 at 37 °C for 2 h. The peptide content and profile of the apical compartment were monitored before (0 h) and after incubation (2 h), and those of the basal compartment were analyzed after a 2-h incubation.

Peptide quantification was carried out using reversed-phase high-performance liquid chromatography (RP-HPLC) on a Phenomenex Jupiter™ C12 Proteo 90 Å HPLC column (250 mm x 4.6 mm, 4 µm; Phenomenex®, Torrance, USA) equipped with an Agilent 1260 Infinity HPLC system (Agilent Technology, Mississauga, Canada). The mobile phases, 0.1% TFA in water (A) and 0.1% TFA in ACN (B), were set at a flow rate of 1 mL/min with the following gradient: 0-20% B for 30 min, 20-95% B for 5 min, hold at 95% B for 5 min and return to 0% B at 10 min at 40˚C. Chromatographic separation was carried out at 40˚C. The absorbance was monitored at UV_{214} and UV_{280} nm. To establish an external standard curve, the desalted and

lyophilized PM-1-70 or PM-1-121 samples dissolved in HBSS at various peptide concentrations (0-20 mg/mL estimated from differences between total and free amino acid contents as described above) were first analyzed on RP-HPLC. Values of the overall peak area monitored at UV_{214} nm for each sample were plotted against the respective amounts of peptides to establish the external standard curve. The peptide contents of sample aliquots collected from the apical (10 µL) and basolateral (100 µL) compartments were then estimated by determining their overall peak area and comparing these values to the established standard curve. Peptide permeability was calculated according to the following equation:

$$\text{Permeability (\%)} = \frac{\text{Peptides detected in basal side after 2 – h incubation (µg)}}{\text{Peptides detected in apical side at 0 h (µg)}} \text{ x } 100$$

Permeated peptides (in the basolateral compartment) of PM-1-70 or PM-1-121 were pooled and lyophilized. Lyophilized samples were then dissolved in DI water and analyzed for ACE inhibitory activity as described in detail above at a final concentration of 0.28 mg/mL in the reaction mixture. Each separated peak collected from RP-HPLC was also lyophilized and analyzed for ACE inhibitory activity at the same final concentration of 0.28 mg/mL. The apical fractions were called AF1-AF6 and basal fractions BF1-BF6.

5.3.8 Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

The *de novo* peptide sequencing of permeated peptides 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. Peptides were eluted using 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) for 13 min in a nanocolumn (Acclaim PepMap 100 C_{18}, 3 mm, 100 Å, 75 mm i.d. x 150 mm) at a flow rate of 300 nl/min. The LC-MS/MS analyses were performed using a full scan in positive ion MS/MS mode and an acquisition range of m/z 50–1990. The mass spectra were exported to BioTools (version 3.1, Bruker Daltonik GmbH), and peptide sequences were carried out using *de novo* sequencing. Identification of protein precursors was carried out using BIOPEP (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep) (Iwaniak, Dziuba, and Niklewicz, 2005) and UniProtKB (http://www.uniprot.org/peptidesearch/) (Boutet, Lieberherr, Tognolli, Schneider, and Bairoch, 2007) based on the chicken protein database (*Gallus gallus*). A search of reported ACE inhibitory peptides was also carried out through BIOPEP and the Anti-Hypertensive Inhibiting Peptide database (AHTPDB) (Kumar et al., 2015).

5.3.9 ACE inhibition profile

Transported peptides identified to be APP, LVK, LVQ, GHK, TGY, EYC, AVF, GLK and YTG were synthesized with 98% purity (GL Biochem, Shanghai, Ltd, Shanghai, China). The masses of these peptides were confirmed by the manufacturer using liquid chromatography coupled to a mass spectrometer (LC-MS/ESI). The concentrations of peptides required for 50% inhibition of the enzyme activity (IC_{50}) of all synthetic peptides were determined. Since APP was found to be the most potent ACE inhibitor, its mode of inhibition was determined. Various concentrations of HHL (0.5, 1, 3, 5 and 7 mM, dissolved in reaction buffer) were incubated with ACE in the absence or presence (0 - 200 µM) of APP at 37 °C. The inhibition kinetics of ACE in the presence of APP was determined based on the Lineweaver–Burk plot. The inhibitor constant K_i was calculated by plotting $1/V_{max}$ versus the concentrations of APP.

5.3.10 Molecular docking of APP

The peptide APP structure was created in a MOL2 file using Discovery Studio Visualizer 2017 (Biovia, 2016). Molecular docking between APP and ACE was carried out using the GOLD program version 4.1 (Jones, Willett, Glen, Leach, and Taylor, 1997). The crystal structure of human ACE, PDB code: 1O86, was obtained from the Protein Data Bank (PDB). All water molecules were deleted, and the hydrogen atoms were added to the structure in the GOLD setup procedure. The other parameters were set as default. The Genetic algorithm (GA) operation was applied for 50 runs. The *ChemScore fitness function* was selected for this docking. The docking results were visualized and analyzed by Discovery Studio Visualizer 2017.

5.3.11 Statistical analyses

All experiments were conducted in triplicate, and the results were analyzed by one-way analysis of variance (ANOVA). Significant differences in mean values were analyzed by Duncan's multiple range mean comparison test within the 95% confidence interval using SPSS 17.0 software (SPSS Inc, Chicago, IL, USA).

5.4 Results and discussion

5.4.1 ACE inhibitory activities of digests

Mild thermal heating at 70˚C improved the ACE inhibitory activity of both breast and thigh **(Figure 5.1a)**. Maximal ACE inhibitory activity was found in breast cooked at 70˚C for 30 min (B/H-70). Digests of breast showed higher ACE inhibitory activity than those of thigh ($p < 0.05$), regardless of the thermal treatment applied, except for a 121˚C treatment that resulted in comparable inhibitory activity ($p > 0.05$). Our results demonstrate that extreme thermal treatment of chicken muscle results in a

reduction of ACE inhibitory potency of the digest. In contrast, mild thermal treatment (60-80˚C) enhanced protein unfolding, which consequently increased susceptibility to protease action and shorter peptide products (Bax et al., 2012; Soladoye et al., 2015). Sangsawad et al. (2017) reported that the digest of Korat chicken breast cooked at 70˚C for 30 min contained shorter peptides with molecular weight <1 kDa. In addition, several reports have shown that peptides containing 2–12 amino acids exhibited higher ACE inhibitory activity than longer counterparts (Norris and FitzGerald, 2013). In contrast, extreme thermal treatment (>100˚C) leads to modification of amino acid residues, including disulfide bridges, sulfoxidation, carbonylation, hydroxylation, nitrosylation, and chlorination (Soladoye et al., 2015; Tang, 2008). Such modifications could lead to alteration of protease recognition sites, causing lower protein digestibility and a lower proportion of small peptides to be released. Prolonged thermal treatment can also lead to protein cross-linking (Davis and Williams, 1998; Soladoye et al., 2015). Akillioglu and Karakaya (2009) reported that extreme thermal treatment at 121˚C for 15, 30 and 50 min reduced the ACE inhibitory activity of pinto bean and green lentil digests. Our study demonstrated that the extreme thermal treatment of chicken breast and thigh at 121˚C for 30 min negatively affected the ACE inhibitory activity of their digests. Heating at 70˚C for 30 min is the preferred cooking method to generate ACE inhibitory peptides upon GI digestion of chicken breast and thigh.

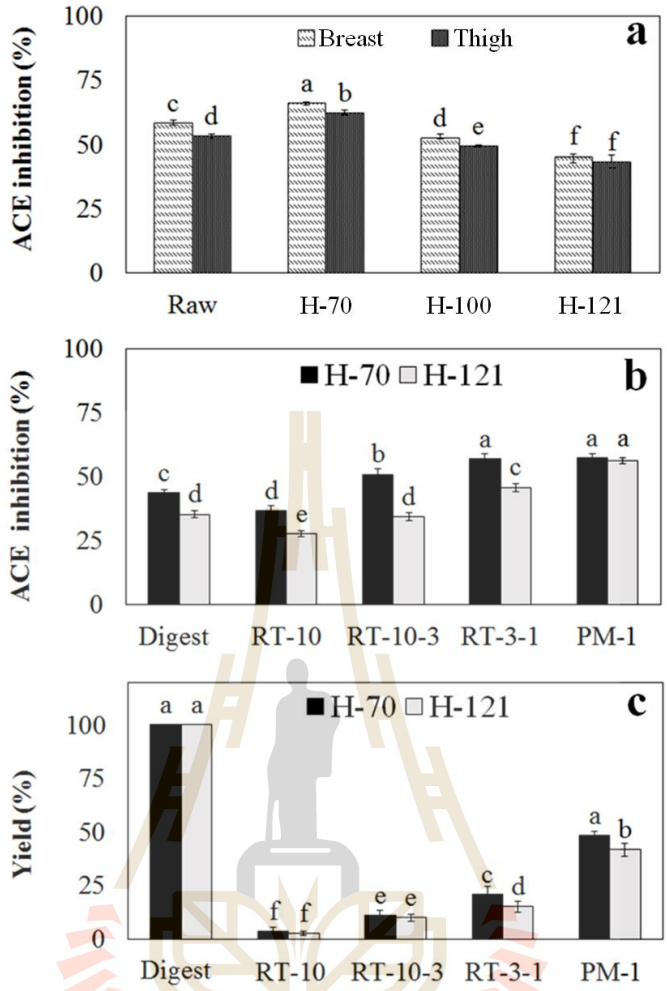

Figure 5.1 ACE inhibitory activity of Korat chicken breast and thigh digests (a) and ultrafiltration retentate (RT) and permeate (PM) fractions of breast digest (b), and peptide yields of ultrafiltration fractions (c). The ACE inhibitory activity was assayed at final concentration of 0.28 mg/mL in reaction mixture. Values are shown as mean ± standard deviation of three replicates. Superscript letters represent significantly different values ($p < 0.05$).

Furthermore, higher ACE inhibitory activity could be obtained from digests of cooked breast compared to thigh. De Oliveira et al. (2016) reported that chicken breast contained a higher protein content and lower collagen and lipid contents than thigh.

Breast muscle is mainly composed of muscle fiber type IIB (92.8 - 96.1%), whereas thigh contains about 66.0 - 72.2% type IIB (Jaturasitha et al., 2008). Due to these differences, the extent of proteolysis by GI proteases is different.

After sequential UF fractionation, maximal ACE inhibitory activity was found in the PM-1 fraction for both samples ($p < 0.05$, **Figure 5.1b)**. This result confirmed that smaller peptides exhibit better ACE inhibition. The highest peptide yield was also observed in these two fractions, indicating that both digests contained low-molecular-weight compounds (**Figure 5.1c).** However, the B/H-70 digest had a higher yield in the PM-1 fraction than did the B/H-121 digest. It might be assumed that the digestibility of cooked chicken breast at mild thermal treatment was higher than that of samples cooked under autoclave conditions. Based on this result, it could be assumed that consumption of chicken breast heated at 70˚C for 30 min would result in shorter peptides, which could partly contribute to higher ACE inhibitory activity.

5.4.2 Permeability of peptides across Caco-2 cell monolayers

The desalted and lyophilized PM-1-70 and PM-1-121 samples contained 57.75% and 54.97% peptide, respectively (**Table 5.1**). These samples did not show cytotoxicity on Caco-2 cells at peptide concentrations up to 15 mg/mL. Therefore, a peptide concentration of 15 mg/mL was used for transport assays. Peptide profiles of the apical and basal compartments are illustrated in **Figures 5.2a and 2b**. The apical layer of both samples at 0 h showed six sharp peaks, and comparable profiles were also

Table 5.1 Amino acid composition of desalted and lyophilized PM-1 samples (g/100 g of sample)[a].

	PM-1-70			PM-1-121		
	Total AA	Free AA	Peptides	Total AA	Free AA	Peptides
Asp + Asn	6.00	0.31	5.69	5.49	0.22	5.27
Glu + Gln	9.54	0.97	8.57	9.14	0.64	8.50
Ser	3.09	0.19	2.90	2.89	0.15	2.74
Gly	3.08	0.15	2.93	2.81	0.14	2.67
His	3.01	0.33	2.68	2.98	0.29	2.69
Arg	6.35	3.63	2.72	6.42	3.71	2.71
Thr	4.91	0.34	4.57	4.39	0.27	4.12
Ala	4.52	0.28	4.24	4.31	0.24	4.07
Pro	1.60	0.04	1.56	1.50	0.02	1.48
Tyr	3.47	1.65	1.82	3.27	1.56	1.71
Val	4.38	0.75	3.63	4.05	0.73	3.32
Met	2.57	0.64	1.93	2.52	0.58	1.94
Cys	0.10	0.09	0.01	0.04	0.07	0.00
Ile	3.62	0.50	3.12	3.30	0.44	2.86
Leu	7.52	2.89	4.63	7.18	2.67	4.51
Phe	4.35	2.30	2.05	4.07	2.10	1.97
Trp	0.00	0.59	0.00	0.00	0.52	0.00
Lys	7.42	2.13	5.29	7.22	2.25	4.97
Sum	**75.53**	**17.78**	**57.75**	**71.58**	**16.61**	**54.97**

[a] Acid hydrolysis resulted in 18 amino acids. Free amino acid were determined by analyses of samples without acid hydrolysis. AA, amino acids.

observed after a 2-h incubation, indicating only minimal modification of peptides in the samples by surface brush border membrane peptidases. In the basal compartment of both samples, only 4 peaks were detected after a 2-h incubation, with smaller peak areas compared to the respective peptide profiles of the apical compartment at 2 h. The permeability of PM-1-70 and its ACE inhibitory activity appeared to be greater than that of PM-1-121 ($p < 0.05$**, Figures 5.3a and b**). It is well established that protein oxidation occurs to a greater extent under the extreme thermal treatment via

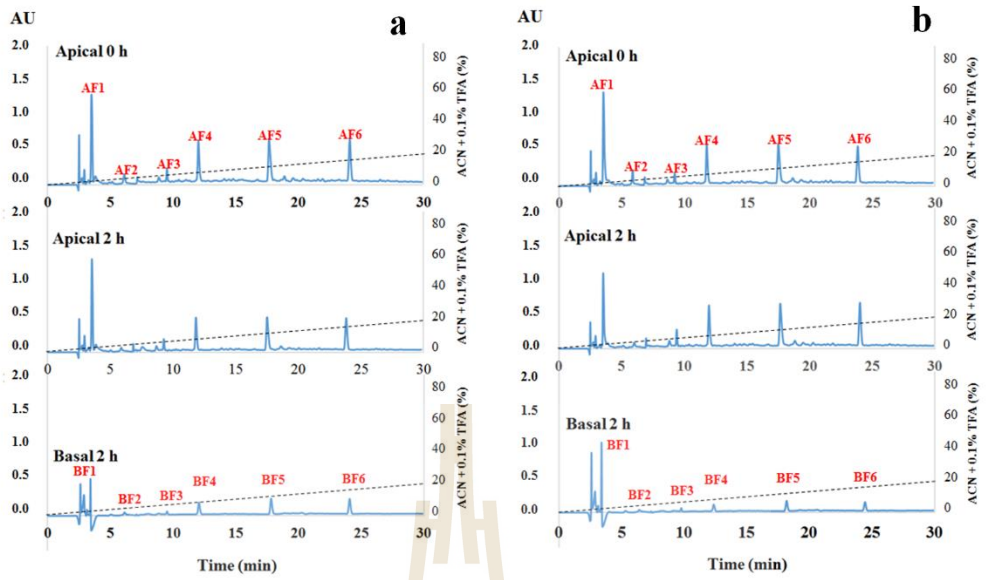

Figure 5.2 RP-HPLC chromatograms of PM-1-70 (a) and PM-1-121 (b), analyzed from the apical compartment at 0 h and 2 h, and the basolateral compartment after 2 h incubation. AF and BF, fractions from apical and basal compartments, respectively.

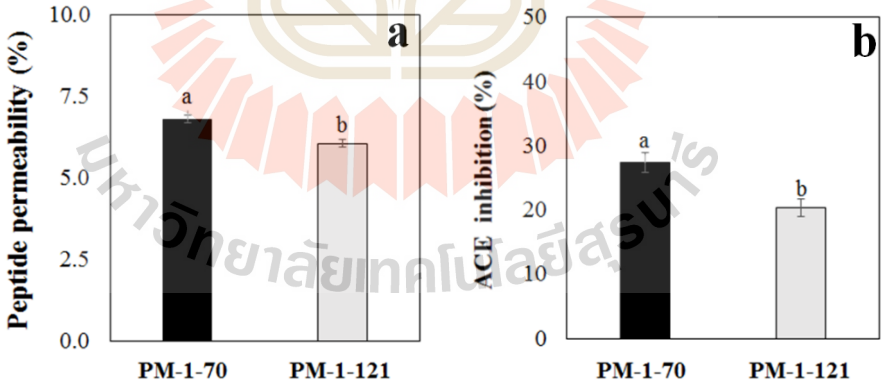

Figure 5.3 Overall peptide permeability (A) and ACE inhibition of PM-1-70 and PM-1-121 permeates (B). The ACE inhibitory activity was assayed at final concentration of 0.28 mg/mL in the reaction mixture. Values shown are the mean ± standard deviation of three replicates. Superscript letters represent significantly different values ($p < 0.05$).

cross-linking and amino acid side chain modifications (Davis and Williams, 1998; Soladoye et al., 2015). Our results revealed that extreme thermal treatment of chicken breast also leads to reduced peptide permeability and ACE inhibitory activity. Further studies are needed to clarify the specific protein modification induced by extreme thermal treatment that hampers peptide permeability.

Based on the current results, consumption of chicken meat cooked under mild thermal treatment (70˚C for 30 min) provided higher peptide permeability as well as ACE inhibitory activity than meat cooked with extreme thermal treatment (121˚C for 30 min). The reported permeability of peptides is relatively low and varies among studies. Peptide contents in digests of tilapia muscle and Pacific hake were 14.8% and 2.80%, respectively (Samaranayaka et al., 2010; Toopcham et al., 2017). In addition, the permeability of peptides with molecular weights of about 0.5 – 1.6 kDa was found to be 9.54 – 10.66% (Bo and Bo, 2017). Roberts et al. (1999) explained that the permeability of peptides decreases as chain length increases. It should be noted that Caco-2 cell monolayers are known to be tighter than *in vivo* human intestinal cells (Vermeirssen et al., 2005), thus, *in vivo* peptide absorption is expected to be higher than that observed with Caco-2 cell monolayers.

The apical and basal compartments of both PM-1-70 and PM-1-121 showed 6 peaks on RP-HPLC chromatograms **(**BF1-BF6, **Figures 5.2a and b).** The BF-1 peptide of the PM-1-121 sample showed higher permeability than that of PM-1-70, whereas peptides of BF4-BF6 of PM-1-70 exhibited higher permeability than those of PM-1-121 (**Figure 5.4a).** Peptides in BF4-BF6 were likely to be more hydrophobic than those that eluted earlier (BF1-BF3). Because the total transported peptides of PM-1-70 had

higher ACE inhibition activity than PM-1-70 (**Figure 5.3b)**, individual permeated peptides of PM-1-70 were investigated for their ACE inhibitory activity. The BF4 fraction exhibited the highest ACE inhibition ($p < 0.05$), and more hydrophobic peptides (BF5 and BF6) showed much less inhibitory activity (**Figure 5.4b).** Therefore, peptides in fraction BF4 were subjected to identification sequencing.

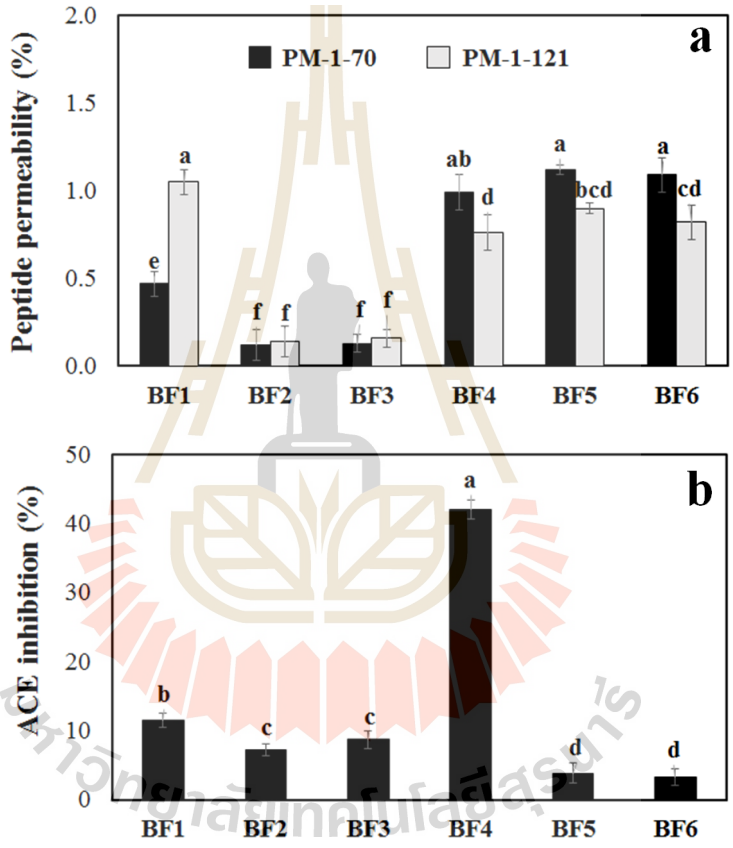

Figure 5.4 Permeability of peptide fractions separated from RP-HPLC of PM-1-70 and PM-1-121 (A) and ACE inhibitory activity of permeated peptide fractions of PM-1-70 (B). BF1-6 refers to Figure 2. ACE inhibitory activity was assayed at final concentration of 0.28 mg/mL in the reaction mixture. Values are shown as mean ± standard deviation of three replicates. Superscript letters represent significant difference ($p < 0.05$).

5.4.3 Identification of transported peptides

Identification of transported peptides in fraction BF4 revealed 9 tripeptides derived from myosin and titin **(Table 5.2)**. The identified peptides showed variable ACE inhibitory activity, with APP being the most potent inhibitor (IC_{50} of 7.78 µM). This was consistent with the results of Jing et al. (2013) who reported that APP showed high ACE inhibitory activity, with an IC_{50} of 4.6 µM. The MS/MS spectrum of APP is shown in **Figure 5.5a**. Myosin, tropomyosin, troponin and actin have been reported as sources of ACE inhibitor peptides (Satake et al., 2002). ACE inhibitory peptides released from chicken myosin included ITTNP, MNPPK, MNP, NPP and ITT with IC_{50} values ranging from 66 to 945 µM (Terashima et al., 2010). In addition, the tripeptides RPR and KAPVA were released from pork nebulin and titin, showing IC_{50} of 382 µM and 46.56 µM, respectively (Soladoye et al., 2015). However, these peptides were identified from protein hydrolysates and have not been investigated for their permeability through epithelial cell. In the present study, the tripeptide APP was present in the basolateral layer, but it is unknown whether it was generated from GI digestion or produced via proteolysis during permeation through intestinal cells by brush border peptidases. Generally, peptides containing 4-9 amino acid residues are likely to be hydrolyzed by brush border peptidases before being transported through cells (Gallego et al., 2016; Isabelle ME, Xiu Min, David, and Eunice CY, 2017; Miguel et al., 2008; Vermeirssen et al., 2004). In contrast, dipeptides are readily absorbed across the brush border membrane by a specific peptide transport system, PepT1 (Daniel, 2004; Vermeirssen et al., 2004). For tripeptides, transport of both intact and hydrolyzed products has been reported (Miguel, Gómez-Ruiz, Recio, and Aleixandre, 2010; Satake et al., 2002).

Table 5.2 IC_{50} values and amino acid sequences of permeated peptides of fraction-BF4 from the PM-1 permeate of B/H-70 digest, based on algorithm for peptide sequencing *de novo* and sources of parent proteins.

Sequence	Mass (Da)	Parent protein	Database[a]	Database ID	IC_{50} (µM) [b]	Previously reported sequences
APP	283.32	Myosin	UniProtKB	P13538, P02565,P10587, P16419, Q90688, Q05623	7.78 ± 0.53	AP[cd], PP[cd], APP[d]
		Myosin	Biopep	1123		
LVK	358.48	Myosin	UniProtKB	Q90688, Q9I8D, Q5ZLA6, Q02440	46.75 ± 1.72	LV[d], VK[cd]
LVQ	358.43	Myosin	UniProtKB	P11799, P29616	54.6 ± 4.68	LV[d], VQ[d], LVQ[d]
		Connectin (titin)	Biopep	1118		
GHK	340.38	Connectin (titin)	Biopep	1076	74.2 ± 7.2	GH[c], HK[c]
		Myosin	UniProtKB	Q02440		
TGY	339.34	Myosin	UniProtKB	P16419, P10587	123.21 ± 7.76	TG[c], GY[cd]
EYC	413.45	Myosin	UniProtKB	Q90688, Q02440	217.58 ± 6.47	EY[cd]
		Actinin	UniProtKB	P05094, Q90734		
AVF	335.4	Myosin	UniProtKB	P16419, Q90688	406.33 ± 22.79	AV[cd], VF[cd], AVF[d]
GLK	316.4	Myosin	UniProtKB	P16419, Q02440	415.35 ± 18.99	GL[cd]
YTG	339.34	Connectin (titin)	Biopep	1076	1178.41 ± 72.53	TG[cd]
		Tubulin	UniProtKB	P09652, P09206, P09203, P09207		

[a] BIOPEP protein database: http://www.uwm.edu.pl/biochemia/index.php/pl/biopep and UniProtKB database: peptide search, http://www.uniprot.org/peptidesearch/ , assessed 9/4/2017.
[b] Average and standard deviation of triplicates.
[c] BIOPEP database: http://www.uwm.edu.pl/biochemia/index.php/pl/biopep assessed 9/4/2017.
[d] AHTPDB peptide database: http://crdd.osdd.net/raghava/ahtpdb/pep.php assessed 9/4/2017.

Thus, further study is needed to identify the formation pathway of APP. Although APP is a transported peptide, it might be further hydrolyzed by exopeptidases in the blood stream before reaching the target organs including vascular tissues, heart, and kidney, where it can lower blood pressure (Toto, Rinner, and Ram, 2004). Based on both the BIOPEP and AHTPDB databases, AP and PP, which are hydrolyzed products of APP, still exert ACE inhibitory activity with IC_{50} of 29 µM and 2.28 mM, respectively (**Table 5.2**). Therefore, APP would likely be a potent ACE inhibitor derived from Korat chicken cooked at 70˚C for 30 min, which deserves further investigation *in vivo*.

5.4.4 Mode of action and molecular docking of APP

The transported peptide APP is a non-competitive ACE inhibitor with K_i of 0.93 µM **(Figures 5.5b and c**), suggesting that the peptide can bind either free enzyme or an enzyme-substrate complex. Such a binding leads to conformational changes and a decrease in substrate affinity at the active site (Segel and Segel, 1976). ACE inhibitory peptides have been reported with competitive, noncompetitive, uncompetitive and mixed modes of action. Most ACE inhibitory peptides act as competitive inhibitors, including MKR, RGY and VAW derived from hen egg white lysozyme hydrolysate with K_i values of 16.30, 52.10 and 2.40 µM, respectively (Rao et al., 2012). Mixed-mode inhibitors include FEDYVPLSCF and FNVPLYE from salmon byproduct hydrolysate with K_i values of 10.48 and 77.84 µM, respectively (Ahn, Jeon, Kim, and Je, 2012). In addition, FLEK and MEKHS from cupuassu seed protein hydrolysate with K_i values of 18.50 and 13.31 µM (da Cruz, Pimenta, de Melo, and Nascimento, 2016) and VWDPPKFD from salmon protein hydrolysate with a K_i of 19.52 µM were found to be noncompetitive inhibitors (Ahn et al., 2012). It should be mentioned that these

peptides were identified from protein hydrolysates; therefore, their permeability through epithelial cell is still in question.

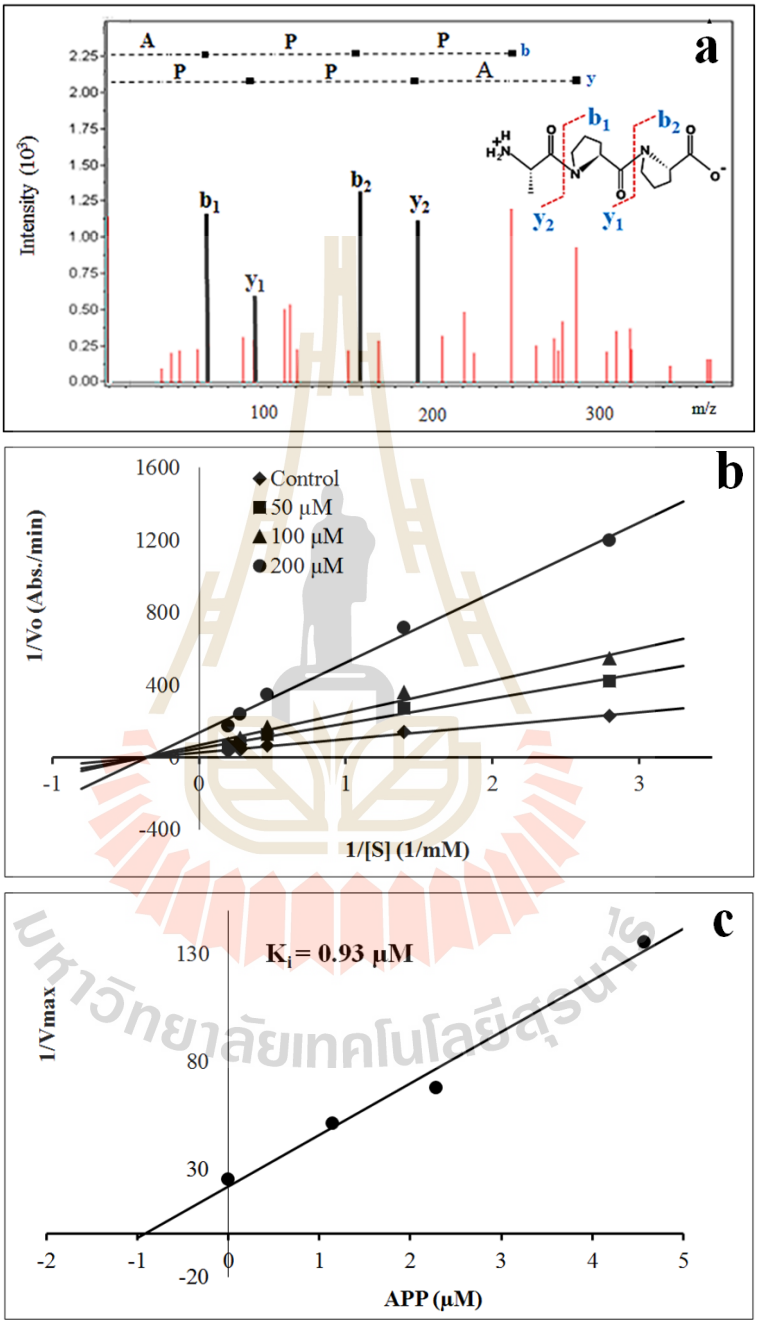

Figure 5.5 APP-MS/MS spectrum of sequence determined *de novo* (a), kinetics study of the ACE inhibition profile of APP (b), and secondary plot of non-competitive inhibition (c).

Based on its K_i value, the transported peptide APP appeared to bind to ACE more effectively than those previously identified peptides. Even though several transported ACE inhibitory peptides, including VGPV and GPRGF, have been characterized as non-competitive inhibitors, their K_i values have not been reported. Comparison of K_i values among transported peptides is therefore not possible.

Tripeptides containing PP at the C-terminus (XPP) have been reported to be effective ACE inhibitors with IC_{50} values ranging from 4.60 to 368.14 µM, while tripeptides with the AXP sequence exhibited IC_{50} values of 2.54-610 µM (http://crdd.osdd.net/raghava/ahtpdb). Thus, PP at the C-terminal end and A at the N-terminal end of tripeptides critically contribute to the potency of ACE inhibition. Our observation was in agreement with Liu et al. (2014) who reported that hydrophobic amino acids at the C-terminus, namely L, P, F, W, and Y, significantly increased ACE binding affinity. Moreover, Norris and FitzGerald reported that the presence of hydrophobic P residues at one or more positions in the C-terminal tripeptide positively influenced ACE inhibitory activity (Norris and FitzGerald, 2013).

To understand how APP binds to ACE, molecular docking simulation was performed. The docking mode of APP with the highest Dock Score was 16.67 kJ/mol **(Figure 5.6a**). Interaction between APP and ACE in the presence of HHL involved 2 hydrogen bonds, 11 van der Waal interactions and an attractive charge interaction **(Figure 5.6b)**. In the ACE-HHL binding site, the 7 amino acid residues involved were Y523, H513, H353, Y520, K511, Q281 and A354, and the catalytic site is composed of a zinc ion and E384 with a water molecule. In addition, the zinc ion is tetrahedrally

coordinated by H383, H387 and E411. The C-terminal P of APP interacted with R522 of ACE via an electrostatic interaction, whereas the N-terminal A of APP formed a conventional hydrogen bond with A356. In addition, the 2nd residue P of APP formed a non-classical hydrogen bond with the –CH of the pyrrolidine ring and O of E411. Non-classical hydrogen bonds between –CH···Y, where Y represents arene or oxygen, have been reported to be potentially important in interactions in biological systems (Liu et al., 2014). These interactions of APP and ACE distorted the tetrahedral geometry around the zinc ion, leading to a decrease in ACE catalytic function.

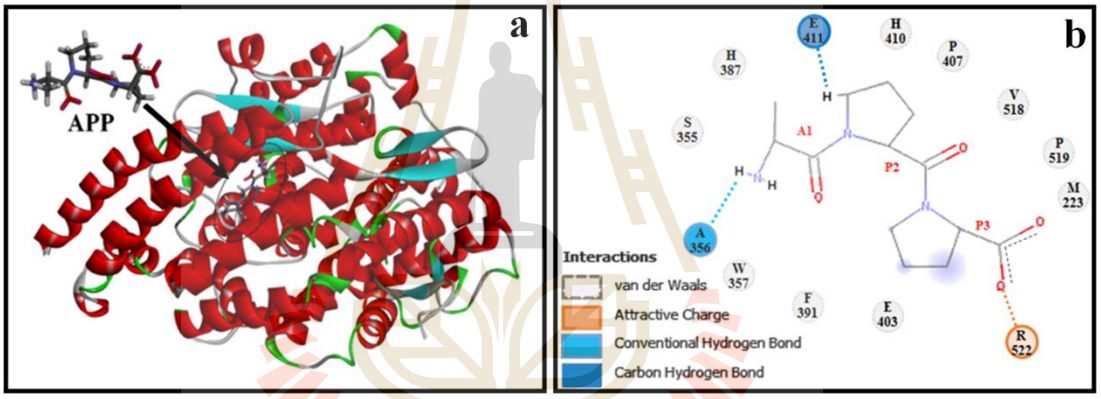

Figure 5.6 Docking simulation of APP binding with ACE, predicted 3D structure of APP-ACE complex (A) and interaction between APP and the residues of ACE (B).

5.5 Conclusion

Korat chicken breast cooked at H-70 exhibited maximal ACE inhibitory activity in the peptides generated upon *in vitro* GI digestion and showed higher peptide permeability through Caco-2 cell monolayers and higher ACE inhibitory activity than did those cooked at H-121. Among the nine permeated peptides, APP exerted the highest inhibition and was derived from myosin. APP was a non-competitive ACE

inhibitor with K_i of 0.93 μM. It formed hydrogen bonds, attractive electrostatic charge interactions, and van der Waals interactions with ACE, leading to reduced ACE activity. Our study demonstrated that cooked Korat chicken breast is a promising precursor source of ACE inhibitor peptides that are permeable. Further *in vivo* studies are necessary to prove the efficacy of digests in lowering blood pressure and their transport route.

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CHAPTER VI

TRANSPORT AND STRUCTURAL TRANSFORMATION OF CHICKEN ANGIOTENSIN I-CONVERTING ENZYME (ACE) PEPTIDES THROUGH CACO-2 CELL MONOLAYERS

6.1 Abstract

Permeability of angiotensin-1 converting enzyme (ACE) inhibitory peptides (KPLLCS, ELFTT, and KPLL) identified from the *in vitro* gastrointestinal digest of cooked chicken breast was investigated using the Caco-2 cell model system. KPLLCS was originally the most effective ACE inhibitor with IC_{50} of 0.37±0.12 µM, but it was degraded during permeation through Caco-2 cells. KPLL showed the highest permeability as an intact form, but it was partially degraded to KP and LL during permeation. ACE inhibitory activity of permeated KPLL was highest of 56%. KPLL and KP possessed a mixed inhibitor characteristic, while LL was a non-competitive inhibitor Based on molecular docking, K at N-terminus of KPLL is a key structure contributing to ACE inhibition as it can occupy the active site of ACE. Health benefits of an ACE inhibitory peptide greatly depend on permeability of the intact form.

Keywords: Bioactive peptide, angiotensin converting enzyme, permeability, kinetics

6.2 Introduction

Antihypertensive peptides inhibiting ACE have been extensively studied as they showed potential antihypertensive properties *in vivo*, which could be further developed to health-promoting food products. Most studies have been focused on production of ACE inhibitory peptides from different sources of food proteins by commercial enzymes, and purification and identification of these peptides. However, when peptides are consumed, they are further digested by gastrointestinal (GI) enzymes, leading to structural modification (Vermeirssen, Van Camp, & Verstraete, 2004). Further structural transformation takes place during permeation through intestinal epithelial cells upon hydrolysis by brush border proteases (BBPs) (Vermeirssen et al., 2004). It is likely that transported peptides could eventually exhibit different amino acid sequences and perhaps ACE inhibitory activity from those parent peptides originally identified in the protein hydrolysate. Thus, identified peptides reported in the literature might not be the "true" inhibitor affecting ACE *in vivo*.

It has been reported that tripeptides can be hydrolyzed by BBPs, such as aminotripeptidases or dipeptidases, resulting in fragmentation of tripeptides and formation of free amino acids and dipeptides (Ganapathy, Ganapathy, & Leibach, 1999; Vermeirssen et al., 2004). In addition, dipeptides are absorbed intact across the brush border membrane by a specific peptide transport system (Vermeirssen et al., 2004). The changes of oligopeptides during absorption are varied, depending on the specific peptide sequence. For example, based on studies using the Caco-2 cell model system, RPPGFSPFR and YAEER (Miguel et al., 2008), KPVAAP (Gallego et al., 2016), LPYPY (Isabelle ME, Xiu Min, David, & Eunice CY, 2017), and VLPVPQK (Vij,

Reddi, Kapila, & Kapila, 2016) were reported to be resistant to BBPs hydrolysis and can be transported intact. In contrast, LHLPLP (Quirós, Dávalos, Lasunción, Ramos, & Recio, 2008), VPP (Satake et al., 2002) and IPI (Isabelle ME et al., 2017) were partially hydrolyzed by BBPs, leading to changes of ACE inhibitory activity as compared to their parent counterparts. The relationship between peptide structure and fate of modification during peptide transportation has not been clearly established. Thus, it is necessary to elucidate transformation of the peptide of interest during *in vitro* intestinal absorption as the transported peptide would likely be physiologically important.

It has been recently found that cooked breast of Korat chicken, which is a crossbreed between a male Thai indigenous chicken and a female broiler, released ACE inhibitory peptides upon *in vitro* GI digestion (Sangsawad, Roytrakul, & Yongsawatdigul, 2017). This implied that chicken breast is a source of health-promoting peptides. The most potent ACE inhibitory peptides were identified to be KPLLCS, KPLL and ELFTT. However, their permeability through intestinal epithelium have not been determined. Structural changes of peptide during intestinal permeation inevitably affect ACE inhibitory activity. Identification of permeated peptides exhibiting ACE inhibitory activity would reveal "true" inhibitors that could reach blood stream and exert an antihypertensive action at the target tissues.

Molecular docking is widely used to predict the interaction between enzyme and inhibitors. This is a computational simulation approach that can be used as a tool to explain structure-activity relationship of ACE inhibitor peptides (Goodsell, 2009). It has been recently shown that ACE inhibitory peptides bind with ACE through various

interactions, including hydrogen bonds, hydrophobic, van der Waals, electrostatic and metal acceptor interaction (Li et al., 2014). Inhibition mechanism of peptides derived from in vitro GI digestion of cooked chicken meat has not been well established. Understandings of ACE inhibition of these peptides at the molecular level would be of critical for further development in nutraceutical and pharmaceutical products of these peptides.

The objective of this study was to investigate permeability of the three ACE inhibitory peptides identified from Korat chicken breast by using the Caco-2 cell monolayer model and to identify the potent transported ACE inhibitory peptides. In addition, the inhibition profile and binding interaction between peptide and ACE were investigated by using molecular docking simulation.

6.3 Materials and methods

6.3.1 Materials

Caco-2 (ATCC accession no. HTB-37) cells at passages 18 were obtained from the American Type Culture Collection (Manassas, VA, USA). Cell culture inserts (24-wells, high pore density polyethylene terephthalate, 0.4 μm pore size, 100±10 x 10^6/cm^2 pore density, 0.3 cm^2 grown surface) (Mississauga, ON, Canada). Eagle's minimum essential mineral (EMEM) medium, fetal bovine serum (FBS), penicillin, streptomycin, Hank's Balanced Salt Solution (HBSS), Dulbecco's Phosphate Buffer Saline (PBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), sodium dodecyl sulfate (SDS) and Lucifer yellow CH dilithium salt were purchased from Invitrogen Canada (Burlington, ON, Canada). Trypsin-EDTA (0.25%) and fetal bovine serum (FBS) were from GibcoTM (Thermo Fisher Scientific, Mississauga, ON, Canada).

Angiotensin-I-converting enzyme (ACE) from rabbit lung, hippuryl-histidyl-leucine tetrahydrate (HHL), hippuryl acid (HA), acetonitrile (ACN), trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Synthetic peptides including KPLLCS, KPLL, ELFTT, KPL, PLL, KP, PL and LL (≥ 98% purity) were purchased from GL Biochem, Shanghai, Ltd. Other chemicals and reagents were of analytical grade.

6.3.2 Determination of ACE inhibitory activity

The ACE inhibitory activity of peptide samples was determined according to Cheung and Li-Chan with some modifications (Cheung and Li-Chan, 2010). Synthetic peptides including KPLLCS, KPLL, ELFTT, KPL, PLL, KP, PL and LL were dissolved in assay buffer (0.05 M Tris-HCl buffer at pH 8.3 containing 0.3 M NaCl). The peptide sample (30 µL) was incubated with of ACE (30 µL of 0.083 U/mL in assay buffer) at 37˚C for 15 min, and then 150 µL of HHL (6.5 mM in assay buffer) was added. After further incubation at 37˚C for 1 h, 250 µL of 1 M HCl was added to stop the reaction of ACE. Then, ethyl acetate (1 mL) was added into the mixtures to extract the HA, after centrifugation, the upper layer (0.7 mL) was collected and dried using a heating block at 120˚C for 30 min. The dried HA was dissolved with 1.3 mL of deionizesd (DI) water and the content of HA was estimated with absorbance at 228 nm. Positive controls were prepared using DI water instead of peptide, while negative controls and samples were prepared by adding HCl before enzyme and peptide. ACE inhibition (%) was calculated as follows:

$$\text{ACE inhibitory activity (\%)} = \left[\frac{(\text{Abs}_{\text{PC}} - \text{Abs}_{\text{NC}}) - (\text{Abs}_{\text{PS}} - \text{Abs}_{\text{NS}})}{(\text{Abs}_{\text{PC}} - \text{Abs}_{\text{NC}})}\right] \text{x } 100$$

where Abs_{PC} is absorbance of the positive control, Abs_{NC} is absorbance of the negative control, Abs_{PS} is absorbance of reaction with peptide sample, and Abs_{NS} is absorbance of the negative sample.

The concentrations of peptides required for 50% inhibition of the enzyme activity (IC_{50}) were calculated from the logarithmic regression equations obtained from plotting the percent ACE inhibition against the sample concentrations. All of the ACE inhibitory assay of peptide were carried out in triplicate.

6.3.3 Caco-2 cells culture and cytotoxic assay

The Caco-2 human colon cancer cell line used in this study were at passages 22-29. Cells were cultured with EMEM medium containing 10% FBS, and antibiotics including 100 µg/mL of penicillin and 100 U/mL of streptomycin. Cells were incubated in fully humidified atmosphere with 5% CO_2 at 37˚C. Cells were subcultured every week using trypsin-EDTA treatment and medium was replaced every 2-3 days. For cytotoxic assay, cells were seeded at a density of 1 x 10^5 cells/cm^2 into 96-well culture plates with medium was replaced every 2-3 days in fully humidified atmosphere with 5% CO_2 at 37˚C. After grown for 21 days, the plates were replaced with the test sample at various concentrations of 0, 2, 4, 6, 8 mM (KPLLCS, KPLL and ELFTT dissolved in EMEM) and incubated at 37 °C in 5% CO_2 for 24 h. MTT was added (100 µL of 0.5 mg/mL in complete EMEM) and incubated in the dark for another 4 h. Then, SDS-HCl solution (100 µL of 10% SDS in 0.01 N HCL) was added for 16 h. Absorbance was measured at 570 nm using Multiskan Spectrum microplate spectrophotometer (Thermo Labsystems, VA, USA).

6.3.4 Caco-2 cell permeability assay

Caco-2 cells were cultured with EMEM medium containing 10% FBS, and antibiotics including 100 µg/mL of penicillin and 100 U/mL of streptomycin. Cells (0.3 mL) were seeded at a density of 2.5 x 10^5 cells/cm^2 into 24-well cell culture inserts, apical side (AP), and EMEM (0.7 mL) was into the basal side (BL). The cells were cultured for 21 days and EMEM was replaced every 2-3 days. Both the apical and basolateral media were removed and cells were gently washed twice with HBSS and then equilibrated at 37˚C for 1 h. Transepithelial electrical resistance (TEER) values were also monitored using a Millicell ERS-2 voltohmmeter (EMD Millipore, Darmstadt, Germany). Cells with TEER value higher than 250 ΩU/cm^2 were chosen for the transport study. Lucifer yellow content was also used to confirm monolayer integrity. The amount of Lucifer yellow transported to the basolateral compartment was monitored using a luminometer (Fluoroskan Ascent, Fisher Scientific UK Ltd., Loughborough, England, UK) at Ex_{425} nm and Em_{530} nm, which should be <10% of the initial concentration. The TEER values and Lucifer yellow content were measured before and after the experiment. For the peptide transport study, 0.3 mL of the test sample (6 mM of KPLLCS, KPLL and ELFTT dissolved in HBSS) was replaced in AP, and 0.7 mL of HBSS was replaced into BL. The plates were incubated under the atmosphere containing 5% CO_2 at 37˚C for 2 h. Subsequently, solutions from both compartments, AP and BL, at 0- and 2-h incubation were collected.

Quantification of peptide in AP and BL was estimated using reversed phase high performance liquid chromatography (RP-HPLC) with Phenomenex Jupiter™ C12 Proteo 90 Å HPLC column (250 mm x 4.6 mm, 4 µ; Phenomenex®, Torrance, USA). Mobile phase A was 0.1% TFA in DI water and B is 0.1% TFA in ACN. Gradient of

elution started at 0-20% of mobile phases B for 30 min, 20-95% of mobile phases B for 5 min, hold in 95% of mobile phases B for 5 min, and returning to 0% of mobile phases B for 10 min. Column temperature was controlled at 40˚C. Absorbance was measured at 214 and 280 nm. The external standard curve was established using synthetic peptides at various concentrations ranging from 0 - 50 µg dissolved in HBSS. Peptide contents of sample collected from AP and BL were then estimated based on the established standard curve. Percentage of peptides remaining and permeability were calculated according to the following equations:

1) $\text{Intact peptide remaining (\%)} = \frac{IP_{AP2}}{IP_{AP0}} \times 100$

2) $\text{Intact peptide transport (\%)} = \frac{IP_{BL2}}{IP_{AP0}} \times 100$

3) $\text{Total peptide transport (\%)} = \frac{TP_{BL2}}{IP_{AP0}} \times 100$

,where IP_{AP0} is the amount of intact peptides detected in the apical compartment at time 0 h (µg), IP_{AP2} is the amount of intact peptides detected in the apical compartment after 2-h incubation (µg), IP_{BL2} is the amount of intact peptides detected in the basal compartment after 2-h incubation (µg), and TP_{BL2} is total peptides (intact and fragment products) detected in basal compartment after 2-h incubation (µg). In addition, transported peptides in the BL compartment (300 µL) after 2-h incubation were collected and lyophilized. Lyophilized samples were then dissolved in DI water (30 µL) and analyzed for ACE inhibitory activity in triplicate as described above.

6.3.5 Degradation profiles of peptide

Since transported peptides from the parent peptide of KPLL exhibited the highest ACE inhibitory activity, they were selected for elucidating degradation profiles. Five synthetic peptides including, KPL, PLL, KP, PL, and LL, were used as external standards to identify breakdown products in the basolateral and apical solutions. They were solubilized in HBSS and quantitatively analyzed by RP-HPLC as described above. In addition, IC_{50} values of all possible degrading peptides were determined.

6.3.6 ACE inhibition profile

Due to the transported peptides of KPLL presented the highest ACE inhibitory activity and its degradation products including KP and LL exhibited ACE inhibitory activity, they were investigated for mode of inhibition. In the reaction assay, peptide samples (0, 50, 100 and 200 mM) dissolved in reaction buffer were incubated with various concentrations of HHL (0.5, 1, 3, 5 and 7 mM) dissolved in reaction buffer, and incubated at 37˚C for 1 h. ACE activity was determined as described above. The inhibitory kinetics was investigated by the Lineweaver–Burk plot (1/absorbance versus 1/HHL) and the inhibitor constant (K_i) was calculated by plotting between $1/V_{max}$ and the concentration of peptide.

6.3.7 Molecular docking

Molecular docking between peptides and ACE was carried out using GOLD program version 4.1 (Jones, Willett, Glen, Leach, and Taylor, 1997), and the peptide structure was created in MOL2 file by Discovery Studio Visualizer 2017 (Biovia, 2016). Three-dimensional structure of human ACE, PDB code: 1O86, was obtained from the Protein Data Bank (PDB). The hydrogen atoms and all water molecules were deleted and added in the structure at GOLD setup procedure and other parameters were

set as default. The docking results was visualized and analyzed by Discovery Studio Visualizer 2017. Genetic algorithm (GA) operations was applied for 50 runs. In addition, the *ChemScore fitness function* was selected for this docking.

6.3.8 Data analysis

All assays were conducted in triplicate. The results were analyzed by one-way analysis of variance (ANOVA) followed by Duncan's new multiple range tests and considered statistically significance when $p<0.05$. It was carried out for determination using SPSS 17.0 software (SPSS Inc, Chicago, USA).

6.4 Results and discussion

6.4.1 Permeability of peptides across Caco-2 cell monolayers

KPLLCS, KPLL and ELFTT did not show cytotoxicity in Caco-2 cells at the tested concentrations up to 6 mM ($p>0.05$; data not shown). Similarly, milk peptides (LKPTPEGDL, LPYPY, IPIQY and WR) and ovotransferrin peptide (IRW) at concentrations of 1-6 mM and 1-5 mM, respectively, also had no cytotoxicity on Caco-2 cells (Bejjani & Wu, 2013; Ding et al., 2014; Isabelle ME et al., 2017). Therefore, KPLLCS, KPLL and ELFTT were tested at 6 mM in the transport experiment.

These 3 peptides were degraded perhaps by cell surface enzymes during 2-h incubation as evidenced by HPLC chromatograms of the apical layer after 2 h incubation (AP-2, **Figure 6.1 a-c**.). ELFTT showed the highest percentage of intact peptide remaining (~85%) and the lowest percentage of intact peptide transported (0.07%). This result suggested that ELFTT was resistant to hydrolysis by BBPs and exhibited very limited permeability (**Table 6.1**). In contrast, only ~38% of KPLL was

remained after 2 h incubation, while KPLLCS was almost completely hydrolyzed (~3% of intact peptide remained), indicating that KPLLCS appeared to be very susceptible to hydrolysis by BBPs. Permeability of intact KPLL was found to be the highest (**Table 6.1, Figure 6.1c)**. Caco-2 cell has been reported to contain a large number of BBPs including Endopeptidase-24.11, Endopeptidase-24.18, Endopeptidase-3, Enteropeptidase, aminopeptidase N, aminopeptidase A, aminopeptidase P, aminopeptidase W, and dipeptidyl peptidase IV (DPP-IV) (Howell, Kenny, & Turner, 1992; Mahato, Narang, Thoma, & Miller, 2003). Substrate specificity of Endopeptidase-24.11 and Endopeptidase-24.18 is hydrophobic amino acids and aromatic amino acid, respectively, while Aminopeptidase A prefers A and E (Mahato, Narang, Thoma, & Miller, 2003). Therefore, ELFTT is likely to be hydrolyzed by

Table 6.1 Peptide permeability and ACE inhibitory activity of intact peptides and permeated peptides.

Parameters	**KPLLCS**	**ELFTT**	**KPLL**
IC_{50} (μM)	0.37 ± 0.12^{c}	6.35 ± 1.06^{b}	11.98 ± 1.65^{a}
Intact peptide remaining (%)[A]	3.42 ± 1.04^{c}	85.29 ± 3.98^{a}	37.81 ± 2.64^{b}
Intact peptide transport (%)[B]	0.11 ± 0.05^{b}	0.07 ± 0.02^{b}	0.58 ± 0.09^{a}
Total peptide transport (%)[C]	2.67 ± 0.59^{a}	3.26 ± 0.78^{a}	1.85 ± 0.14^{b}
ACE inhibitory activity of overall transported peptides[D]	26.87 ± 0.97^{b}	1.78 ± 1.49^{c}	56.03 ± 0.59^{a}

[A] Percentage of intact peptides remaining in the apical compartment after 2 h incubation.
[B] Percentage of intact peptides transported through Caco-2 cell monolayer after 2 h incubation.
[C] Percentage of the overall transported peptides (intact and fragment products transported through Caco-2 cell monolayer).
[D] ACE inhibitory activity of overall transported peptides in basal compartment after 2 h incubation.
Values are shown as mean ± standard deviation of three replicates. Superscript letters represent significantly different values ($p < 0.05$).

these BBPs due to the presence of L and F as well as E at the N-terminus. In addition, degradation of KPLL and KPLLCS can be initiated by DPP-IV, which shows specificity towards P and A. These results indicated that hydrolysis of bioactive peptides is likely to occur at the brush border membrane before absorption and that the degree of hydrolysis varies with the specific amino acid sequence. Interestingly, it was noted that the attachment of CS at the terminal end of KPLL to form the hexapeptide KPLLCS appeared to promote hydrolysis by BBPs. Our results indicated that the relationship between structure of peptides and the extent of permeability of an intact peptide through Caco-2 cells is varied.

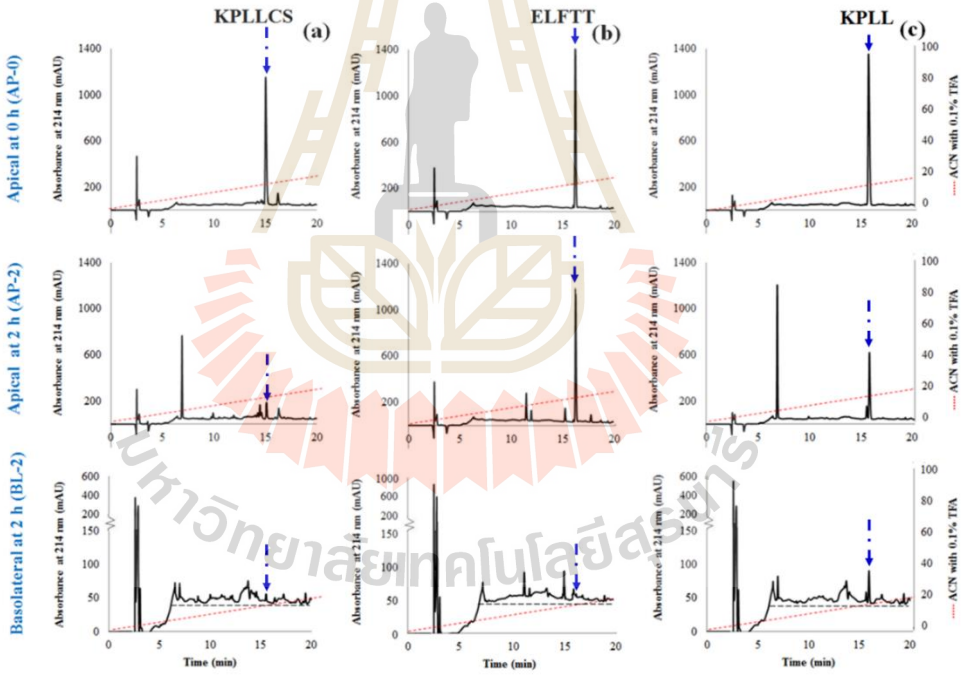

Figure 6.1 RP-HPLC elution profiles of the apical layer at time 0 h (AP-0; top panel) and after 2 h incubation (AP-2; middle panel), and the basal layer after 2 h incubation (BL-2; bottom panel) of KPLLCS (a), KPLL (b) and ELFTT (c) in the Caco-2 cell transport study

When the amounts of total transported peptides (intact and breakdown products) were considered, KPLLCS and ELFTT showed higher permeability than did KPLL (**Table 6.1**). This suggested that KPLLCS and ELFTT were degraded to smaller fragments that passed through Caco-2 cells. However, ACE inhibitory activity of transported peptides appeared to correlate with the amount of permeated intact peptide, but not the amount of total peptides **(Table 6.1)**. This suggested that smaller peptide fragments did not possess significant ACE inhibitory activity. It should also be noted that inhibitory potency of the original intact peptide does not guarantee its physiological significance, particularly if its permeability is limited. In this study, KPLL is originally the least effective ACE inhibitor, but it showed the highest permeability as an intact peptide in conjunction with the highest ACE inhibitory of the Caco-2 permeated peptides. This confirm the significant implication of bioavailability study of food bioactive peptides. In addition, permeability of an intact peptide appeared to be more relevant than that of total peptides. This could be because small peptide fragments that permeated through Caco-2 cells exhibited lesser ACE inhibitory activity. Thus, health benefits of a bioactive peptide do not rely only on inhibitory potency in the food product but also on permeability of the potent peptide in the intact form. However, permeability of total peptides should be considered when peptide fragments formed during permeation exhibit distinct ACE inhibitory activity.

Possible peptide fragments of KPLL are KPL, PLL, KP, PL, and LL with varied retention times on reverse-phase HPLC chromatogram (**Table 6.2)**. Among fragments of KPLL, KPL promoted ACE activity, but was not found in both AP-2 and BL-2. Thus, it was unlikely to be formed physiologically. The intact KPLL and their fragments, KP and LL, were found in both AP-2 and BL-2, while KPL and PLL were

not found in either apical or basal compartments **(Figure 6.2b and c)**. KPLL and its breakdown products, KP and LL, appeared to be the major transported peptides (**Figure 6.2c**). DPP-IV is a cell-surface protease belonging to the prolyl-oligopeptidase family and it selectively removes the N-terminal dipeptide from peptides with proline or alanine in the second position (Lambeir, Durinx, Scharpé, & De Meester, 2003). This enzyme is believed to be the most active in brush border membrane, particularly once the Caco-2 cells are completely differentiated (Bo & Bo, 2017; Howell, Kenny, & Turner, 1992). Thus, DPP-IV might hydrolyze KPLL to KP and LL. These three peptides were able to pass through intestinal epithelial cells, exerting ACE inhibitory activity of permeated peptides. In addition, our previous study showed that KPLL was resistant to plasmin hydrolysis, a blood protease (Sangsawad et al., 2017), therefore, KPLL could be transported peptide reaching the target organ and exerting an antihypertensive effect *in vivo*.

Table 6.2 RP-HPLC retention time and IC_{50} value of KPLL and its fragments that found in the basal compartment.

Sequence	**Mass**	**Retention time (min)**[A]	**IC_{50} (μM)**
KPLL	469.6	16.0	11.8 ± 1.7[a]
KPL	356.5	11.4	Promote ACE activity
PLL	341.5	11.6	4487.1 ± 174.3[c]
KP	243.3	6.9	130.0 ± 3.9[b]
PL	228.3	12.5	8037.8 ± 90.1[d]
LL	244.3	15.8	7870.3 ± 138.6[e]

[A] Retention time of peptides after RP-HPLC.
Values are shown as mean ± standard deviation of three replicates. Superscript letters represent significantly different values ($p < 0.05$).
ND = not detected.

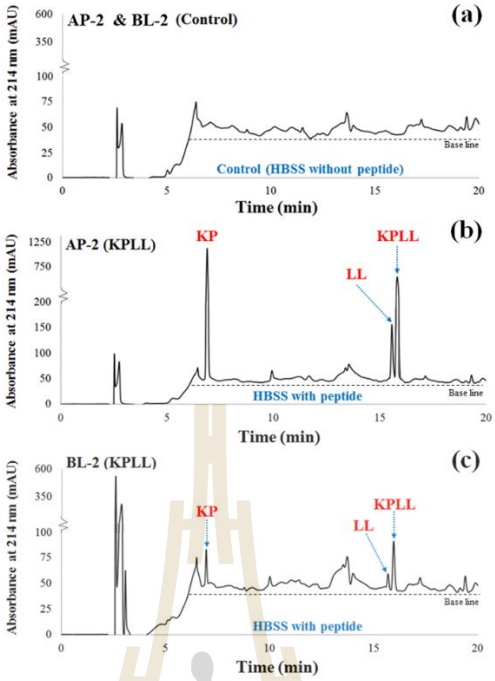

Figure 6.2 RP-HPLC elution profiles of the apical and basal layers after 2 h transport through Caco-2 cell monolayer for control buffer without peptide (a), KPLL peptide profile of the apical layer (AP-2) (b) and KPLL peptide profile of the basal layer (BL-2) (c).

It has been accepted that oligopeptides from GI digestion can be further hydrolyzed by BBPs to free amino acids, di- or tripeptides during transport across intestinal epithelial cells. These di- and tripeptides are absorbed intact across the brush border membrane by a specific peptide transport system via PepT1(Ganapathy et al., 1999; Vermeirssen et al., 2004). Tetra- and penta-peptides are transported through the paracellular tight-junction pathway (Hong, Tanaka, Koyanagi, Shen, & Matsui, 2016). Thus, it is reasonable to assume that KPLL was transported through Caco-2 cell monolayer via paracellular route and their fragments were transported via PepT1. Caco-2 cell monolayers are known to be tighter than *in vivo* human intestinal cells

(Vermeirssen et al., 2004). Thus, *in vivo* peptide absorption is expected to be higher than that obtained from Caco-2 cell monolayers.

6.4.2 Mode of action of KPLL, KP and LL

Since KPLL and its fragments, KP and LL, showed significant permeation through intestinal epithelial cells and exhibited ACE inhibitory activity, they were selected for determining mode of inhibition. KPLL and KP showed a mixed mode inhibition, implying that these peptides bind to ACE at the active site as well as other sites (**Figure 6.3a and b**). In case of binding to the active site, it could hamper substrate binding, while binding at regions outside the catalytic site could lead to enzyme conformational changes that reduced substrate affinity. LL appears to be a non-competitive inhibitor, suggesting that it interacts with free enzyme and/or enzyme-substrate complex with similar affinity (**Figure 6.3c**). The inhibitor constant (K_i) value of KPLL, KP and LL for ACE inhibition were calculated to be 0.09, 3.86 and 56.74 mM, respectively. KPLL exhibited the greatest affinity for ACE as compared to its breakdown fragments.

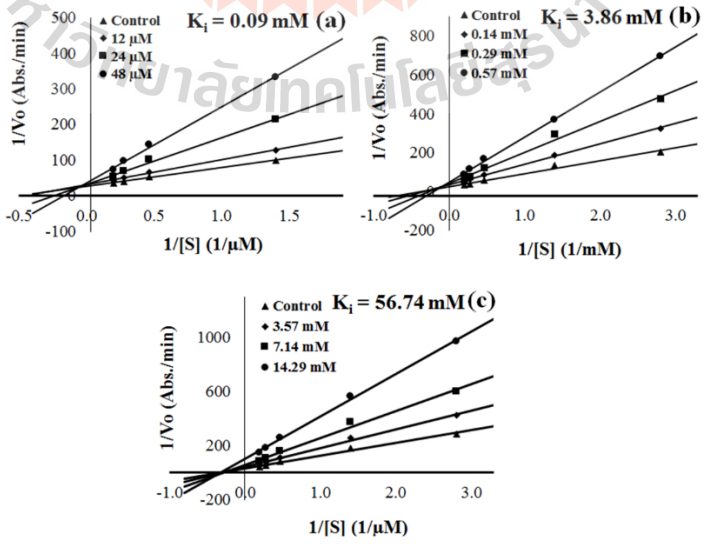

Figure 6.3 ACE inhibition kinetics plots of KPLL (a), KP (b) and LL (c).

Competitive and non-competitive inhibitors have been the most frequently reported ACE inhibitors. Peptides showing mixed mode were FEDYVPLSCF and FNVPLYE, which were derived from salmon byproduct hydrolysate with K_i of 10.48 and 77.84 µM, respectively (Ahn, Jeon, Kim, & Je, 2012). Non-competitive inhibitors included VWDPPKFD derived from salmon protein hydrolysate with K_i of 19.52 µM (Ahn et al., 2012), FLEK and MEKHS derived from cupuassu seed protein hydrolysate with Ki of, 18.50 and 13.31 µM, respectively (Ahn et al., 2012; da Cruz, Pimenta, de Melo, & Nascimento, 2016). It should be noted that these reported peptides have not been investigated for their permeability. Based on K_i value determined from our study, the transported peptide, KPLL, appeared to bind to ACE more effectively than previously reported peptides. It can be assumed that the transported KPLL and its breakdown fragments were likely to reach the target tissues and interact with both active site and non-active sites of ACE.

6.4.3 Molecular docking of KPLL, KP and LL

To explore the molecular binding between transported peptides and ACE, the most stabilized poses of docking simulations were carried out. KPLL and KP were performed with mixed inhibition mode, while LL was modeled based on non-competitive mode. Interactions of these 3 peptides and ACE included hydrogen bonds, hydrophobic interactions, electrostatic interactions, van der Waals and metal acceptor. The predicted 3D structure of peptide-ACE complex for the docking simulation is shown in **Figure 6.4a.** KPLL and KP successfully enter the deep narrow channel of ACE at the same position after docking, while LL was located outside. Generally, ACE-HHL complex involves 7 amino acid residues of Y523, H513, H353, Try520,

K511, Q281 and A354, while the catalytic site is composed of E384 and a zinc ion bound with H383, H387 and E411(tri - coordination) (Wang, Wu, Xu, Xie, & Guo, 2011). Based on the best pose of KPLL- ACE complex, K_1 of KPLL was bound with zinc ion by van der Waals interaction and interacted with E384 through H-bond (**Figure 6.4b**). Thus, K_1 seems to be the key element contributing to ACE inhibition. ACE inhibitors, captopril, enalaprilat and lisinopril, are also bound to zinc ion, rendering significant conformational changes in ACE. Displacement of zinc ion from the active site resulted in significant inhibition of ACE (Ghassem, Arihara, & Babji, 2012; Masuyer, Schwager, Sturrock, Isaac, & Acharya, 2012).

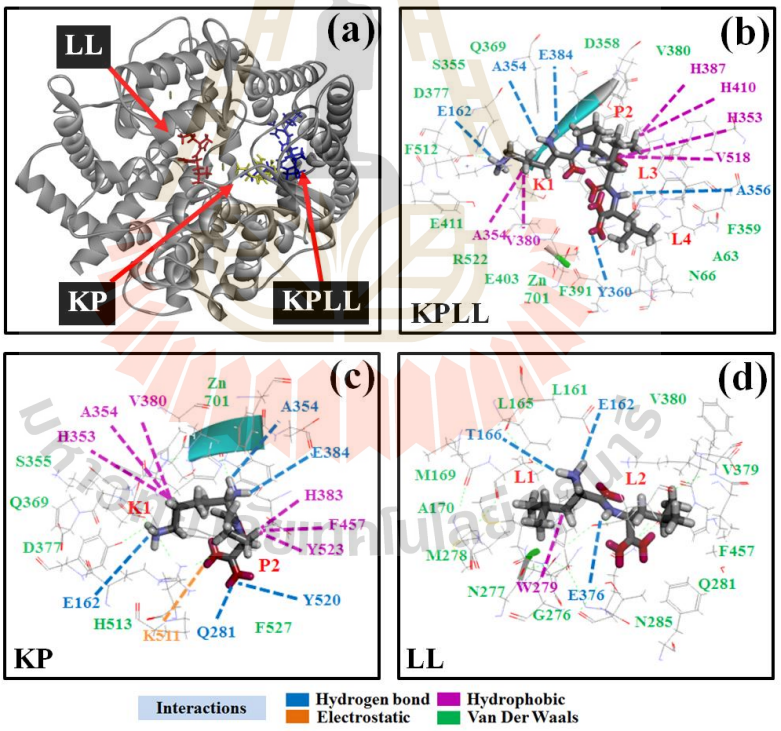

Figure 6.4 Docking simulation of peptide binding with ACE, predicted 3D structure of peptide-ACE complex (a), interactions between peptides and the residues of ACE; KPLL (b), KP (c) and LL (d).

In addition, KPLL also interacted with ACE's binding sites (H353 and A354), tri-coordination for the zinc ion (H387 and E411) and other 13 van der Waals interactions (**Figure 6.4b**). These interactions may cause distortion of the ACE structure, leading to a reduction in ACE activity.

The best pose of KP-ACE complex is illustrated in **Figure 6.4c.** K_1 of KP was also bound with zinc ion by van der Waals interaction and E384 by H-bond. Moreover, KP could interact with 6 binding sites, and other 5 van der Waals interactions. Both KPLL and KP occupied the same position of ACE active site and binding site. However, the best pose of LL-ACE complex showed different binding sites (**Figure 6.4d**). It interacted with ACE at E162, T166, E376 and W279 through H-bond and hydrophobic interactions, and other 12 van der Waals interactions.

6.5 Conclusions

Potency of ACE inhibitory peptides under physiological condition greatly depends on permeability of a potent peptide in an intact form. The transported peptide of KPLL revealed the highest ACE inhibitory activity. KPLL was hydrolyzed by surface brush border enzymes to KP and LL. All intact and peptide fragments were transported through Caco-2 cells. Enzyme inhibition kinetics and molecular docking studies illustrated that KPLL and KP exhibit mixed mode inhibition (K_i of 0.09 and 3.86 mM, respectively), while LL was a non-competitive inhibitor. K_1 at N-terminus of KPLL is a key residue required for ACE inhibition. The tetrapeptide KPLL derived from the digest of Korat chicken breast cooked at 70˚C for 30 min is a promising ACE-inhibitory peptide shown to be permeable in the Caco-2 cell model, and could potentially exert health benefits *in vivo*.

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CHAPTER VII

SUMMARY

Breast of Korat crossbred chicken (Kc) and commercial broiler (Br) were cooked under various conditions, namely heating at 70 °C for 30 min (H-0.5) and 24 h (H-24), autoclaving (AC) at 121°C for 15 min (AC-15) and 60 min (AC-60). The H-0.5 promoted metal chelating activity and angiotensin converting enzyme (ACE) inhibitory activity of Kc digest, and ferric-reducing antioxidant power assay (FRAP) as well as anti-liposome oxidation of Br digest. The Br/H-0.5 and Kc/AC-15 digests exhibited the greatest protective effect in HepG2 cells. Cytoprotection of Kc/AC-15 digest was evidenced by intracellular ROS scavenging activity in a dose-related manner. In addition, digest of Kc/H-0.5 exhibited the highest ACE inhibitory activity. The novel potent ACE inhibitory peptides were identified as KPLLCS, ELFTT and KPLL with IC_{50} values of 2.57, 44.46, and 76.96 μM, respectively. ELFTT and KPLL were stable against plasmin digestion for up to 60 min.

Peptides permeated through 1-kDa ultrafiltration membrane of Korat chicken breast at H-0.5 digest showed higher permeability through Caco-2 cells monolayer and ACE inhibitory activity than did those of AC-60 digest. Among nine permeated peptides, APP exerted the highest inhibition and was derived from myosin. APP was a non-competitive ACE inhibitor with K_i of 0.93 μM. It formed hydrogen bonds, attractive electrostatic charge interaction, and van der Waals interactions with ACE, leading to reduced ACE activity.

When permeability of the most potent ACE inhibitory peptides was studied, KPLL was hydrolyzed by surface brush border enzymes to KP and LL, which were all permeable. Transported peptides of KPLL and its degraded peptides also showed the highest ACE inhibitory activity. Enzyme inhibition kinetics and molecular docking studies illustrated that KPLL and KP exhibited mixed mode inhibition with K_i of 0.09 and 3.86 mM, respectively, while LL was a non-competitive inhibitor. K_1 at N-terminus of KPLL is a key residue required for ACE inhibition.

These results revealed that digest of Kc/H-0.5 was a promising precursor of antioxidant and ACE inhibitory peptides upon GI digestion. ACE inhibitory peptides of digest can be transported through Caco-2 cell monolayers. KPLL was permeable in the Caco-2 cell model, and could potentially exert health benefits. Further *in vivo* studies would be necessary to prove the efficacy of lowering blood pressure of Kc digest.

BIOGRAPHY

Papungkorn Sangsawad was born in January 29, 1987, at Lamphun, Thailand. He studied for his high school diploma at TheeraKarn Banhong School (2001-2006). In 2010, he received Bachelor of Science (Food Science and Technology) with first class honor from Maejo University, Chaing Mai, Thailand. Upon his graduation, he continued his Ph.D. study in Food Science and Technology under the Royal Golden Jubilee Scholarship granted by Thailand Research Fund at Suranaree University of Technology. In 2015, he got a chance to be a visiting scholar at The University of British Columbia, Canada to work on permeability of ACE inhibitory peptides from Korat chicken.

In 2016, he has published the article entitled "Chemical and cellular antioxidant activities of chicken breast muscle subjected to various thermal treatments followed by simulated gastrointestinal digestion" in Journal of Food Science, and "Angiotensin converting enzyme (ACE) inhibitory peptides derived from the simulated *in vitro* gastrointestinal digestion of cooked chicken breast" in Journal of Functional Foods, in 2017. Another work has been submitting to Journal of Agricultural and Food Chemistry under review with the title of "angiotensin converting enzyme (ACE) inhibitory peptides derived from the simulated *in vitro* gastrointestinal digestion of cooked chicken muscles: Permeability and identification of the transported peptides". The final work in the title of "Permeability and structural transformation of angiotensin I-converting enzyme (ACE) peptides through Caco-2 cell permeation" has been preparing to submit on Journal of Functional Foods.

He also presented oral and poster presentation including: 1) RGJ- Ph. D. Congress XV (Chonburi, Thailand, 11-13 June 2014) with poster presentation in the title of the impact of thermal treatments of chicken breast protein on simulated gastrointestinal digestion and formation of antioxidant peptides, 2) RGJ Seminar Series 116 (Khonkaen University, Thailand, 9-10 August 2016) with the best oral presentation in title of ACE inhibitory peptides of chicken breast muscle subjected to various thermal treatments followed by simulated gastrointestinal digestion, and 3) Food Innovation Asia Conference (Bangkok, Thailand, 15 -17 June 2017) with the first runner-up oral presentation in title of cooked Korat-chicken breast as a potential source of antioxidant and angiotensin converting enzyme (ACE) inhibitory peptides upon *in vitro* gastrointestinal digestion.

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