Developing Antihypertensive Peptides from Spent Laying Hen Muscle Proteins

by

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A thesis submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

Food Science and Technology

Department of Agricultural, Food and Nutritional Science

University of Alberta

ABSTRACT

Hypertension is the leading cause of global morbidity and mortality, afflicting > 20% of adults worldwide (~1 in 4 in Canada). Pharmaceutical drugs are the primary treatment for hypertension, but are generally associated with side effects. Recently, food-derived antihypertensive peptides are emerging alternatives. Central to the pathophysiology of hypertension is the renin-angiotensin system, which is regulated by two enzymes, angiotensin-converting enzyme (ACE) and ACE2. ACE converts angiotensin (Ang I) into Ang II that elevates blood pressure (BP) via Ang II type 1 receptor (AT_1R), whereas ACE2 reverts this process by degrading Ang II into Ang (1–7) followed by binding with Mas receptor (MasR).

Spent hens are laying hens reaching the end of egg-laying cycle, with an average yearly production of > 30 million in Canada over the last decade. Despite being a byproduct, spent hen is a rich source of muscle proteins that can be biotransformed into bioactive peptides with antihypertensive properties. The overall objective of this thesis is to purify and characterize novel antihypertensive peptides from spent hen muscle proteins targeting both the ACE-Ang II-AT_1R (by inhibiting ACE) and the ACE2-Ang (1-7)-MasR (by upregulating ACE2) axes.

In the first study, eighteen spent hen muscle protein hydrolysates (SPHs) were prepared and were screened for the ACE inhibitory (ACEi), ACE2 upregulating (ACE2u), antioxidant, and anti-inflammatory activities, as well as their fates during gastrointestinal digestion and transepithelial transport. Three SPHs were screened and fed to spontaneously hypertensive rat (SHR), while only SPH prepared by thermoase PC10F (SPH-T) reduced BP. The second study further investigated the antihypertensive effect of SPH-T at two doses (high dose, 1,000 mg/kg body weight [BW], and low dose, 250 mg/kg BW). Its BP reduction was associated with increased circulating ACE2

and Ang (1-7) but lowered Ang II levels, upregulated vascular expression of ACE2, as well as ameliorated vascular inflammation, oxidative stress, and fibrosis.

The third study aims to purify ACEi peptides and ACE2u peptides from SPH-T. Finally, five potent ACEi peptides, VRP, LKY, VRY, KYKA, and LKYKA (IC_{50} values of 0.034–5.77 μg/mL) and four ACE2u peptides, VKW, VHPKESF, VVHPKESF (V-F), and VAQWRTKYETDAIQR-TEELEEAKKK (upregulated ACE2 expression by 0.52–0.84 folds) were identified. Among them, four with the highest ACEi and/or ACE2u activities from the major muscle proteins were selected for animal study; they were categorized into 3 groups: VRP (ACEi activity), V-F (ACE2u activity), and LKY and VRY (both ACEi and ACE2u activities).

Prior to assessing the *in vivo* efficacies of VRP, LKY, VRY, V-F, their antioxidant and anti-inflammatory effects were evaluated in vascular smooth muscle A7r5 cells (VSMCs) and endothelial EA.hy926 cells (ECs), upon stimulation by Ang II and tumor necrosis factor alpha (TNFα), respectively. All four peptides showed antioxidant activity in VSMCs, whereas only V-F attenuated inflammation, manifested by inhibiting expressions of inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX2) in VSMCs and VCAM-1 expression in ECs. VRP, LKY, and VRY exhibited antioxidant activity by acting as direct free radical scavengers whereas V-F also activated endogenous antioxidant enzymes. The anti-inflammatory effect of V-F was likely through modulation of the nuclear factor kappa B p65 and p38 mitogen-activated protein kinase pathways and was partially dependent on MasR.

VRP, LKY, VRY, and V-F were orally administrated to SHRs at a dose of 15 mg/kg BW; only V-F reduced BP significantly over an 18-day treatment period. Associated with BP reduction were the increased circulating ACE2 and Ang (1-7) but reduced Ang II levels, upregulated vascular

ACE2 and MasR expressions, as well as attenuated vascular inflammation and oxidative stress. Notably, V-F was not gastrointestinal stable and its fragment, VVHPK, was also an ACE2u peptide.

The present study demonstrated the presence of antihypertensive peptides in spent hen muscle proteins, supporting the use of spent hens as antihypertensive functional food ingredients. Furthermore, this study reported for the first time the antihypertensive effect of an ACE2u peptide (V-F) identified using *in vitro* method. The discovery and characterization of ACE2u peptides in this work manifested their feasibilities of being isolated from proteins and uses for hypertension management.

PREFACE

This thesis is an original work done by Hongbing Fan and has been written according to the guidelines for a paper format thesis of the Faculty of Graduate Studies and Research at the University of Alberta. The concept of the thesis was originated from my supervisor Dr. Jianping Wu. The research was funded by the Natural Sciences and Engineering Research Council (NSERC) of Canada and Egg Farmers of Canada and also received financial support from China Scholarship Council (CSC), Alberta Innovates Technology Futures (AITF), the Killam Trusts, and the American Oil Chemists` Society (AOCS) Thomas H. Smouse Memorial Fellowship. The experimental protocols of animal studies in Chapters 3, 4, and 7 of this thesis were approved by the University of Alberta Animal Care and Use Committee (Protocol #AUP00001571) per the guidelines issued by the Canada Council on Animal Care and were adhered to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health.

This thesis consists of eight chapters. Chapter 1 provides a general introduction on the research background and objectives. Chapter 2 provides a detailed literature review regarding spent hen utilization in the egg industry and food-derived antihypertensive peptides; a part of Chapter 2 has been accepted as "Food Peptides in Blood Pressure Regulation" in "*Food Proteins and Peptides: Emerging Biofunctions, Food and Biomaterial Applications*" by the Royal Society of Chemistry. Chapter 3 prepares a spent hen muscle protein hydrolysate using thermoase (SPH-T) with antihypertensive effect in spontaneously hypertensive rats (SHR); the whole chapter has been published as "Spent hen protein hydrolysate with good gastrointestinal stability and permeability in Caco-2 cells shows antihypertensive activity in SHR" in *Foods* [2020, *9*(10), 1384]. Chapter 4 explores the antihypertensive effect of SPH-T and its underlying mechanisms in SHR and is ready for submission. Chapter 5 identifies the angiotensin-converting enzyme (ACE) inhibitory (ACEi)

and ACE2 upregulating (ACE2u) peptides from SPH-T; the whole chapter has been published as "Purification and identification of novel ACE inhibitory and ACE2 upregulating peptides from spent hen muscle proteins" in *Food Chemistry* [2021, *345*, 128867]. Chapter 6 further studies the antioxidant and anti-inflammatory effects of SPH-T derived peptides in vascular cells; a part of this chapter entitled "Spent hen muscle protein-derived RAS regulating peptides show antioxidant activity in vascular cells" has been accepted in *Antioxidants* [2021, *10*(2), 290]. Chapter 7 investigates the antihypertensive effect of SPH-T derived peptides in SHR and is ready for submission. Chapter 8 concludes the key findings and significance of this thesis as well as recommendations for future research.

Dr. Jianping Wu contributed to the experimental design, data interpretation, manuscript edits of the entire thesis. Dr. Sandra T. Davidge contributed to the experimental design (Chapter 4), data interpretation, and manuscript edits (Chapters 4 and 7). Dr. Wang Liao has assisted me in animal husbandry and data collection of Chapters 3 and 4. Dr. Floor Spaans and Mr. Mazhar Pasha have assisted me in the vascular function and immunostaining studies as well as data interpretation of Chapters 4 and 7. Mr. Khushwant S. Bhullar has helped with the antioxidant assay in Chapter 6. Mrs. Sareh Panahi provided technical support in animal work including the surgery (Chapters 3 and 4) and isolation of mesenteric arteries for vascular function studies (Chapters 4 and 7). Dr. Lin-Fu Zhu performed animal surgery in Chapter 7, while Dr. Nan Shang helped with the vascular function studies. I was responsible for the literature search relevant to all the chapters, designing and performing laboratory experiments, data collection, analysis, and interpretation, as well as manuscript drafting, edits, and revisions.

DEDICATION

Dedicated to my beloved family and many friends...

ACKNOWLEDGMENTS

I have received tremendous support and assistance throughout my doctorate program at the University of Alberta.

First and foremost, I would like to express my deepest gratitude to my supervisor Dr. Jianping Wu for providing me the opportunity to pursue my graduate studies in his lab and for his countless mentorship, support, encouragement, and guidance throughout the program. His insightful feedback pushed me to improve my thinking in formulating research questions and experimental designs and brought my work to a higher level. I would like to extend my deepest gratitude to Dr. Sandra T. Davidge and Dr. Leluo Guan for being my supervisory committee members and for their support, guidance, and constructive criticism to my research; special thanks to Dr. Davidge for letting me use the research facilities in her lab. I would also like to express my sincere thanks to Dr. Lingyun Chen for serving as the arm's length examiner and to Dr. Linda Giblin for accepting to be the external examiner for my dissertation defense.

I would like to thank the Natural Sciences and Engineering Research Council of Canada (NSERC) and Egg Farmers of Canada for the funding provided throughout my doctorate program. The financial support received from China Scholarship Council (CSC), Alberta Innovates Technology Futures (AITF), the Killam Trusts, and American Oil Chemists` Society (AOCS) Thomas H. Smouse Memorial Fellowship is greatly appreciated. I also wish to thank the Faculty of Graduate Studies and Research (FGSR), the Faculty of Agricultural, Life and Environmental Sciences (ALES), and the Department of Agricultural, Food and Nutritional Science (AFNS) for their financial support.

I am grateful to Dr. Yuchen Gu and Dr. Wenlin Yu, whose previous work on spent hen proteins has paved the way for this thesis. I also wish to thank Dr. Wang Liao and Mr. Khushwant S.

Bhullar for the collaboration of animal and cell studies. Many thanks to Mrs. Sareh Panahi and Dr. Lin-fu Zhu for providing technical assistance in animal surgery and to Dr. Jude S. Morton, Dr. Floor Spaans, Mrs. Sareh Panahi, Ms. Anita Quon, and Mr. Mazhar Pasha for the technical assistance in vascular biology and tissue immunostaining experiments as well as data interpretation. Thanks should also go to Ms. Jing Zheng for her assistance in collecting and interpreting the mass spectrometry data. Some figures and graphical abstracts such as in Chapters 4 and 7 were created with BioRender.com (with the publishing license), which is also appreciated.

I would like to extend my thanks to the University of Alberta Cardiovascular Research Center for letting me use research facilities for animal work; special thanks to Jody Levasseur and Amy Barr for coordinating the animal housing and blood pressure recording schedules. I am also grateful to the Graduate Program support staff of ANFS, Jody Forslund, Kelly Berdahl, Tonia Harris, and Holly Horvath. Thanks should also go to AFNS Store Managers, Robin Miles and Eduardo Rodriguez, for their assistance in chemical ordering.

I cannot wait to express my thanks to my fellow graduate students and colleagues, both past and present, in Dr. Wu's laboratory: Dr. Subhadeep Chakrabarti, Dr. Jiapei Wang, Dr. Hui Hong, Dr. Qingbiao Xu, Dr. Myoungjin Son, Dr. Meram Chalamaiah, Dr. Nandika Bandara, Dr. Xiaohong Sun, Dr. Yussef Esparza, Dr. Ali Akbari, Dr. Forough Jahandideh, Dr. Jiandong Ren, Dr. Selene Gonzales, Dr. Nan Shang, Dr. Ping Liu, Dr. Meiyu Liu, Dr. Xiaofeng Ren, Dr. Qiufang Liang, Dr. Liang Chen, Dr. Long Ding, Dr. Liying Wang, Dr. Xiao Wang, Qiyi Li, Shreyak Chaplot, Xu Jiang, Sule Keskin Ulug, Harshita Arora, Pauline Helene Duffuler, Manjot Singh Kharbanda, Xiaoyu Bao, Zihan Wang, and many others.

I am deeply indebted to my grandparents, my parents, and my sister Jiamin for their endless love, trust, and encouragement throughout my life. I would also like to thank my parents-in-law

for their love and support. Word cannot express my gratitude towards my loving wife Zhiying and my baby daughter Mishu for their unconditional love and for always being my side. The completion of my dissertation would not have been possible without their support.

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LIST OF ABBREVIATIONS

ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)

ACE: Angiotensin-converting enzyme

ACEi: Angiotensin-converting enzyme inhibitory

ACE2: Angiotensin-converting enzyme 2

ACE2u: Angiotensin-converting enzyme 2 upregulating

ACN: Acetonitrile

Ang I: Angiotensin I

Ang II: Angiotensin II

Ang (1-7): Angiotensin 1-7

AT_1R: Angiotensin II type 1 receptor

AT_2R: Angiotensin II type 2 receptor

AUC: Area under the curve

BCA: bicinchoninic acid

BP: Blood pressure

BW: Body weight

Caco-2: Human colon carcinoma cells

CEC: Cation exchange chromatography

COX2: Cyclooxygenase 2

COVID (-19): Coronavirus disease (2019)

CV: Column volume

CVDs: Cardiovascular diseases

DASH: Dietary Approaches to Stop Hypertension

DBP: Diastolic blood pressure

DH: Degree of hydrolysis

DHE: Dihydroethidium

DMEM: Dulbecco's modified Eagle's medium

DPPH: 2,2-diphenyl-1-picrylhydrazyl

DTT: Dithiothreitol

ECE: Endothelin-converting enzyme

ECs: Endothelial (EA.hy 926) cells

EDHF: Endothelium-derived hyperpolarizing factor

EDTA: Ethylenediaminetetraacetic acid

EDUF: Electrodialysis with ultrafiltration membranes

eNOS: Endothelial nitric oxide synthase

ERK1/2: Extracellular signal-regulated kinase 1/2

ET-1: Endothelin-1

FBS: Fetal bovine serum

HBSS: Hanks balanced salt solution

HEPES: 4-(2−68 hydroxyethyl)-1-piperazineethanesulfonic acid

HHL: N-hippuryl-His-Leu

HR: Heart rate

ICAM-1: Intracellular adhesion molecule-1

IEC: Ion exchange chromatography

IL-1α: Interleukin-1α

IL-15: Interleukin-15

iNOS: Inducible nitric oxide synthase

JNK: c-Jun N-terminal kinase

LC-MS/MS: Liquid chromatography with tandem mass spectrometry

LMW: Low-molecular-weight

L-NAME: N(ω)-nitro-L-arginine methyl ester

MA: Mesenteric artery

MAP: Mean arterial pressure

MasR: Mas receptor

Mch: Methacholine

MCP-1: Monocyte chemoattractant protein-1

MMP: Metalloprotease

NADPH, Nicotinamide adenine dinucleotide phosphate

NEAA: Nonessential amino acids

NF-κB: Nuclear factor kappa B

NO: Nitric oxide

NOS: Nitric oxide synthase

$ONOO^-$: Peroxynitrite anion

p38 MAPK: p38 mitogen-activated protein kinase

PGI_2: Prostaglandin I_2

PE: Phenylephrine

QSAR: Quantitative structure-activity relationship

RAS: Renin-angiotensin system

ROS: Reactive oxygen species

RP-HPLC: Reverse-phase high performance liquid chromatography

RP-UPLC: Reverse-phase ultra-performance liquid chromatography

sACE: Somatic ACE

SBP: Systolic blood pressure

SCW: Subcritical water

SEC: Size exclusion chromatography

SDS: Sodium dodecyl sulfate

SHR: Spontaneously hypertensive rat

SNP: Sodium nitroprusside

SPH: Spent hen muscle protein hydrolysate

SPH-T: Spent hen muscle protein hydrolysate prepared by thermoase PC10F

SPH-P: Spent hen muscle protein hydrolysate prepared by pepsin

SPH-26L: Spent hen muscle protein hydrolysate prepared by Protex 26L

tACE: Testicular ACE

TEER: Transepithelial electrical resistance

TFA: Trifluoroacetic acid

TGFß: Transforming growth factor beta

TNBS: Methane-sulfonic acid, 2,4,6-trinitrobenzenesulfonic acid

TNFα: Tumor necrosis factor alpha

TNF-R1: Tumor necrosis factor alpha receptor 1

TNF-R2: Tumor necrosis factor alpha receptor 2

UV-A: Ultraviolet A

VDCC: Voltage-dependent Ca^{2+} channels

VCAM-1: Vascular cell adhesion molecule-1

VSMCs: Vascular smooth muscle (A7r5) cells

V-F: VVHPKESF

LIST OF AMINO ACIDS

A: Alanine (Ala)

C: Cysteine (Cys)

D: Aspartic acid (Asp)

E: Glutamic acid (Glu)

F: Phenylalanine (Phe)

G: Glycine (Gly)

H: Histidine (His)

I: Isoleucine (Ile)

K: Lysine (Lys)

L: Leucine (Leu)

M: Methionine (Met)

N: Asparagine (Asn)

P: Proline (Pro)

Q: Glutamine (Glu)

R: Arginine (Arg)

S (Ser): Serine

T (Thr): Threonine

V: Valine (Val)

W: Tryptophan (Trp)

Y: Tyrosine (Tyr)

CHAPTER 1 – General Introduction and Objectives

1.1 Hypertension and renin-angiotensin system (RAS)

Hypertension, an important risk factor for cardiovascular diseases (CVDs), afflicts more than 20% of adults worldwide (Whelton et al., 2018; Zhou et al., 2017). The prevalence remains underestimated, given hypertension has been redefined as persistently-elevated systolic and diastolic blood pressure (BP) above 130/80 mm Hg, other than the previous 140/80 mmHg, by the America College of Cardiology and the American Heart Association recently (Whelton et al., 2018). In Canada, the hypertension-related annual cost was estimated to be $20.5 billion in 2020, accounting for over 10% of the health expenditure (Weaver et al., 2015).

The RAS plays a pivotal role in the pathogenesis of hypertension. Within the RAS, angiotensin-converting enzyme (ACE) converts angiotensin (Ang) I to Ang II that binds with the Ang II type 1 receptor (AT_1R), leading to elevated BP. While, ACE2 degrades Ang II into Ang (1-7) that binds with the Mas receptor (MasR), counteracting the detrimental effects of the classical ACE-Ang II-AT_1R axis through the ACE2-Ang (1-7)-MasR axis (Wu, Liao, & Udenigwe, 2017). Inhibition of ACE is the major target for BP regulation and thus is widely used for characterizing food-derived antihypertensive peptides (Wu et al., 2017). Recently, however, an egg-derived, IRW, initially identified as an ACE inhibitory (ACEi) peptide, was proved to exert its antihypertensive effect mainly via upregulating ACE2-Ang (1-7)-MasR axis (Liao, et al., 2019a). Furthermore, a few ACE2 upregulating (ACE2u) peptides have been identified from food proteins (Liao et al., 2019b). These indicated a new mechanism of antihypertensive peptides (ACE2u peptides) that is via upregulating the ACE2-Ang (1-7)-MasR axis and the feasibility of identifying ACE2u peptides from food proteins. Thus, ACE2u peptides are an emerging direction for developing novel antihypertensive peptides.

1.1.1 ACE

ACE, discovered in 1956, is a key enzyme in the RAS which produces the potent vasopressor Ang II (Skeggs, Kahn, & Shumway, 1956). There are two isoforms of ACE: testicular ACE (tACE) and somatic ACE (sACE). tACE is present solely in germinal cells in the male testis, while sACE is expressed ubiquitously in various tissues, cells, and extracellular fluids, and is responsible for the physiological role in the RAS (Natesh et al., 2004). Over-activated ACE elevates BP via the ACE-Ang II-AT_1R axis. Many drugs targeting ACE inhibition have been developed as antihypertensive therapies, such as captopril, lisinopril, and enalapril (Menard & Patchett, 2001). Owing to their side effects, however, food-derived ACEi peptides have gained increasing interest as alternatives in recent years. Currently, potent ACEi peptides are generally composed of a hydrophobic N-terminus and a C-terminus with hydrophobic, positively charged, and aromatic or cyclic amino acids, being different in structural features for di/tripeptides and larger peptides (Fan et al., 2019; Iwaniak, Minkiewicz, & Darewicz, 2014; Jimsheena & Gowda, 2010; Wu, Aluko, & Nakai, 2006). Many ACEi peptides identified using *in vitro* methods have been confirmed their efficacies in reducing BP in spontaneously hypertensive rat (SHR) (Ehlers et al., 2011; Majumder et al., 2013; Majumder et al., 2015; Yu et al., 2014).

1.1.2 ACE2

ACE2, a homolog of ACE, was first identified in the human heart in 2000 (Donoghue et al., 2000). Later studies indicated that it was also expressed in many other tissues such as kidney, liver, brain, and blood vessels (Patel et al., 2014). As a protective arm of the RAS, ACE2 counteracts the harmful effects activated by the ACE-Ang II-AT_1R axis (Santos et al., 2003; Vickers et al., 2002). ACE2 also cleaves Ang I to Ang (1-9) which can be further converted by ACE to Ang (1-7), however, it is much less efficient than the ACE2-Ang (1-7)-MasR axis (Vickers et al., 2002).

ACE2 deficiency in ACE2 knockout mice exacerbated Ang II-induced cardiac and vascular pathological responses including hypertension, oxidative stress, fibrosis, and remodeling (Alghamri et al., 2013; Zhong et al., 2010), which could be blunted by the supplementation of recombinant human ACE2 (Zhong et al., 2010). ACE2 level is significantly lower in SHR than normotensive Wistar-Kyoto rats (Ferreira et al., 2011; Tikellis et al., 2006; Yang et al., 2013); increased ACE2 expression could attenuate oxidative stress, hypertension, and hypertension-linked pathophysiological changes in SHR (Díez-Freire et al., 2006; Lo et al., 2013). These findings demonstrated the protective roles of ACE2 in hypertension and cardiovascular functions under pathological conditions. Diminazene and xanthene are two ACE2 activators identified by the conformation-based structural selection (Kulemina & Ostrov, 2011; Prada et al., 2008), although whether their physiological functions are ACE2-dependent are still controversial (Haber et al., 2014). A few bioactive peptides reduced BP associated with ACE2 upregulation at both gene or protein levels in SHR (He et al., 2019; Liao et al., 2019a; Zheng et al., 2017).

1.2 General introduction on antihypertensive peptides

Food proteins are the major sources of bioactive peptides. Bioactive peptides are latent in food proteins but can be released using various technologies and exert numerous bioactivities such as antioxidant, anti-inflammatory, and antihypertensive activities, among others (Udenigwe & Aluko, 2012). Among them, antihypertensive peptides have attracted substantial attention due to their possible applications as alternatives to antihypertensive drugs with lower side effects (Martínez-Maqueda et al., 2012). Most of the currently-identified antihypertensive peptides were initially characterized as ACEi peptides, but were later found to reduce BP through multiple mechanisms, such as improved endothelial function, ameliorated inflammation, attenuated oxidative stress, and upregulated ACE2 (Wu et al., 2017). Upregulation of the ACE2-Ang (1-7)-MasR axis by

upregulating ACE2 was the most-recently identified *in vivo* mechanism of antihypertensive peptides (Liao et al., 2019a). Besides, it should be noted that some antihypertensive peptides possess multifunctional properties, which jointly contribute to their *in vivo* antihypertensive activities (Li et al., 2019; Liao et al., 2019a; Liao, Fan, & Wu, 2018).

1.3 Spent hen utilization

Spent hens are the laying hens reaching the end of the laying cycle and therefore require disposal in the egg industry (Ferreira et al., 2011). Over the past decade, an average of more than 30 million spent hens are produced each year in Canada and are the producers' liability for their disposal (AAFC, 2020). Processing spent hen for food or feed uses is of little market value, due to the high processing cost, low meat yield, and poor meat quality. Instead, they are mostly disposed by burial, composting, and incineration, which cause environmental and animal welfare concerns (Wang, Wu, & Betti, 2013; Yu, Field, & Wu, 2018a). Therefore, finding more sustainable uses for spent hens is relevant. Despite being a byproduct or waste in the egg industry, spent hen is an inexpensive source of proteins and lipids. Over the past decade, various value-added uses have been developed for spent hen utilization, including protein- or lipid-based biomaterials such as adhesives, plastics, and bionanocomposites, as well as bioactive peptide-based functional food ingredients with antioxidant and anti-inflammatory properties (Esparza et al., 2018; Gu et al., 2019; Hong et al., 2017; Wang & Wu, 2012; Yu et al., 2018a; Yu, Field, & Wu, 2018b; Zubair, Wu, & Ullah, 2019).

1.4 Hypothesis and objectives

Many studies have reported that muscle proteins are excellent sources of antihypertensive peptides (Toldrá et al., 2018; Udenigwe & Howard, 2013). A previous bioinformatics study

demonstrated the superiority of chicken muscle proteins in producing ACEi peptide precursors than many other food protein sources including milk, egg, soybean, and fish, indicating the potential of chicken muscle proteins as a promising starting material to prepare antihypertensive peptides (Gu, Majumder, & Wu, 2011). Recently, Yu, Field, & Wu (2018a) prepared a spent hen muscle protein hydrolysate with anti-inflammatory properties; an ameliorated inflammation is also reported to be beneficial to BP reduction. Therefore, we hypothesized that spent hen muscle proteins are a source of antihypertensive peptides. The overall objectives are to isolate and identify the responsible antihypertensive peptides from spent hen muscle proteins and to understand their underlying antihypertensive mechanisms. The specific objectives of this project are:

1. To prepare a spent hen muscle protein hydrolysate (SPH) with antihypertensive activity.
2. To purify and identify ACEi peptides and ACE2u peptides from the prepared SPH above.
3. To evaluate the modulatory effects of the identified peptides in vascular cell models.
4. To investigate the antihypertensive effect of the identified peptides and their underlying antihypertensive mechanisms.

1.5 Chapter format

Two cell lines, including vascular smooth muscle A7r5 cells (VSMCs) and endothelial EA.hy 926 cells (ECs), as well as an animal model for human essential hypertension, SHR, were used to evaluate the antihypertensive activity of peptides in this study. The following chapters were designed to achieve the specific objectives of this study, and the brief descriptions of each chapter are as follows:

Chapter 2 provides a literature review on the production status of spent hens in the Canadian egg industry including their conventional and current value-added uses. Antihypertensive peptides

derived from food proteins, including their production, identification, bioactivity, and underlying mechanisms were discussed.

Chapter 3 prepares and screens an SPH with antihypertensive activity. Eighteen SPHs were prepared and their *in vitro* ACEi, ACE2u, antioxidant and anti-inflammatory activities were studied. ACEi activity was determined using a biochemical assay; ACE2u, antioxidant, and anti-inflammatory activities were assessed in VSMCs or ECs. The fates of these activities in the digestive tract were evaluated using simulated gastrointestinal digestion and an intestinal permeability cell model, human colon carcinoma cells (Caco-2). After screening using the above parameters, three SPHs with *in vitro* antihypertensive potential were orally administrated to SHR (n=3), and the SPH prepared by thermoase PC10F (SPH-T) reduced BP significantly. Objective 1 is partially addressed in this chapter.

Chapter 4 further studies the antihypertensive effect of SPH-T and explores the underlying mechanisms. More animals (n=6-7) were included to evaluate the BP-lowering activity of SPH-T. Effects of SPH-T on the RAS components, vascular function, vascular inflammation, fibrosis, and oxidative stress were studied. Objective 1 is fully addressed in this chapter.

Chapter 5 isolated ACEi peptides and/or ACE2u peptides from SPH-T. The conventional activity-guided fractionation method including ultrafiltration and chromatographic technologies was applied; 5 ACEi peptides and 4 ACE2u peptides were identified. Only peptides with high activity and short peptide length from the major muscle proteins were considered for the subsequent animal work. Finally, 4 peptides with ACEi and/or ACE2u activities were selected for *in vivo* trials in SHR. Objective 2 is addressed in this chapter.

Chapter 6 evaluates the regulatory effects of the identified ACEi or ACE2u peptides in two vascular cells, VSMCs and ECs, upon stimulation by Ang II and tumor necrosis factor alpha

(TNFα), respectively. Their antioxidant effects were assessed in VSMCs; the anti-inflammatory effects were evaluated in both VSMCs and ECs. The mechanisms of these peptides underlying their antioxidant and anti-inflammatory effects were studied. Their regulatory effects on intracellular signaling events were determined; the involvement of downregulating the Ang II-AT_1R axis and upregulating the ACE2-Ang (1-7)-MasR axis was also investigated. Objective 3 is addressed in this chapter.

Chapter 7 studies the antihypertensive effects of the identified ACEi or ACE2u peptides in SHR. Peptides were orally administrated to the animals and BP was recorded. Effects of the peptides on BP reduction, the RAS components, vascular inflammation, and oxidative stress were assessed. Possible reasons for peptides failing to reduce BP were also discussed. Objective 4 is addressed in this chapter.

Chapter 8 concludes the key findings and the anticipated significance of the present study. Limitations and the recommended future studies were proposed.

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CHAPTER 2 – Literature Review [1]

2.1 Overview of spent hens

2.1.1 Production status in Canada

In 2019, there were 1,172 registered egg farms in Canada, generating $1.2 billion of revenue, contributing 1.9% of cash receipts to farming operation in Canada (AAFC, 2020). In 2019, Canadian per capita consumption of eggs reached 21.4 dozen, representing a 34% increase over the last ten years, leading to a growing demand for lay hens for egg production. At the end of the egg-laying circle (normally ~1 year), these hens turn to be "spent" as the egg productivity reduces, being a major byproduct that requires disposal in the egg industry. From 2005 to 2019, the production of spent hen has been increasing from 26 million to 35 million in the Canadian egg industry (Figure 2.1), as compared with its US counterpart from 350 million to 400 million (AAFC, 2020; Shahbandeh, 2020). Due to a very limited market value of processing spent hens for food use, they are mostly disposed by landfilling (burial, composting, and incineration), which raises animal welfare and environmental concerns; besides, the producers are liable for paying the cost of transportation and disposal. Hence, finding new sustainable solutions for spent hens while generating additional revenue to the egg industry would be welcomed.

2.1.2 Conventional disposal of spent hens

Spent hens are not considered for food use in Canada; however, they are widely accepted as a regular meat product in Asian countries. Spent hen as raw meat usually needs additional tenderization to be consumed due to the objectionable toughness and the presence of residual bone

[1] Part of this chapter has been accepted as "Food peptides in blood pressure regulation" in "*Food Proteins and Peptides: Emerging Biofunctions, Food and Biomaterial Applications*" by the Royal Society of Chemistry.

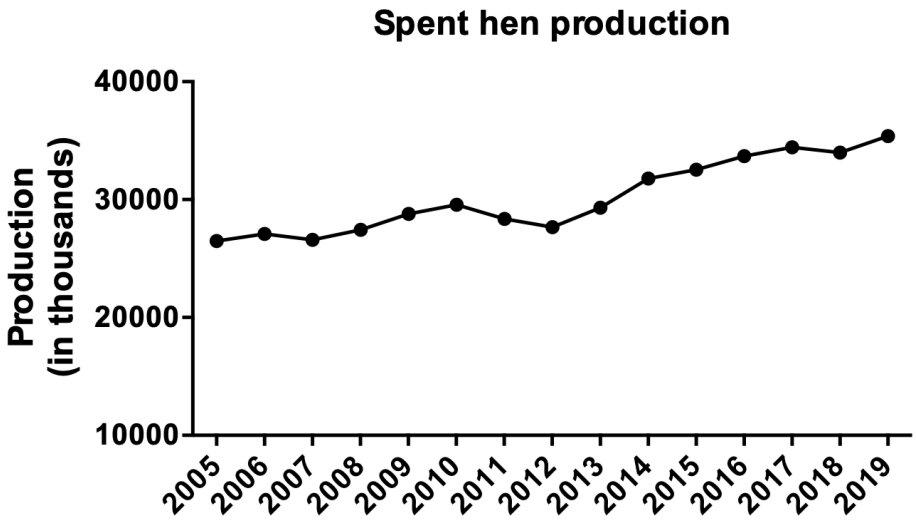

Figure 2.1 Spent hen production in Canada from 2005 to 2019. Data were obtained from *Agriculture and Agri-Food Canada - Chicken (Chicks) and Turkey (Poults) Reports*. Assessed date: 30 December 2020.

fragments. Quite a few natural tenderizers have been reported to improve texture, tenderness, and sensory attributes of spent hen meat, such as papaya leaves, pineapple rind powder, and kiwifruit proteases, among others (Abdalla et al., 2013; Kantale et al., 2019; Sharma & Vaidya, 2018). Other than being consumed raw, spent hens can also be developed into various processed meat products or snacks or be incorporated into other food products as base components or additives (Hur, Choi, & Jin, 2016; Lee et al., 2003; Singh et al., 2001; Sorapukdee, Uesakulrungrueng, & Pilasombut, 2016). Besides, spent hens have been popularly used to make chicken soup. For example, the addition of 25% of spent hen meat shred in an instant soup mix provided extra nutrition without impairing the sensory qualities; the soup could be aerobically stored at ambient temperature for a period of up to 90 days. Chicken soup has been reported to mitigate inflammation by inhibiting neutrophil chemotaxis and has a long history of being used to treat colds and upper respiratory infections (Lipman, 2003; Rennard et al., 2000). Recently, some researchers recommended

homemade chicken soup as a potential folk remedy to promote immune function against the coronavirus disease 2019 (Rennard, Kalil, & Casaburi, 2020).

Spent hens can be converted into protein meal as animal feed or as pet food. Previous research demonstrated that rendered spent hen meal was acceptable as a protein and nutrient source for commercial broiler from hatch to 6 weeks of age. Other than being a part of the broiler diet, incorporating 5-10% of spent hen meal into a regular corn basal diet improved early postmolt performance of laying hens, while incorporating 10% of the meal did not alter the egg quality and acceptability (Koelkebeck et al., 2001; Williams & Damron, 1999). A spent hen hard tissue meal made by feathers, bones, and connective tissues maintained nitrogen metabolism in goats, similarly to that of traditional protein sources (Freeman et al., 2009). A few trials have also been conducted to develop spent hen meal into pet foods. For example, the soft tissues of spent hens, including striated muscle, viscera, and other organ tissues, were used to make a high-protein and low-ash food product, being a good option for pet food (Aldrich, 2006; Krestel-Rickert, 2001).

2.1.3 New value-added applications of spent hens

In Canada, spent hens are conventionally disposed by euthanasia and landfilling, causing environmental and animal welfare concerns (Wang, Wu, & Betti, 2013; Yu, Field, & Wu, 2018a). Recently, more value-added uses have been or are being explored. As a rich source of animal proteins (approximately 56% protein on a dry weight basis), spent hens can be bio-transformed into low-molecular-weight (LMW) peptides possessing health beneficial effects or into bio-based biomaterials as potential substitutes for synthetic materials (Figure 2.2) (Hong et al., 2018; Park, Bae, & Hettiarachchy, 2000; Safder, Temelli, & Ullah, 2019). The newly-developed applications with value addition of spent hens align with the global trend of valorization of agricultural byproducts (McHugh, 2019).

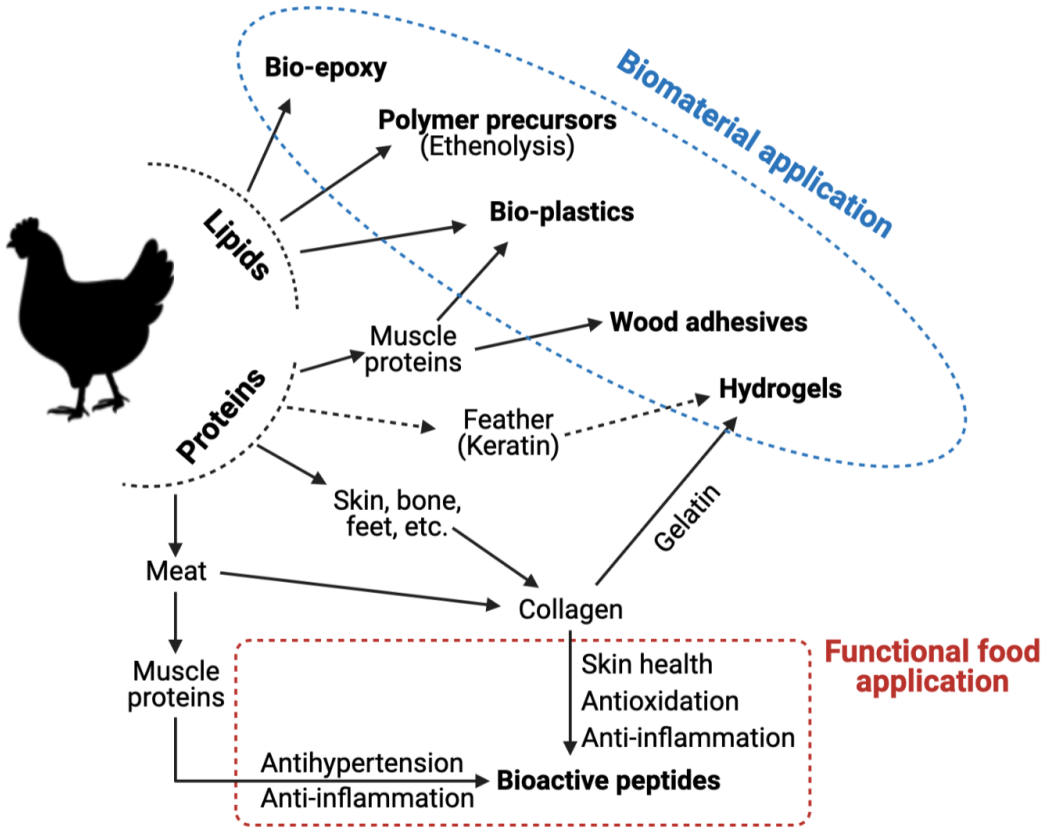

Figure 2.2 Current biomaterial and functional food applications of spent hen-derived proteins and lipids.

2.1.3.1 Functional foods

A special focus has been put on developing bioactive peptides from spent hen proteins as functional food ingredients, such as peptides with renin-inhibitory, angiotensin-converting enzyme (ACE) inhibitory (ACEi), antioxidant, anti-inflammatory, and anti-aging activities. Kumar et al. (2020) prepared a spent hen meat protein hydrolysate using flavourzyme or alcalase and found that the flavourzyme-digested hydrolysate possessed higher protein recovery and antioxidant activity. In another study, a spent hen meat protein hydrolysate prepared by pepsin and pancreatin inhibited renin and ACE activities, reduced plasma oxidation, and exerted antihypertensive effect in spontaneously hypertensive rat (SHR) (Udenigwe et al., 2017). Meat proteins comprise mainly

muscle proteins and collagens. Muscle proteins have been used to prepare antioxidant, ACEi, and anti-inflammatory peptides. For example, a previous study identified an actin-derived ACEi peptide, IWHHT, which can be further digested into IWH and IW in the digestive tract; further analysis demonstrated that IWHHT possessed also antioxidant and anti-inflammatory activities, whereas IWH and IW possessed either ACEi, antioxidant or anti-inflammatory property (Gu et al., 2019). A spent hen muscle protein hydrolysate prepared by Protex 50FP (a food-grade protease) exhibited *in vitro* inhibitory activity of interleukin-6, a pro-inflammatory cytokine, however, a subsequent 3-week feeding trial in Sprague-Dawley rats did not show an *in vivo* interleukin-6 inhibition; instead, it promoted the production of an anti-inflammatory cytokine, interleukin-10 (Yu et al., 2018a; Yu, Field, & Wu, 2018b). Collagens from terrestrial animal sources such as spent hens confront a big technical challenge for being utilized, due to the difficulty in breaking down the highly-crosslinked collagen into LMW peptides. Recently, new technological advancements have been achieved. It was found that treatment with pepsin significantly improved LMW peptide production by papain hydrolysis by removing telopeptides of collagen molecules (Hong et al., 2017). Subsequently, the same researchers found that pre-treatment with formic acid further increased LMW peptide production under the same conditions (Hong et al., 2018). Furthermore, the prepared LMW peptides exhibited anti-aging activity in human dermal fibroblasts upon ultraviolet A (UV-A) exposure (Wang, Hong, & Wu, 2019). In another study, 4 proteases (protease M, alcalase, Protex 50FP, and Protex 51FP) were used to produce spent hen collagen hydrolysates, with antioxidant, anti-inflammatory, proliferative, or type I procollagen synthetic activity in human dermal fibroblasts (Offengenden, Chakrabarti, & Wu, 2018). Spent hen collagens from the skin have also been used to produce antioxidant peptides and ACEi peptides (Nadalian et al., 2015; Yusop et al., 2016). Besides, spent hen-derived bioactive peptides have

been applied as additives into other food products. For example, a spent hen meat protein hydrolysate prepared by alcalase, flavourzyme, neutrase, protamex, pepsin, and trypsin was added in crab meat analogue, providing the product with antioxidant and ACEi activities (Jin et al., 2016). Table 2.1 represents the recent functional food applications of spent hens. These findings demonstrated the presence of bioactive peptides with various health benefits in spent hen proteins.

2.1.3.2 Biomaterials

Given the raw materials of synthetic materials excessively rely on fossil resources, which is vulnerable to changes in global policies and politics, biobased materials have attracted considerable interest from researchers and industrial observers. Besides, the production of synthetic materials raises concerns about environmental security and sustainability. Recently, spent hens were used as starting materials for preparing biobased wood adhesives and plastics (Wang & Wu, 2012a; Zubair, Wu, & Ullah, 2019). Spent hen muscle proteins were modified by sodium dodecyl sulfate (SDS) (0.5–5%) or urea (1–8 M) to produce protein-based adhesives; the optimized incorporation rates were 3% for SDS and 3 M for urea, respectively. Use of either modification agent enhanced protein unfolding and thus exposed more secondary structures that strengthen protein-wood bonding; the prepared adhesives were applicable in both dry and wet environments (Wang et al., 2012a). The muscle proteins have also been used to prepare bionanocomposite films for food packaging application, with the addition of glycerol (as plasticizer), chitosan (as crosslinker), and nanoclay (as nano-reinforcement); the product showed satisfactory thermal, thermomechanical, and barrier properties (Zubair et al., 2019). Likewise, spent hen collagen was used to prepare hydrogels for tissue engineering, which promoted the proliferation of human dermal fibroblasts, supporting its wound healing application (Esparza et al., 2018). Feather keratin can also be valorized as biomaterials such as hydrogels for wound healing

application and sorbents for wastewater treatment (Esparza et al., 2018; Zahara et al., 2021). Except for spent hen proteins, the derived lipids can be modified by various agents to produce various biomaterials. For example, fatty acid methyl esters prepared by transesterification of spent hen-derived triglycerides were used to generate linear α-olefins by ethenolysis, which are raw materials for polyethylene production for synthesizing oxo alcohols and making poly-α-olefins used in synthetic lubricants (Chatterjee & Jensen, 2017; Pradhan, Arshad, & Ullah, 2020). Spent hen lipids can also be modified to make bio-plasticizers. Safder et al. (2019) extracted lipids from spent hens followed by converting them into bio-epoxy by epoxidation for bio-plasticizer production. The recent biomaterials derived from spent hen proteins or lipids are shown in Table 2.2.

2.2 Antihypertensive peptides from food proteins

2.2.1 Hypertension and antihypertensive peptides

High blood pressure (BP), also known as hypertension, is an important risk factor for cardiovascular diseases (CVDs), the leading cause of mortality and morbidity worldwide. Taking the US as an example, the prevalence of hypertension among adults older than 18 years old was largely unchanged (nearly 30%) over the past two decades (Pazoki et al., 2018). Treatment of hypertension with medications significantly improved the symptom; however, still one-third of treated hypertensives did not have their BP controlled (Zhou et al., 2018). Also, the long-term use of pharmacological treatment is associated with significant adverse effects (Khanna, Lefkowitz, & White, 2008). Various nonpharmacological interventions have been reported to be effective in lowering BP, such as increased physical activity, sodium reduction, potassium supplementation, the Dietary Approaches to Stop Hypertension (DASH) diet, among others (Whelton et al., 2018).

Hypertension can be classified into primary (essential) or secondary hypertension. Primary or essential hypertension arises in middle or old age, accounting for more than 90% of hypertension. Primary hypertension has no a clear etiology and involves complex genetic and environmental factors (Poulter, Prabhakaran, & Caulfield, 2015); its molecular pathogeneses involve such as overactive renin-angiotensin system (RAS), dysregulated kallikrein kinin-system, increased sympathetic nervous system, endothelial dysfunction, aberrant inflammation, and oxidative stress (Majumder & Wu, 2015c). Among them, the RAS is thought to play a pivotal role. Within the RAS, renin converts angiotensinogen into angiotensin (Ang) I, which can be further converted into Ang II by ACE; Ang II binds with Ang II type 1 receptor (AT_1R), leading to elevated blood pressure. On the contrary, another key regulatory enzyme, ACE2, a homology of ACE, degrades Ang II into Ang (1-7), which binds with Mas receptor (MasR) and counteracts the pathological responses caused by the ACE-Ang II-AT_1R axis; the ACE2-Ang (1-7)-MasR axis is an emerging player in the RAS (Mendoza-Torres et al., 2015). Figure 2.3 presents a schematic with these two arms in the RAS. Contribution of the other factors to the pathogenesis of hypertension has been elucidated in Majumder et al. (2015c).

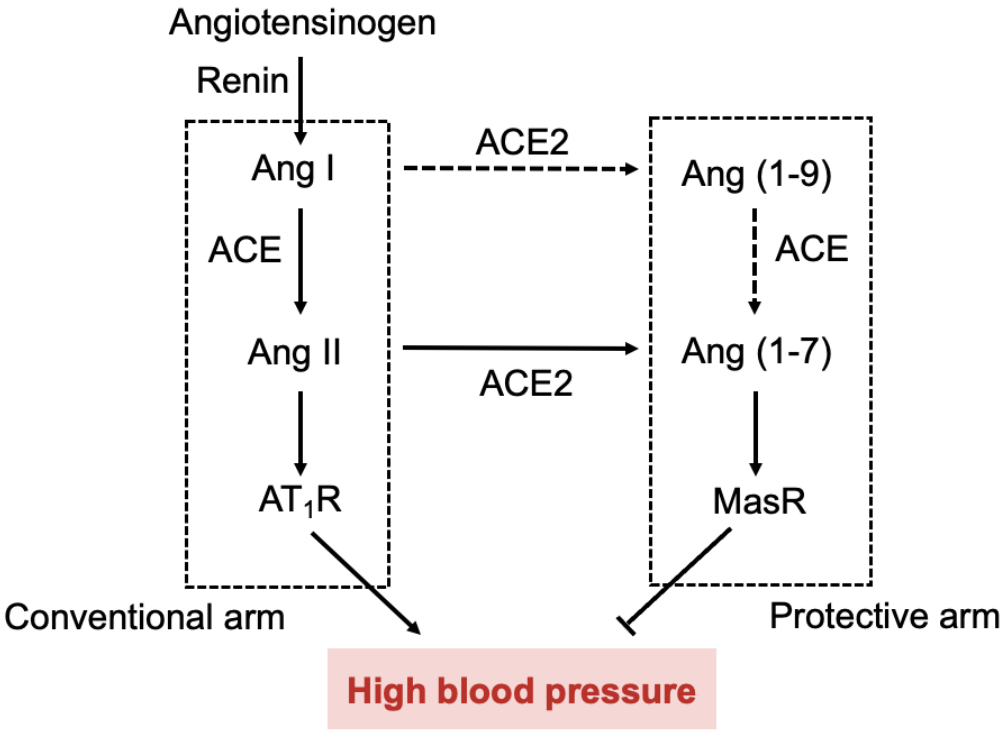

Figure 2.3 The renin-angiotensin system (RAS). In the conventional arm of the RAS, ACE converts angiotensin (Ang) I to Ang II that increases blood pressure via Ang II type 1 receptor (AT_1R). In the protective arm, ACE2 degrades Ang II to Ang (1-7) that reduces blood pressure via Mas receptor (MasR). ACE2 also degrades Ang I to Ang (1-9) (dashed line), representing a minor action of ACE2, with much lower efficiency than the ACE2-Ang (1-7) axis.

Food-derived antihypertensive peptides have gained considerable interest as an emerging therapy for hypertension due to less side effect and lower cost than pharmaceutical drugs. Antihypertensive peptides are short amino acid sequences (usually < 20 residues) latent in food proteins but can be functional as regulatory compounds to exert physiological benefits upon release. They can be produced during microbial fermentation, food processing, and enzymatic hydrolysis, as well as during gastrointestinal digestion (Udenigwe & Aluko, 2012b). Purification and identification of these peptides are usually through multiple-step fractionation processes involving various separation technologies guided by a specific bioactivity, mostly ACEi activity. Other

activities such as ACE2 upregulating (ACE2u) and AT_1R downregulating peptides have recently been reported (Chen et al., 2020; Liao et al., 2019b). The rapid development of bioinformatics and the establishment of quantitative structure-activity relationship (QSAR) enable the prediction of potent peptides from food proteins using *in silico* digestion, which significantly improves the efficiency in discovering new antihypertensive peptides. Use of some new technologies such as electrodialysis with ultrafiltration membranes (EDUF) or a combination of fractionation and gastrointestinal digestion also helps to identify peptides with specific molecular weight, charges, or gastrointestinal-resistant ability (Fan et al., 2019; Firdaous et al., 2009). By using these techniques, over the past decades, a tremendous number of antihypertensive peptides are identified from various food proteins, including cereals, legumes, nuts, seafood, meat, milk, egg, and other foodstuffs (Aluko, 2015).

2.2.2 Production and purification of antihypertensive peptides

2.2.2.1 Peptide production

Bioactive peptides are encrypted in food proteins that require certain strategies to release; These consist of *in vitro* processes including microbial fermentation, food processing, and enzymatic hydrolysis, as well as *in vivo* bioconversion such as gastrointestinal digestion and intestinal absorption (Udenigwe et al., 2012b; Xu et al., 2019). Microbial fermentation has long been used in producing many conventional foods such as sourdough, sausage, soy, and dairy products (Gallego et al., 2018; Gibbs et al., 2004; Nakamura et al., 1995; Zhao et al., 2013). For example, in fermented sausages, microbial proteases generate a large number of peptides and free amino acids, contributing to the characteristic flavor while also liberating ACEi peptides (Gallego et al., 2018). Sour milk fermented by *Lactobacillus helveticus* exerts antihypertensive effect in

both animal and human trials, from which two antihypertensive peptides, IPP and VPP, have been identified and characterized (Aihara et al., 2005; Nakamura et al., 1995). ACEi peptides can also be produced during food manufacturing processes such as dry-curing (meat) and ripening (meat and cheese) (Gómez-Ruiz et al., 2006; Hernández-Ledesma, del Mar Contreras, & Recio, 2011; Saito et al., 2000; Toldrá et al., 2018). Peptides are also produced by digestive proteases present in the gastrointestinal tract during oral ingestion of proteins; however, it is hard to monitor these processes *in vivo*, which are usually mimicked using *in vitro* digestive models with exogenous gastrointestinal proteases. Indeed, exogenous gastrointestinal proteases such as pepsin and trypsin have widely been used to prepare antihypertensive peptides (Aluko, 2015). For example, ACEi peptides have been produced by gastrointestinal proteases from various foods such as chicken, pork, squid, fish, milk, insect, egg, and among others (Alemán, Gómez-Guillén, & Montero, 2013; Basirico et al., 2015; Chalamaiah et al., 2015; Escudero et al., 2012b; Majumder et al., 2013b; Sangsawad, Roytrakul, & Yongsawatdigul, 2017; Wu et al., 2015; You & Wu, 2011). While, many studies have reported being lack of efficiency of gastrointestinal proteases in hydrolyzing certain proteins (Liu et al., 2017). Hence, non-gastrointestinal proteases (*e.g.* thermolysin, alcalase, etc.) have been applied, either in isolation or in combination, to assist the production of antihypertensive peptides; these proteases are mostly from microbial or plant sources that possess broader cleavage sites than gastrointestinal proteases (Aluko, 2015; Liao et al., 2018; Liu et al., 2017). Non-gastrointestinal proteases can also assist new antihypertensive peptide production from their parent peptides in the gastrointestinal tract. For instance, the release of ovotransferrin-derived IRW, IQW, and LKP, as well as actin-derived IWH and IW, require thermolysin to pre-digest the proteins prior to peptic hydrolysis (Fan et al., 2018b; Fujita, Yokoyama, & Yoshikawa, 2000; Gu et al., 2019;

Majumder & Wu, 2010). Currently, enzymatic hydrolysis dominates the production of antihypertensive peptides from food proteins (Table 2.3-2.4).

There is no restriction of specific proteins or enzymes for the generation of antihypertensive peptides; however, those producing a high yield of LMW peptides are preferred, due to the smaller size of potent antihypertensive peptides (Aluko, 2015; Huang, Davidge, & Wu, 2013). A wide range of foodstuffs from various plant, animal, and marine sources are used for peptide production (Table 2.3-2.4). The initiatives behind the selection of these proteins are to pursue value addition of underutilized proteins or protein-rich by-products (González-García, Marina, & García, 2014; Pokora et al., 2014; Santana et al., 2016; Udenigwe et al., 2017), or to liberate specific peptide sequences of pharmacological interest from target proteins (Gu et al., 2019; Gu, Majumder, & Wu, 2011; Gu & Wu, 2016; Majumder et al., 2010; Udenigwe et al., 2012b). Proteins can be hydrolyzed by a single enzyme or by more than one simultaneously or sequentially. These enzymes can be in purified or crude form (Huang et al., 2011; Lin et al., 2011; Tavares et al., 2012). Enzyme combinations are particularly effective in releasing target sequences using *in silico* digestion assisted by QSAR, with significantly reduced cost and labor intensity (Gu et al., 2011). However, the three-dimensional structure of proteins may present as a barrier leading to a failure for peptide generation, therefore pre-treatments to denature proteins may be applied. Heating has been among the most commonly used, typically at 80-95 °C for 10-15 min, but the time and temperature vary among different types of proteins (Fan et al., 2019; Majumder & Wu, 2009). Besides, under certain circumstances, extra pre-treatments may be applied to further enhance the hydrolysis. For example, boiling for 5 min and high pressure at 600 Mpa for 15 min remarkably improved degree of hydrolysis and ACEi activity of bovine collagen hydrolysates (Zhang, Olsen, Grossi, & Otte, 2013). Pre-treatments with heating, sonication, high pressure, and reducing agents promoted the release

of renin-inhibitory or ACEi peptides from pea, rice, lentil, corn, and wheat proteins (Chao et al., 2013; Garcia-Mora et al., 2015; Jia et al., 2010; Majumder et al., 2010; Yang et al., 2017; Zhou et al., 2013).

Emerging technologies such as subcritical water (SCW) hydrolysis have recently been used for peptide production. It is a cost-effective and eco-friendly method (100-374 °C, 0.1-22 Mpa), to extract proteins and produce peptides or amino acids from food proteins (Garcia-Moscoso et al., 2013; Marcet et al., 2014; Powell, Bowra, & Cooper, 2016; Sereewatthanawut et al., 2008; Shitu, Izhar, & Tahir, 2015). In a value-added study on ice-cream wastewater using SCW, the highest ACEi activity of the hydrolysate was achieved at 230 °C for 240 min (Enteshari & Martínez-Monteagudo, 2018). In another study investigating ACEi activity of SCW extract of an insect, *Protaetia brevitarsis larvae*, the activity increased with temperature within 200 °C but decreased subsequently (Bae & Lee, 2020). It is suggested that ACEi activity of the SCW extract of proteins reduced at high temperature with prolonged time. SCW parameters including reaction temperature, time, solid to water ratio, pH, and solute characteristics require to be optimized to obtain the highest extraction efficiency (Shitu et al., 2015). To date, most studies on SCW hydrolysis of proteins focus on generating amino acids other than the immediate peptides. This is due to a lack of specificity in cleaving peptide bonds and also a very limited cleavage of disulfide bonds. Thus, pre-treatments such as reduction and alkylation of proteins are suggested to further improve protein sequence coverage (Powell et al., 2016). The recent advancement of new technologies such as high hydrostatic pressure and microwave-assisted extraction for the production of bioactive peptides has been recently reviewed in Ulug, Jahandideh, and Wu (2020).

2.2.2.2 Peptide purification and identification

Once the *in vitro* antihypertensive potential of a protein hydrolysate is characterized, the next step is to purify and identify the responsible peptides therein. The active peptide sequences can be enriched through a step-by-step fractionation process using various separation technologies guided by a bioactivity assay (mostly ACEi assay). A line of membrane and chromatographic technologies have been widely used including ultrafiltration, size exclusion chromatography, ion exchange chromatography, and reverse-phase high-performance liquid chromatography (RP-HPLC), which separate peptides based on varying physicochemical properties such as size, charge, hydrophobicity, etc. Peptides of smaller size are thought to be more active in ACE inhibition than larger ones, due to the enhanced accessibility to the ACE active site, a deep narrow binding channel (Fan, Liao, & Wu, 2018a). Fractionation based on peptide size generally involves ultrafiltration with molecular weight cut-off membranes of typically 1, 3, 5, and 10 kDa and size exclusion chromatography such as gel filtration chromatography (Alemán et al., 2013; Banerjee & Shanthi, 2012; Bravo et al., 2019; Fan et al., 2019; Shi et al., 2014; Wu, Aluko, & Muir, 2008; Wu et al., 2015; Zhang et al., 2017; Zou et al., 2014). Ion exchange chromatography separates peptides based on their different charge properties including cation or anion exchange chromatography (Fan et al., 2019; Majumder & Wu, 2011; Sangsawad et al., 2017; Wu, Du, Jia, & Kuang, 2016; You et al., 2011). Fractionation may also start with Sep-pak C18 cartridge which preliminarily and quickly separate peptides with varying hydrophobicity (Chen et al., 2020; Liao et al., 2019b; Yamamoto, Maeno, & Takano, 1999). After initial separation using size exclusion and ion exchange chromatography that are belong to the fast protein liquid chromatography (FPLC), peptides can be further fractionated on a high-performance liquid chromatography (HPLC) system based on hydrophobicity (using a reverse-phase C_{18} column) (Pontis, 2017). The HPLC fractionation

involves preparative and analytical columns that usually require more than one round to obtain the highly-purified fractions (Alemán et al., 2013; Fan et al., 2019; Katayama et al., 2007; Liao et al., 2019b; Nakano et al., 2006; Wu et al., 2016; Wu et al., 2015; Zhang et al., 2017). FPLC and HPLC can be used alternatively during the fractionation (Liao et al., 2019b; Wu et al., 2008), under certain circumstances, however, using either of them alone may be sufficient for the fractionation (Banerjee et al., 2012; Jimsheena & Gowda, 2011; Majumder et al., 2009; Mane & Jamdar, 2017; Rao, Ju et al., 2012; Tanzadehpanah et al., 2013). Whether to use these fractionation technologies in isolation or combination and how many rounds are needed depend on the physicochemical properties of the peptides produced. The activity-guided fractionation can also be coupled with gastrointestinal digestion to identify gastrointestinal-resistant peptides or prodrug-type peptides that require gastrointestinal proteases for a further release (Fan et al., 2019).

Some technical limitations such as low selectivity and membrane fouling of ultrafiltration and the high cost of chromatographic technology may somehow restrict the use of enzymatic hydrolysis. A patented EDUF has been developed that allows selective separation of peptides with specific charges and molecular weight (Bazinet et al., 2008; Firdaous et al., 2009). For example, an antihypertensive peptide, VW, was successfully recovered in a study separating bioactive peptides from alfalfa white protein hydrolysate using EDUF (Firdaous et al., 2009). A fraction rich in arginine was obtained from flaxseed protein hydrolysate using EDUF with significant BP-lowering activity in SHR (Udenigwe et al., 2012a). Another study on flaxseed protein hydrolysate yielded a cationic fraction rich in positively-charged amino acids (histidine, lysine, and arginine) with molecular weight of 300-400 Da reduced BP in SHR (Doyen et al., 2014). Similar findings were also observed during selective fractionation of cationic peptide fragments from rapeseed and dairy protein hydrolysates (He et al., 2016; Lapointe et al., 2006; Poulin, Amiot, & Bazinet, 2006,

2007). EDUF has a special potential for large-scale fractionation of bioactive peptides (Firdaous et al., 2009; Nasri, 2017).

The common steps of production, purification, and identification of antihypertensive peptides using both the conventional activity-guided and QSAR-assisted approaches are presented in Figure 2.4.

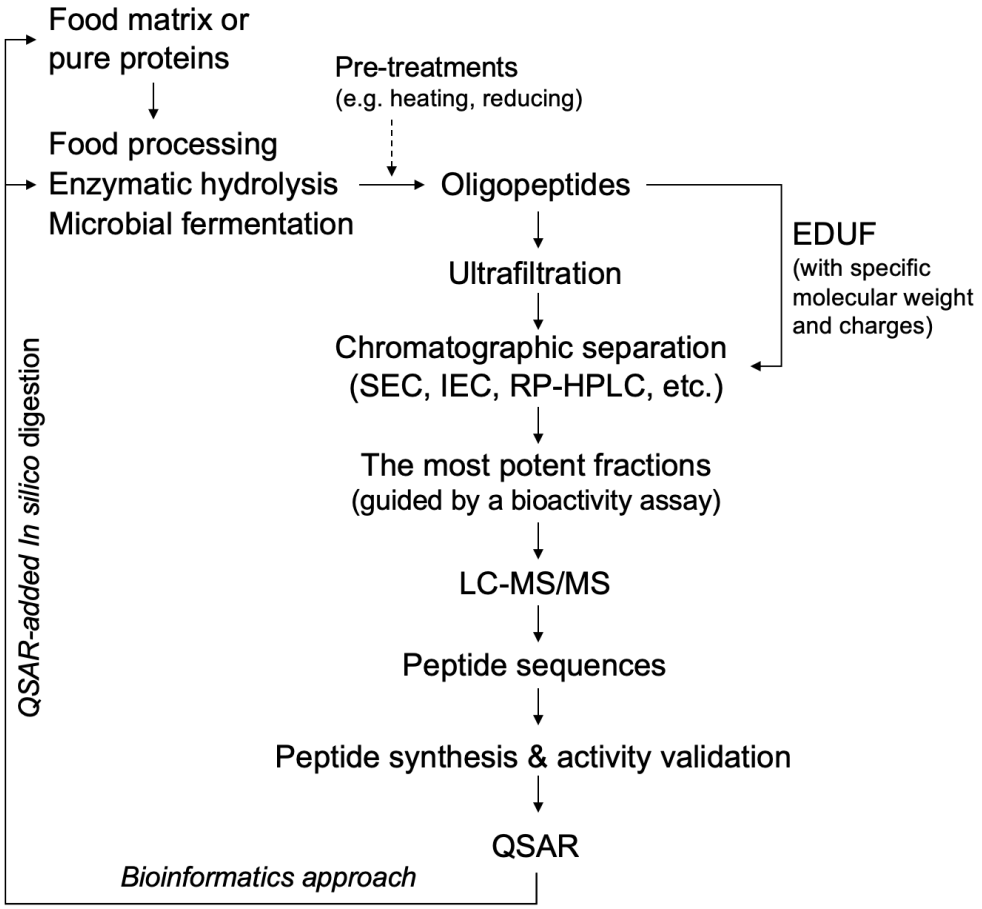

Figure 2.4 Production, purification, and identification of food-derived antihypertensive peptides. SEC, size exclusion chromatography, IEC, ion exchange chromatography, RP-HPLC, reverse-phase-high performance liquid chromatography, EDUF, electrodialysis with ultrafiltration membranes, LC-MS/MS, liquid chromatography with tandem mass spectrometry, QSAR, quantitative structure-activity relationship.

2.2.3 Antihypertensive hydrolysates and peptides from food proteins

Peptides that are characterized by *in vitro* antihypertensive assays require validation *in vivo*, since many physiological processes may lead to a loss of their activities especially during oral administration (Fujita et al., 2000; Jensen et al., 2014; Vermeirssen et al., 2004). Protein hydrolysates derived from salmon, cod, and haddock exhibited potent *in vitro* ACEi activities but failed to reduce BP through oral administration (Jensen et al., 2014). Three potent ACEi peptides (IC_{50} values of 0.55-1.2 μM), FKGRYYP, FFGRCVSP, and ERKIKVYL derived from chicken muscle or egg did not cause BP reduction in SHR through either oral or intravenous administration (Fujita et al., 2000). One factor is due to the presence of numerous proteases and peptidases in the gastrointestinal tract that influences the bioavailability of peptides; another factor may be due to the presence of different categories of ACEi peptides (*e.g.* substrate-, prodrug-, or true-type) (Fujita et al., 2000). Categorization of ACEi peptides can be easily conducted by comparing ACEi activity before and after pre-incubation with ACE. A substrate-type peptide, LRIPVA derived from spinach, did not show antihypertensive effect in SHR, irrespective of its potent *in vitro* ACEi activity (IC_{50} value of 0.38 μM); this peptide can be degraded to LRIP (2.2 mM), IP (1.8 mM), LR (0.9 mM) by ACE (Yang et al., 2003). Prodrug-type inhibitors such as IWHHT, LKPNM (from bonito or chicken muscle), MRWRD, IAYKPAG (from spinach), KRVIQY (porcine skeletal muscle), have shown antihypertensive effect in SHR, which were due to their respective peptide fragments after degradation by ACE, gastrointestinal proteases, or others (Fujita et al., 2000; Muguruma et al., 2009; Yang et al., 2003). It was reported that only prodrug-type and true ACEi peptides are likely to exert *in vivo* antihypertensive activity; therefore, it is always recommended to determine at least the gastrointestinal stability and category of ACEi peptides before being administrated to animals (Fujita et al., 2000).

Table 2.3-2.4 list hydrolysates or peptides reducing BP from various food protein sources, such as meat, egg, milk, cereal, legume, oilseed, and seafood. Both gastrointestinal (*e.g.* pepsin, trypsin, etc.) and non-gastrointestinal proteases (*e.g.* thermolysin, alcalase, etc.) have been applied. Exogenous gastrointestinal proteases showed great efficiencies in producing antihypertensive peptides from egg, whey, seed, and marine proteins (Abubakar et al., 1998; Alashi et al., 2014; Girgih et al., 2014b; Li et al., 2014; Liu et al., 2012; Miguel et al., 2005, 2006b; Wang et al., 2008; Zhang et al., 2009). For example, a single oral administration of 100 or 400 mg/kg body weight (BW) of pepsin-digested egg white hydrolysate to SHR significantly reduced systolic blood pressure (SBP) by 39 or 44 mmHg after 6 h (Miguel et al., 2005, 2006b). Chicken muscle proteins prepared by pepsin or pepsin + pancreatin reduced SBP by 27-36 mmHg within 2 h after a single dose of 200 mg/kg BW (Udenigwe et al., 2017). Besides, non-gastrointestinal proteases such as thermolysin exhibited a great potential in producing antihypertensive peptides from various food proteins, including porcine skeletal muscle, egg, fish muscle, cereals, legumes, oilseeds, and nut proteins (Fujita, Yamagami, & Ohshima, 2001; He et al., 2013a; Li et al., 2014; Liao et al., 2018; Miyoshi et al., 1995; Nakano et al., 2006; Nakashima et al., 2002; Nwachukwu, Girgih, Malomo, Onuh, & Aluko, 2014). Non-gastrointestinal and gastrointestinal proteases can be combined to produce antihypertensive peptides (Jahandideh et al., 2016; Liu et al., 2012; Udenigwe et al., 2012a; Yamada et al., 2013). Endogenous gastrointestinal proteases may play similar roles as exogenous ones, since thermolysin-digested egg white hydrolysate showed a comparable antihypertensive effect to that of the (thermolysin + pepsin)-digested one (Fan et al., 2019; Jahandideh et al., 2016). Moreover, orally-administrating aqueous protein extracts of bamboo shoots and mushrooms caused significant SBP reduction (Jang et al., 2011; Liu et al., 2013). Antihypertensive peptides are widely distributed in various foodstuffs.

With the exception of several studies on intravenously administrating pure peptides, a majority of protein hydrolysates or peptides are given to SHR by oral administration (Fujita et al., 2000; Lee et al., 2006; Miyoshi et al., 1991; Scruggs et al., 2004). These peptides are mostly characterized as potent ACEi peptides, with only a few selected based on renin-inhibitory activity (Fitzgerald et al., 2014; He et al., 2013b). The doses of hydrolysates are within a wide range varying from one to several thousand mg/kg BW, while those of peptides are within a smaller range less than one hundred mg/kg BW (Table 2.3-2.4). Most animal works are short-term studies (within 24 h) through a single administration. Despite BP reduction can be seen within minutes or hours for certain peptides, others may take days or weeks (Majumder et al., 2015a; Nakamura, Masuda, & Takano, 1996; Takano, 2002). Therefore, long-term studies are warranted given hypertension is a chronic disease. As for pure peptides, there is no apparent correlation between peptide size and BP reduction, although larger peptides are believed to be less bioavailable and thus a lower activity. Some large peptides belonging to pro-drug type peptides may require gastrointestinal proteases to liberate the true active sequences, such as FRADHPFL (RADHP), YAEERYPIL (YPI), and IVGRPRHQG (IVGRPR) (Fujita et al., 2000; Miguel et al., 2006a). However, many others are indeed substrates of trypsin, such as KRQKYDI, DKVGINYW, RPKHPIKHQ, VKKVLGNP, and QVGPLIGRYCG (Bravo et al., 2019; Katayama et al., 2008; Katayama et al., 2007; Saito et al., 2000; Tavares et al., 2012). Besides, some large peptides may exert an antihypertensive effect, without being absorbed, through other mechanisms other than ACE inhibition (Mas-Capdevila et al., 2020; Wu, Liao, & Udenigwe, 2017). Nevertheless, peptides identified by *in vitro* assays always require confirmation of their *in vivo* activities; for large peptides, the roles of the fragmentary peptides through possible bioconversion of endogenous proteases/peptidases also need to be considered.

2.2.4 Mechanisms of action of antihypertensive peptides

Food-derived peptides have demonstrated antihypertensive effects through multiple mechanisms, including modulation of the RAS, improvement of vascular function, blocking of Ca^{2+} channel, attenuation of oxidative stress, and opioid-like activity. The exemplified peptides along with their mechanisms of action underlying BP reduction are listed in Table 2.5; a simplified overview of mechanisms of antihypertensive peptides was shown in Figure 2.5.

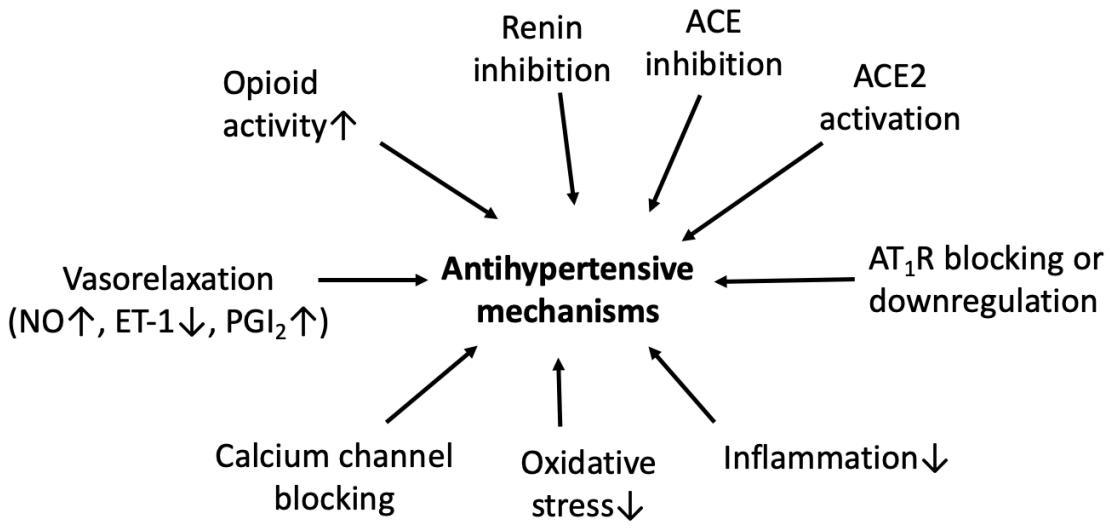

Figure 2.5 Primary mechanisms of action of antihypertensive peptides in spontaneously hypertensive rats. ACE, angiotensin-converting enzyme; ACE2, angiotensin-converting enzyme2; AT_1R, angiotensin II type 1 receptor; ET-1, endothelin-1; NO, nitric oxide; PGI_2, prostaglandin I_2.

2.2.4.1 Modulation of the RAS components

BP reduction by antihypertensive peptides targeting the RAS is mainly through downregulation of the ACE-Ang II-AT_1R axis and/or upregulation of the ACE2-Ang (1-7)-MasR axis (Mendoza-Torres et al., 2015). A hemp seed hydrolysate produced by pepsin and pancreatin lowered BP by reducing serum renin and ACE levels (Girgih et al., 2014a). Orally administrating

egg white hydrolysate produced by thermolysin and pepsin to SHR at low (250 mg/day/kg BW) and high (1,000 mg/day/kg BW) doses for 12 days reduced BP, associated with a lower vascular ACE expression, albeit no change in serum Ang II level. This finding implied the importance of local RAS in regulating BP (Jahandideh et al., 2016). Two lactoferrin-derived peptides, DPYKLRP and LRP, reduced circulating ACE activity and Ang II level (García-Tejedor et al., 2014). In another study on whey protein hydrolysate, both circulating and local ACE activities (lung, kidney, and abdominal aorta) were reduced in a dose-dependent manner, consistent with BP reduction in SHR (Wang et al., 2012b). The two famous milk peptides, IPP and VPP, also demonstrated the importance of ACE inhibition in relaxing mesenteric arteries of SHR (Jäkälä et al., 2009). Increasing evidence has demonstrated the antihypertensive actions of peptides were beyond ACE inhibition (Wu et al., 2017). Two egg-derived antihypertensive peptides, YAEERYPIL and RADHPFL, inhibited both Ang I and Ang II-induced vasoconstriction, indicating that the antihypertensive effects were mediated not only through ACE inhibition but also blocking the downstream actions of Ang II (Miguel et al., 2010). Another egg-derived peptide, IRW, reduced circulating Ang II level in SHR by increasing ACE2 level (Liao, Fan, Davidge, & Wu, 2019a; Majumder et al., 2013a; Majumder et al., 2015b). There are a few peptides that have been reported to regulate both the ACE-Ang II-AT_1R and ACE2-Ang (1-7)-MasR axes. An egg-derived peptide, RVPSL, reduced BP through decreasing renin, ACE, and AT_1R levels in kidneys or serum in SHR (Yu et al., 2014). IQP and VEP derived from blue algae (*Spirulina platensis*) and the papain-digested algae hydrolysate reduced BP, associated with upregulating the ACE-Ang II-AT_1R axis while downregulating the ACE2-Ang (1-7)-MasR axis in kidneys (Zheng et al., 2017). Three rapeseed-derived peptides LY, RALP, and GHS decreased renin and ACE levels, while increased the expressions of ACE2, Ang (1-7), and MasR in myocardium (He et al., 2019). The

egg white hydrolysate produced by thermolysin and pepsin also enhanced vascular expression of AT_1R (Jahandideh et al., 2016).

2.2.4.2 Improvement of vascular function

Signaling between endothelium and vascular smooth muscle layer maintains the vascular tone. The endothelium plays a fundamental role in maintaining vascular homeostasis and regulating vascular function via the complementary actions of endothelial cell-derived vasoactive factors: vasodilators including nitric oxide (NO), prostanoids, and endothelium-derived hyperpolarizing factor (EDHF), and vasoconstrictor (endothelin-1 [ET-1], thromboxane A_2) (Sandoo et al., 2010). Whether vascular relaxation is endothelium-dependent or independent can be examined through *ex vivo* studies by denuding endothelium or applying various vasoconstricting and vasodilatory molecules.

NO is the major player of endothelium-dependent vasorelaxation. Jahandideh et al. (2016) reported that an egg white hydrolysate enhanced endothelium-dependent relaxation of mesenteric arteries of SHR, mediated by NO, and endothelium-independent relaxation by an exogenous NO donor, sodium nitroprusside (SNP). A lactoferrin hydrolysate produced by pepsin (< 3 kDa) elicited mesenteric arterial vasorelaxation in similar mechanisms (García-Tejedor et al. 2017). Asides from the hydrolysates, some milk- and egg-derived peptides, such as DPYKLRP, IRW, IQW, LKP, enhanced vasorelaxation, in which IRW, IQW, LKP enhanced endothelium-dependent vasorelaxation while DPYKLRP improved that in both endothelium-dependent and independent manners (García-Tejedor et al., 2017; Majumder et al., 2013a; Majumder et al., 2015a). In a recent study in SHR, a chicken-derived nonapeptide, AVFQHNCQE, reduced BP via NO-mediated vasorelaxation by intra-peritoneally infusing L-NAME after one-hour oral administration of the peptide (Mas-Capdevila et al., 2019). Prostanoids such as prostaglandin I_2 (PGI_2) also participate

in endothelium-dependent vasorelaxation. A rapeseed-derived peptide, RIY, exerted antihypertensive activity through PGI_2 in the mesenteric artery of SHR (Yamada et al., 2010). Lactoferrin-derived peptides appeared to enhance prostanoids-mediated dilation, although which specific prostanoids have been involved were unknown (García-Tejedor et al., 2017); these peptides might also elicit EDHF to further dilate the vessel, since the enhanced vasodilation occurred by increasing K^+ channel conductance and subsequent K^+ efflux. Indeed, the vasodilation mediated by EDHF is still scantly reported. It should be noted that some peptides can elicit endothelium-dependent vasorelaxation via multiple mechanisms involving NO, prostanoids, and EDHF simultaneously (García-Tejedor et al., 2017).

ET-1 is produced by endothelin-converting enzyme (ECE) from big ET-1 in the endothelium and causes vasoconstriction by binding with ET-1 receptors (type A or B) on the vascular smooth muscle layer (Sandoo et al., 2010). There is scant evidence on regulating ECE activity and ET-1 level in SHR, but findings from other animals (*e.g.* rabbit) and cell studies can provide some clues. For example, an β-lactoglobulin-derived ACEi peptide, ALPMHIR, reduced both basal level and thrombin-stimulated ET-1 releases in endothelial cells (Maes et al., 2004). Fernández-Musoles et al. (2010) reported that eight lactoferricin B-derived ACEi peptides showed *in vitro* ECE inhibitory effect with big ET-1 as substrate; *ex vivo* vascular functional studies in rabbit carotid artery enhanced vasorelaxation induced by big ET-1 not ET-1, indicating that the mechanism was through ECE inhibition. Future studies are warranted to elucidate the role of ECE and ET-1 in BP reduction exerted by antihypertensive peptides in SHR.

2.2.4.3 Antioxidative stress

Excessive oxidative stress accelerates the development of hypertension. Reactive oxygen species (ROS) can directly alter vascular tone through diminishing NO bioavailability or signaling

transduction (Taniyama & Griendling, 2003). Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is the major producer of ROS in the vasculature (Schulz, Gori, & Münzel, 2011). Oral administration of an egg white hydrolysate at a dose of 1,000 mg/kg BW over 12 days significantly diminished oxidative stress in the aorta in SHR, which might contribute to the BP reduction (Jahandideh et al., 2016); its derived antihypertensive peptides, IRW and IQW, also reduced BP through improving vascular oxidative stress (Majumder et al., 2013a; Majumder et al., 2015a). A chicken-derived peptide, AVFQHNCQE, lowered BP in SHR and improved oxidative status by downregulating NADPH oxidase in the aorta and glutathione in the liver in SHR (Mas-Capdevila et al., 2019). Improvement of oxidative stress contributes to vasorelaxation and thus to the antihypertensive activity of food-derived bioactive peptides.

2.2.4.4 Opioid-like activity

Opioid receptors are widely distributed in the central nervous system and peripheral tissues (Peng, Sarkar, & Chang, 2012). It has been reported that morphine, an opioid agonist, induced endothelium-dependent vasorelaxation mediated by NO in rat aortic rings (Stefano et al., 1995). A milk α-lactalbumin derived peptide, YGLF, reduced BP in SHR significantly, but the trend was reversed by administrating an opioid receptor antagonist, naloxone (Nurminen et al., 2000). This suggested that YGLF reduced BP through an opioid-like activity. Another milk peptide, YLLF, also exerted NO-mediated endothelium-dependent relaxation through opioid-like activity in mesenteric arteries of SHR (Sipola et al., 2002). Similar results were observed for AVFQHNCQE, which exerted BP reduction via possibly opioid receptors in the gastrointestinal tract, other than being absorbed (Mas-Capdevila et al., 2019; Mas-Capdevila et al., 2020); the mechanism underlying BP reduction by activation of opioid receptors may be due to the production of NO by the peptide which caused vasorelaxation.

2.2.4.5 Voltage-dependent Ca^{2+} channels (VDCC) blocker

Both vasoconstrictors (*e.g.* ET-1, Ang II) and vasodilators (*e.g.* NO, PGI_2) regulate vascular tone through influencing Ca^{2+} level in the vascular smooth muscle cells (VSMC). Increased Ca^{2+} level in the VSMC leads to vasoconstriction, resulted from the intracellular Ca^{2+} release from the endoplasmic reticulum or influx of extracellular Ca^{2+} via VDCC (Ghosh et al., 2017). Therefore, blocking VDCC contributes to vasorelaxation and thus reduces BP. A peptide, VY, exerted an antiproliferative effect in Ang II-induced VSMC by serving as a VDCC blocker (Matsui et al., 2005). Similarly, another anti-atherosclerotic peptide, WH, demonstrated antiproliferative activity by acting as a VDCC blocker in both VSMC and isolated thoracic aorta of Wistar Kyoto rats (Wang et al., 2010). More *in vivo* evidence is yet to be obtained with regards to the contribution of VDCC blockage to BP reduction by antihypertensive peptides.

2.2.5 Human trials of antihypertensive peptides

Table 2.6 lists some trials of antihypertensive peptides in human subjects. Most research on the efficacy of antihypertensive peptides in human subjects has been focusing on dairy products especially those containing IPP and VPP (Boelsma & Kloek, 2008). In a randomized, placebo-controlled, double-blind study with 70 Caucasian subjects with prehypertension or stage 1 hypertension, daily consumption of two 500-mg milk protein hydrolysate capsules (each enriched with 7.5 mg IPP) lowered SBP by 3.8 mmHg in stage 1 hypertensive subjects but did not change SBP in prehypertensive subjects (Boelsma et al., 2010). Consumption of 150-mL yogurt containing IPP and VPP for 12 weeks caused SBP reduction of 6.1 mmHg in hypertensive subjects (Nakamura et al., 2004). Also, a daily intake of 150-mL fermented milk product by *Lactobacillus helveticus* LBK-16H reduced SBP by 6.7 mmHg over 21 weeks (Seppo et al., 2003). In addition to dairy products, peptides from other food sources were also able to regulate BP in hypertensive

subjects. A thermolysin-digested pea protein hydrolysate, which had previously reduced BP in both SHR and the Han:SPRD-*cy* rat (a chronic kidney disease model), lowered BP by 5-6 mmHg within 2-3 weeks after being orally administrated at 3 g per day to 7 volunteers (4 females and 3 males, 30-55 year, with SBP of 125-170 mmHg) (Li et al., 2011). A sardine muscle-derived peptide, VY, reduced SBP by 9.3 mmHg after 4 weeks of oral intake (6 mg per day) in 29 mild hypertensive subjects; the plasma level of Ang I was increased while those of Ang II and aldosterone were decreased, suggesting a role of ACE inhibition being occurred (Kawasaki et al., 2000). The antihypertensive activity of VY has also been confirmed in a larger number of 63 hypertensive volunteers (Kawasaki et al., 2002). LKPNM, an ACEi peptide derived from the bonito muscle, was responsible for the antihypertensive effect of the thermolysin-digested bonito muscle protein hydrolysate. Orally administrating the hydrolysate (< 3 kDa) to borderline and mildly hypertensive subjects induced a 12 mmHg SBP reduction after 5 weeks of consumption (1.5 g per day). The treatment group and placebo group were then exchanged for another 5 weeks. BP of the new treatment group started decreasing while that of the new placebo group slowly returned to the baseline level, with a difference in SBP of 7 mmHg at the close of the study (Fujita et al., 2001). However, an increasing inconsistency was observed in the antihypertensive effect of lactopeptides in hypertensive subjects among different populations. For example, oral administration of lactopeptides did not affect BP in Dutch and Danish subjects (Engberink et al., 2008; Usinger et al., 2010). Furthermore, BP reduction appeared to be more evident in Asians than Caucasians by lactopeptides in a meta-analysis study (Cicero et al., 2011). It was speculated that the lack of robust, randomized controlled studies may lead to the varying antihypertensive effects among different human intervention trials (Fekete, Givens, & Lovegrove, 2013). Therefore, more well-designed clinical trials are warranted to confirm the antihypertensive effect of food-derived peptides.

2.3 General conclusions

Spent hens present as a major byproduct in the egg industry but are a rich source of multifunctional peptides, especially with ACEi, antioxidant, and anti-inflammatory activities, which play important roles in BP regulation. Continuous development of health-beneficial compounds from spent hens will provide new sustainable solutions for alleviating the burden of waste disposal of the egg industry with extra revenue and contribute to less expensive alternatives for hypertensive individuals. Food-derived peptides have gained substantial interest as an emerging option for the prevention or treatment of hypertension over the past decades. Research on antihypertensive peptides has focused on their production, purification, identification, bioavailability, efficacy, and mechanisms of action. Understanding the health-beneficial effects of antihypertensive peptides provides the scientific basis for their development into functional food ingredients or nutraceuticals. Yet there are still a few challenges impeding the translation of lab work for bedside applications:

1. Enzymatic hydrolysis remains the primary technology for producing antihypertensive peptides, which has a low yield and is generally time-consuming and costly and also very limited choices of food-graded enzymes. However, more novel technologies are being developed to reduce the production cost and improve production efficiency, such as subcritical water hydrolysis, high hydrostatic pressure, and microwave-assisted extraction, among others (Ulug et al., 2020).
2. Given the high production cost of pure peptides, protein hydrolysates produced by food-grade proteases are the possible product forms for the commercialization of antihypertensive peptides. However, developing manufacturing processes with lower cost without comprising the bioactivities remains a challenge. Continuous enzymatic hydrolysis

using immobilized enzymes may be an option to improve the efficiency of large-scale production (Cui et al., 2011; Kapel et al., 2006). Other technologies like the EDUF that avoids ultrafiltration membrane fouling may be a good option especially for the enrichment of specific peptide sequences (*e.g.* those containing arginine, the precursor of NO) with enhanced antihypertensive effect (Firdaous et al., 2009; Nasri, 2017).

3. Off-flavors may present as a big barrier for the organoleptic attributes of the protein hydrolysates or peptides, such as the bitterness and others such as the fishy flavor of marine foods. Some techniques such as Millard reaction and encapsulation may be applied and optimized to remove the off-flavors without affecting the bioavailability and bioactivity (Fu et al., 2019; Yang et al., 2012).
4. While there are numerous peptides identified based on *in vitro* assays mainly ACEi activity, only a small fraction of them have been tested for the *in vivo* BP reduction in animals; only a handful of peptides have been validated in human hypertensive subjects (Boelsma et al., 2010; Kawasaki et al., 2000; Li et al., 2011). Therefore, randomized clinical trials are suggested to support the translational studies of antihypertensive peptides. Concerning the peptides showing antihypertensive effects in animals, more studies are warranted to reveal the mechanisms of action of antihypertensive peptides.
5. Bioavailability plays a crucial role in regulating *in vivo* efficacy of antihypertensive peptides (Xu et al., 2019); however, some peptides are reported to reduce BP without absorption (Mas-Capdevila et al., 2020). Thus, it is very interesting to study the interaction between peptides and unknown receptors in the gastrointestinal tract to further understand the *in vivo* mechanisms of antihypertensive peptides. Apart from these, gut microbiota may

also need to be considered, given their roles in the development of CVDs (Tang & Hazen, 2014).

This project aims to prepare a spent hen muscle protein hydrolysate with antihypertensive activity and to identify and characterize the responsible antihypertensive peptides therein. The identified peptides may provide new insights into the *in vivo* mechanisms of antihypertensive peptides. These studies will provide new value-added uses for the disposal of spent hens and generate extra revenue for the Canadian egg industry.

2.4 References

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Table 2.1 Functional food application of spent hens

Bioactive peptides (properties)	Portion	Processing conditions*	Product characterization	References
Antioxidant peptides	Meat proteins	- Prepared by flavourzyme or alcalase simultaneously or sequentially (E/S 1-3%, 50-55 °C, pH 6.5-7.5 for up to 6 h)	- A sequential hydrolysis by alcalase and flavourzyme showed higher degree of hydrolysis - Flavourzyme-digested hydrolysate showed higher protein recovery and antioxidant activity (DPPH-scavenging activity, FRAP, and ferrous ion chelating activity)	(Kumar et al., 2020)
Antioxidant, ACEi, and anti-inflammatory peptides (IWHHT, IWH, IW)	Muscle proteins	- IWHHT was prepared by thermolysin (E/S 0.5%, 60 °C, pH 8 for 3h) - IWH and IW were gastrointestinal digests of IWHHT	- IWHHT/IW had ACE IC_{50} values of 9.93/2.0 μM - IWHHT, IWH, and IW reduced basal oxidative stress in endothelial cells (DHE staining assay) - IWHHT and IWH attenuated TNFα-induced inflammation (reduced VCAM-1 expression by 40%-60%) in endothelial cells - IWHHT, IWH, and IW were transported intact in Caco-2 cell monolayers	(Fan, Xu, Hong, & Wu, 2018b; Gu et al., 2019)
Antioxidant and ACEi peptides	Meat proteins	- Prepared by alcalase (55 °C, pH 7.0), flavourzyme (50 °C, pH 7.0), neutrase (50 °C, pH 6.0), protamex (40 °C, pH 7.0), pepsin (37 °C, pH 3.0) and trypsin (37 °C, pH 8.0) for up to 6 h (E/S, 1%) - Added hydrolysates into crab meat analogue	- Incorporation of 1.5% of the hydrolysate increased DPPH- and hydroxyl radical-scavenging activities of crab meat analogue - Incorporation of 1.0% of the hydrolysate increased ACEi activity of crab meat analogue	(Jin et al., 2016)
Antioxidant peptides	Skin (Elastin)	- Prepared by alcalase (pH 8.5, 60°C) and elastase (pH 8.5, 37 °C) for 2, 4, 8, 12, 16 or 24 h	- DPPH-scavenging activity (16-50%) - ABTS-scavenging activity (60-79%) - Fe^{2+} chelating activities (50-77%)	(Nadalian et al., 2015)
Anti-inflammatory peptides (WPW, FLWGKSY, AGLLGLL, SFMNVKHWPW, AFMNVKHWPW, TFLPMLQHIS ASLSTFQQMWITK	Muscle proteins	- Prepared by Protex 50FP (E/S 4%, 50 °C, pH 3.0 for 3h)	- The hydrolysate increased interleukin-10 level in Sprague-Dawley rats - The hydrolysate and derived peptides showed in *vitro* interleukin-6 inhibitory activity in endotoxin-activated macrophage-like U937 cells	(Yu et al., 2018a,b)

Antioxidant, anti-inflammatory, proliferative, and type I collagen synthetic activities	Collagen	- Prepared by protease M (pH 3.0), alcalase (pH 8.0), Protex 50FP (pH 3.0), Protex 51FP (pH 7.5), by an individual enzyme (2 h) or in combination (2h for each enzyme) (E/S 2%, 50 °C) (10 hydrolysates)	In TNFα-stimulated human dermal fibroblasts - Five hydrolysates reduced oxidative stress - Six hydrolysates reduced inflammation (inhibited ICAM-1 and VCAM-1 expressions) - Two hydrolysates promoted cellular proliferation - One hydrolysate increased type I procollagen synthesis	(Offengenden et al., 2018)
Renin, ACEi, antioxidant, and antihypertensive activities	Meat proteins	- Prepared by pepsin (E/S 1%, pH 2.0) at 37 °C for 1.5 h - Prepared by pepsin (E/S 1%, pH 2.0, 1.5 h) and then by pancreatin (E/S 1%, pH 7.5, 3 h) at 37 °C	- Renin inhibition (IC_{50} value: 0.34 – 0 .52 mg/mL) - ACE inhibition (IC_{50} value: 0.42 –0.64 mg/mL) - Bovine plasma oxidation-inhibitory activity (plasma sulfhydryl content and FRAP) - Reduced systolic blood pressure by 26.5 and 36.8 mmHg in SHR	(Udenigwe et al., 2017)
ACEi activity	Skin (Elastin)	- Prepared by alcalase (pH 8.5, 60°C) and elastase (pH 8.5, 37 °C) for 2, 4, 8, 12, 16 or 24 h	- Both elastin hydrolysate and its fraction (< 3 kDa) exhibited ACEi activity	(Yusop et al., 2016)
LWM peptides	Collagen	- Pepsin (E/S 1%, pH 2.0) for 24 h of pre-treatment followed by hydrolysis by papain (E/S 2%, pH 6.0, 6h)	- Pepsin treatment enhanced production of LMW collagen peptides (to 32.59%) by removing telopeptides and reduces cross-links	(Hong et al., 2017)
LMW peptides	Collagen	- Formic acid treatment of pepsin-(E/S 1%, pH 2.0, for 24 h) or heat-soluble collagens, before hydrolysis by papain (E/S 2%, pH 6.0, 6h)	- Formic acid treatment enhanced production of LMW collagen peptides (from 36.32% to 43.34%) for pepsin-soluble or (33.79%-48.92%) for heat-soluble collagen by removing telopeptides and reducing cross-links	(Hong et al., 2018)
Anti-aging of LWM peptides	Collagen	- Produced by papain hydrolysis after formic acid and pepsin pre-treatments (Hong et al., 2018)	In human dermal fibroblasts with UVA-exposure - Increased cell viability and type I procollagen production - Reduced apoptotic genes - Reduced oxidative stress (DHE staining assay) - Reduced MMP-1 and MMP-9 production	(Wang et al., 2019)

* Processing condition includes enzymatical hydrolysis parameters (enzyme/substrate (E/S), temperature (T), and pH value). ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); ACEi, angiotensin-converting enzyme (ACE) inhibitory (ACEi); DHE, dihydroethidium; DPPH, 2,2-diphenyl-1-picrylhydrazyl; FRAP, ferric reducing antioxidant power; ICAM-1, intracellular adhesion molecule-1; MMP, metalloprotease; LMW, low-molecular-weight; SHR, spontaneously hypertensive rat; TNFα, tumor necrosis factor alpha; UV-A, ultraviolet A; VCAM-1, vascular cell adhesion molecule-1.

Table 2.2 Biomaterial applications of spent hens

Application (properties)	Portion	Processing conditions	Product characterization	References
Wood adhesive	Muscle proteins	- Extracted muscle proteins were modified by 3% sodium dodecyl sulfate or 3M urea	- Treatment with sodium dodecyl sulfate or urea promoted protein unfolding, exposing more secondary structures that strengthen protein-wood bonding - Interaction between proteins and modification agents enhanced mechanical interlocking - The protein-based adhesive can be used in both dry and wet environments	(Wang et al., 2012a)
Hydrogel	Collagen (Gelatin)	- Gelatin was made from extracted collagen - Gelatin scaffold was formed after Glutaraldehyde crosslinking	- Properties of gelatin scaffold: porosity (90%), pore size (range of 104 – 244 μm), and water uptake (1149%) - The scaffold promoted proliferation of human dermal fibroblasts for wound healing	(Esparza et al., 2018)
Polymer precursors (Ethenolysis)	Lipids	- Ethenolysis was achieved by a microwave-assisted solvent-free approach with catalysts	- A renewable lipidic source for ethenolysis	(Pradhan et al., 2020)
Bio-epoxy	Lipids	- Lipids were extracted using supercritical CO_2 - Bio-epoxy was produced by epoxidation of the extracted lipids	- An spent hen lipid-derived bio-epoxy material produced using supercritical CO_2 extraction and solvent-free epoxidation of extracted lipids	(Safder et al., 2019)
Bionanocomposite films	Muscle proteins	- Compression molding using glycerol (plasticizer), chitosan (cross-linker), and nanoclay (nanoparticles)	- Produced a protein-based exfoliated bionanocomposite film with improved thermal, thermomechanical, and barrier properties - The bionanocomposite film may be used as food packaging materials	(Zubair et al., 2019)
Bionanocomposite films	Lipids	- The bionanocomposite film was prepared by compression molding using spent hen-derived fatty acids and nanoclay	- Produced a lipid-based bionanocomposite film with enhanced thermal stability and flame retardancy compared to the neat homopolymer	(Safder, Temelli, & Ullah, 2020)

Table 2.3 Blood pressure lowering activity of food protein hydrolysates in spontaneously hypertensive rats

Protein sources	Proteases	SBP (ΔmmHg)	Dose (mg/kg BW)	Reference
Spanish dry-cured ham extract	Aqueous extract	–27 (6 h) –38 (6 h) –24 (6 h)	1.48 (O) 4.56 (O) 8.7 (O)	(Escudero, Aristoy, Nishimura, Arihara, & Toldra, 2012a)
Porcine skeletal muscle	Thermolysin	–16 (6 h) –23 (8 h)	210 (O) 2,100 (O)	(Nakashima et al., 2002)
Bovine gelatin	Thermolysin	–17 (6 h)	300 (O)	(Herregods et al., 2011)
Chicken muscle	Pepsin Pepsin + Pancreatin	–27 (2 h) –36 (2 h)	200 (O) 200 (O)	(Udenigwe et al., 2017)
Kacang goat meat	Protamex+ Flavourzyme	–19 (6 h) –27 (6 h)	10 (O) 100 (O)	(Mirdhayati, Hermanianto, Wijaya, Sajuthi, & Arihara, 2016)
Whole egg	Fried	–23 (3 d) –20 (18 d)	1,000 (O) 1,000 (O)	(Jahandideh et al., 2014; Majumder et al., 2013b)
Egg white	Pepsin	–44 (6 h) –42 (20 w) –39 (6 h)	400 (O) 1,000 (O) 100 (O)	(Miguel et al., 2005, 2006b)
	Pepsin Pepsin + Corolase PP	–28 (6 h) –20 (4 h)	100 (O) 100 (O)	(Miguel, Alonso, Salaices, Aleixandre, & Lopez-Fandino, 2007a; Miguel, Manso, Aleixandre, Alonso, Salaices, & Lopez-Fandino, 2007c)
	Thermolysin + pepsin	–30 (12 d)	1000 (O)	(Jahandideh et al., 2016)
Egg yolk	Newlase F from *Rhizopus*	–20 (12w)	500 (O)	(Yoshii, Tachi, Ohba, Sakamura, Takeyama, & Itani, 2001)
Sour milk	*Lactobacillus helveticus* + *Saccharomyces cerevisiae*	–35 (4 h) –20 (14 w)	5 mL (O) 2.5% diet (O)	(Nakamura et al., 1996; Nakamura et al., 1995; Takano, 2002)
Bovine whey	Alcalase	–34 (8 h) –30 (15 d)	240 (O) 240 (O)	(Wang, Wang, Cheng, Zhou, Tang, & Mao, 2012b)
Bovine whey	Trypsin Proteinase K Actinase E	–51 (6 h) –55 (6 h) –55 (6 h)	8 (O) 8 (O) 8 (O)	(Abubakar et al., 1998)
Bovine whey	Crude aequous extract of *C. carduncul*us (< 3 kDa)	–20 (4 h) –22 (4 h)	400 (O) 400 (O)	(Tavares et al., 2012)
	Protease D3	–22.5 (2 h)	500 (O)	(Kodera & Nio, 2006)

	Fermented product (WE80BG)	–24.5 (8 h)	2 (O)	(Murakami et al., 2004)
Bovine casein	AS1.398 Neutral protease	–23 (5 h) –14 (4 w) –18 (4 w)	300 (O) 100 (O) 300 (O)	(Jiang, Tian, Brodkorb, & Huo, 2010)
	Protease D3	–27.5 (2 h)	500 (O)	(Kodera et al., 2006)
	Subtilisin + Bacillolysin + Trypsin	–15 (2 h) –23 (2 h) –38 (2 h) –12 (4 w) –12 (4 w)	5 (O) 10 (O) 100 (O) 10 (O) 25 (O)	(Yamada et al., 2013)
	Yeast (*K. lactis* KI3) Yeast (D. *hansenii* Dh4) Yeast (*K. marxianus* Km2)	–11 (2 h) –21 (2 h) –22 (2 h)	200 (O) 200 (O) 200 (O)	(García-Tejedor, Padilla, Salom, Belloch, & Manzanares, 2013)
Bovine Lactoferrin	Yeast (*K. lactis* KI6) Yeast (*D. hansenii* Dh4) Yeast (*K. marxianus* Km2)	–12 (2 h) –18 (1 h) –24 (1 h)	200 (O) 200 (O) 200 (O)	(García-Tejedor et al., 2013)
Jellyfish (*Rhopilema esculentum*)	Pepsin + Papain	–31 (2 h) –33 (4 h) –34 (2 h) –59 (5 w) –62 (5 w) –69 (5 w)	200 (O) 400 (O) 800 (O) 200 (O) 400 (O) 800 (O)	(Liu et al., 2012)
Shrimp (*Acetes Chinensis*)	Pepsin (< 3 kDa)	–20 (6 h) –28 (6 h) –40 (6 w)	300 (O) 600 (O) 900 (O)	(Zhang et al., 2009)
Oyster	Pepsin (gel filtrated fraction)	–13 (2 h) –20 (2 h) –26 (4 w)	20 (O) 100 (O) 20 (O)	(Wang et al., 2008)
Bonito	Thermolysin	–5 (4 h) –10 (4 h) –15 (6 h)	250 (O) 500 (O) 1,000 (O)	(Fujita et al., 2001)
Corn zein	Thermolysin	–15 (6 h)	5,000 (O)	(Miyoshi et al., 1995)
Corn	Alcalase + Neutrase	–40 (8 w) –35 (8 w) –45 (8 w)	450 (O) 1350 (O) 4050 (O)	(Lin et al., 2011)
Corn	Alcalase (< 3 kDa)	–26 (2 h)	100 (O)	(Huang et al., 2011)

		–34 (30 d)	100 (O)	
Wheat gluten	Protease D3	–27.5 (2 h)	500 (O)	(Kodera et al., 2006)
Buckwheat	Lactic-fermented	–40 (6 h)	1 (O)	(Koyama, Naramoto, Nakajima, Aoyama, Watanabe, & Nakamura, 2013)
Rapeseed	Thermolysin	–9 (8 h)	100 (O)	(He et al., 2013a)
	Proteinase K	–5 (8 h)	100 (O)	
	Alcalase	–24 (8 h)	100 (O)	
	Flavourzyme	–17 (6 h)	100 (O)	
	Pepsin + Pancreatin	–21 (24 h)	100 (O)	
Mung bean	Alcalase	–30 (6 h)	600 (O)	(Li, Shi, Liu, & Le, 2006)
Mung bean	Bromelain	–40 (24 h)	20 (O)	(Sonklin, Alashi, Laohakunjit, Kerdchoechuen, & Aluko, 2020)
Soybean	Protease D3	–22.5 (2 h)	100 (O)	(Kodera et al., 2006)
		–27.5 (2 h)	500 (O)	
		–37.5 (2 h)	1,000 (O)	
Pea	Thermolysin	–13 (4 h)	100 (O)	(Li et al., 2011)
		–19 (4 h)	200 (O)	
		–30 (8 w)	0.5% of diet (O)	
		–30 (8 w)	1.0% of diet (O)	
Sesame	Thermolysin	–25 (6 h)	1 (O)	(Nakano et al., 2006)
		–30 (8 h)	10 (O)	
		–11.6 (2 w)	1 (O)	
		–18.7 (2 w)	10 (O)	
Rapeseed	Subtilisin	–5 (4 h)	150 (O)	(Marczak et al., 2003)
		–10 (4 h)	250 (O)	
		–15 (4 h)	500 (O)	
Canola protein isolate	Alcalase	–34.5 (4 h)	200 (O)	(Alashi et al., 2014)
	Chymotrypsin	–16.3 (6 h)	200 (O)	
	Pepsin	–23.7 (4 h)	200 (O)	
	Pancreatin	–15.1 (6 h)	200 (O)	
Hemp seed isolate	Pepsin + Pancreatin	–25 (4 w)	1% of diet (O)	(Girgih, Alashi, He, Malomo, & Aluko, 2014a)
	Pepsin + Pancreatin	–25 (4 w)	0.5% of diet (O)	
Hemp seed isolate	Pepsin + Pancreatin	–30 (8 h)	200 (O)	(Girgih, Udenigwe, Li, Adebiyi, & Aluko, 2011)
Flaxseed protein isolate	Thermoase	–30 (4 h)	200 (O)	(Nwachukwu et al., 2014)

	Thermoase (3-5 kDa)	–38 (8 h)	200 (O)	
Flaxseed	Trypsin + Pronase	–18 (2 h)	200 (O)	(Udenigwe et al., 2012a)
Pistachio (*Pistacia vera L.*) kernel	Pepsin	–12 (6 h)	1,000 (O)	(Li et al., 2014)
	Trypsin	–25 (6 h)	1,000 (O)	
	Pepsin + Trypsin	–25 (6 h)	1,000 (O)	
Apricot almond meal	Neutrase + N120P	–17.2 (2 h)	400 (O)	(Wang, Tian, & Wang, 2011)
		–20.8 (2 h)	800 (O)	
		–12.5 (4 w)	400 (O)	
		–12.5 (4 w)	800 (O)	
Red Seaweed (*Palmaria palmata*)	Papain	–34 (2 h)	50 (O)	(Fitzgerald et al., 2014)
Bamboo shoot	Aqueous extract	–11 (4 h)	20 (O)	(Liu et al., 2013)
		–20 (4 h)	50 (O)	
		–27 (5 h)	100 (O)	
Mushroom (*Pleurotus cornucopiae*)	Aqueous extract	–37 (1 h)	600 (O)	(Jang et al., 2011)
Sweet potato	Thermoase PC10F + Protease S + Proleather FG-F	–16 (8 h)	100 (O)	(Ishiguro, Sameshima, Kume, Ikeda, Matsumoto, & Yoshimoto, 2012)

BW, body weight; SBP, systolic blood pressure; values in the dose column indicate the dosage of oral (O) or intravenous (IV) administration, respectively; values in the SBP column indicate the maximum SBP reduction within the time period in the parentheses (within 24 h) or the endpoint SBP reduction for long term studies within days/weeks; comments in the parentheses of the proteases column specified the hydrolysate samples fed to animals.

Table 2.4 Blood pressure lowering activity of food-derived antihypertensive peptides in spontaneously hypertensive rats

Protein sources	Proteases	Sequences	SBP (ΔmmHg)	Dose (mg/kg BW)	Reference
Spanish dry-cured ham	Aqueous extract	AAATP	–26 (8 h)	1 (O)	(Escudero, Mora, Fraser, Aristoy, Arihara, & Toldra, 2013)
Porcine meat	Pepsin + Pancreatin	RPR	–33 (6 h)	1 (O)	(Escudero et al., 2012b)
		KAPVA	–34 (6 h)	1 (O)	
		PTPVP	–26 (6 h)	1 (O)	
Porcine skeletal muscle (e.g. myosin, troponin)	Pepsin	KRQKYDI	–10 (3 h)	10 (O)	(Katayama et al., 2008)
	Thermolysin	MNPPK	–23 (6 h)	1 (O)	(Nakashima et al., 2002)
		MNP	–20 (6 h)	1 (O)	
		PPK	–25 (6 h)	1 (O)	
		ITTNP	–21 (6 h)	1 (O)	
	Pepsin	VKKVLGNP	–24 (3 h)	10 (O)	(Katayama et al., 2007)
	Pepsin	KRVIQY	–25 (6 h)	10 (O)	(Muguruma et al., 2009)
		VKAGF	–20 (6 h)	10 (O)	
Chicken Feet	Protamex	AVFQHNCQE	–25 (6 h)	10 (O)	(Bravo et al., 2019)
		QVGPLIGRYCG	–11 (6 h)	10 (O)	
Chicken muscle	Thermolysin	IKW	–50 (4 h)	10 (IV)	(Fujita et al., 2000)
			–17 (4 h)	60 (O)	
		LKP	–75 (4 h)	10 (IV)	
			–18 (4 h)	60 (O)	
Kacang goat meat	Protamex + Flavourzyme	FQPS	–11 (8 h)	2.4 (O)	(Mirdhayati et al., 2016)
Egg white	Pepsin	IVF	–32 (6 h)	4 (O)	(Miguel et al., 2006a; Miguel, Alvarez, Lopez-Fandino, Alonso, & Salaices, 2007b; Miguel et al., 2005; Miguel et al., 2007c)
		ESIINF	–30 (6 h)	10 (O)	
		RDILNQ	–15 (2 h)	10 (O)	
		RADHPFL	–34 (6 h)	2 (O)	
		YAEERYPIL	–32 (6 h)	2 (O)	
		YRGGLEPINF	–30 (4 h)	10 (O)	
	Pepsin + Corolase PP	RADHP	–30 (4 h)	2 (O)	
		YPI	–40 (2 h)	2 (O)	
		YAEER	–17 (6 h)	2 (O)	
Egg white	Alcalase	QIGLF	–11 (10 h)	50 (O)	(Yu, Zhao, Ding, Wang, Chen, & Liu, 2017)
			–14 (6 d)	50 (O)	
			–37 (4 w)	50 (O)	
		RVPSL	–20 (4 w)	50 (O)	(Yu, Yin, Zhao, Chen, & Liu, 2014)

Egg ovalbumin	Pepsin	FRADHPFL LW	–18 (4 h) –22 (2 h) –45 (2 h)	25 (O) 60 (O) 10 (IV)	(Fujita, Sasaki, & Yoshikawa, 1995; Fujita et al., 2000)
	Chymotrypsin	RADHPF	–15 (6 h) –20 (20 min)	20 (O) 5 (IV)	(Matoba, Usui, Fujita, & Yoshikawa, 1999; Scruggs et al., 2004)
	Pepsin	LW	–45 (2 h) –22 (2 h)	10 (IV) 60 (O)	(Fujita et al., 2000)
Egg ovotransferrin	Chymotrypsin	KVREGTTY KVREGT	–21 (40 min) –24 (20 min)	1 nmol/kg (IV) 1 nmol/kg (IV)	(Lee et al., 2006)
	Thermolysin + pepsin	IRW IQW LKP	–40 (18 d) –21 (18 d) –30 (18 d)	15 (O) 15 (O) 15 (O)	(Majumder et al., 2015a)
Sour milk	*L. helveticus* + *S. cerevisiae*	VPP IPP	–32 (4 h) –28 (8 h)	0.6 (O) 0.3 (O)	(Nakamura et al., 1995)
	L. helveticus CPN4	YP	–27.4 (6 h)	1 (O)	(Yamamoto et al., 1999)
Bovine whey	*C. cardunculus*	DKVGINYW DAQSAPLRVY KGYGGVSLPEW	–14 (2 h) –10 (6 h) –20 (6 h)	5 (O) 5 (O) 5 (O)	(Tavares et al., 2012)
	Fermented product (WE80BG)	ALPM	–21.4 (8 h)	1 (O)	(Murakami et al., 2004)
	Proteinase K	VYPFPG GKP IPA FP VYP	–22 (6 h) –26 (6 h) –31 (6 h) –27 (6 h) –21 (6 h)	8 (O) 8 (O) 8 (O) 8 (O) 8 (O)	(Abubakar et al., 1998)
Bovine casein	Subtilisin + Bacillolysin + Trypsin	MKP	–23 (8 h)	0.1 (O)	(Yamada et al., 2013)
	A proteinase from L. helveticus CP790	KVLPVPQ	–24 (6 h)	2 (O)	(Maeno, Yamamoto, & Takano, 1996)
Milk casein	From casein sources, *e.g.* caprine kefir, ovine and dairy products	LVYPFTGPIPN HLPLP IAK YAKPVA WQVLPNAVPAK HPHPHLSF KKYNVPQL	–28 (4 h) –24 (4 h) –21 (4 h) –23 (4 h) –18 (4 h) –16 (4 h) –12 (4 h)	10 (O) 7 (O) 4 (O) 6 (O) 7 (O) 10 (O) 10 (O)	(Miguel, Gómez-Ruiz, Recio, & Aleixandre, 2010)
Bovine Lactoferrin		DPYKLRP	–27 (1 h)	10 (O)	

	Yeast (*K. marxianus*)	PYKLRP YKLRP KLRP GILRP	–22 (2 h) –21 (2 h) –13 (1 h) –20 (3 h)	10 (O) 10 (O) 10 (O) 10 (O)	(García-Tejedor, Sánchez-Rivera, Castelló-Ruiz, Recio, Salom, & Manzanares, 2014)
	Yeast (*K. marxianus*) + Corolase PP	LRP	–27 (2 h)	10 (O)	
Gouda Cheese		RPKHPIKHQ YPFPGPIPN	–9.3 (6 h) –7.0 (6 h)	6.1-7.5 (O) 6.1-7.5 (O)	(Saito et al., 2000)
Cuttlefish	Crude enzymes from *B. mojavensis* A21	VELYP	–20 (6 h)	10 (O)	(Balti, Bougatef, Sila, Guillochon, Dhulster, & Nedjar-Arroume, 2015)
	Endogenous hepatopancreas *Crude extract from B. mojavensis* A21	MAW VYAP VIIF AHSY	–13 (2 h) –22 (6 h) –19 (4 h) –14 (4 h)	10 (O) 10 (O) 10 (O) 10 (O)	(Balti, Bougatef, Guillochon, Dhulster, Nasri, & Nedjar-Arroume, 2012; Balti, Nedjar-Arroume, Adje, Guillochon, & Nasri, 2010; Balti, Nedjar-Arroume, Bougatef, Guillochon, & Nasri, 2010)
Tuna muscle	Pepsin	WPEAAELMMEVDP	–15 (3 h)	10 (O)	(Qian, Je, & Kim, 2007)
Bonito muscle	Thermolysin	FQP	–12.6 (6 h)	2.0 (O)	(Mirdhayati et al., 2016; Yokoyama, Chiba, & Yoshikawa, 1992)
Yellowfin sole	Chymotrypsin	MIFPGAGGPEL	–38 (6 h)	10 (O)	(Jung et al., 2006)
Bonito muscle	Thermolysin	IY IW IKP LKP IWH IVGRPR LKPNM IWHHT IVGRPRHQG	–45/–19 (2 h) –55/–22 (2 h) –70/–20 (6 h) –75/–18 (4 h) –70/–30 (4 h) –25/–17 (6 h) –80/–23 (6 h) –60/–26 (6 h) –0 /–14 (8 h)	10 (IV)/60 (O) 10 (IV)/60 (O) 10 (IV)/60 (O) 10 (IV)/60 (O) 10 (IV)/60 (O) 10 (IV)/60 (O) 10 (IV)/60 (O) 10 (IV)/60 (O) 10 (IV)/60 (O)	(Fujita, Yokoyama, Yasumoto, & Yoshikawa, 1995; Fujita et al., 2000)
Cotton leafworm *(Spodoptera littoralis)*	Pepsin + Trypsin + Chymotrypsin Pepsin + Trypsin + Chymotrypsin + mucosal peptidases	AVF VF	–9 (4 h) –19 (6 h)	5 (O) 6 (O)	(Vercruysse, Van Camp, Morel, Rougé, Herregods, & Smagghe, 2010)
Corn zein	Thermolysin	LRP	–15 (2 min)	30 (IV)	(Miyoshi et al., 1991)
Corn gluten	Alcalase	AW	–9.5 (2 h)	50 (O)	(Yang, Tao, Liu, & Liu, 2007)

Rice	Chymotrypsin	IHRF	–18 (7 h) –39 (7 h)	5 (O) 15 (O)	(Kontani et al., 2014)
	Alcalase + Trypsin	VNP	–29 (4 h)	5 (O)	(Chen, Liu, Ye, Cai, Ji, & Wu, 2013)
		VWP	–38 (4 h)	5 (O)	
Buckwheat	Lactic-fermented	DVWY	–55 (6 h)	0.1 (O)	(Koyama et al., 2013)
		FDART	–30 (6 h)	0.1 (O)	
		FQ	–33 (9 h)	0.1 (O)	
		VAE	–24 (6 h)	0.1 (O)	
		VVG	–28 (9 h)	0.1 (O)	
		WTFR	–30 (6 h)	0.1 (O)	
Mung bean	Bromelain	LPRL	–25 (24 h)	20 (O)	(Sonklin et al., 2020)
		YADLVE	–47 (24 h)	20 (O)	
		LRLESF	–30 (24 h)	20 (O)	
		HLNVVHEN	–38 (24 h)	20 (O)	
		PGSGCAGTDL	–25 (24 h)	20 (O)	
Pea protein isolate	Thermolysin	WMP	–39 (8 h)	30 (O)	(Aluko, Wu, & Aukema, 2014)
		ADMFPF	–25 (24 h)	30 (O)	
Rapeseed Rapeseed	Subtilisin	IY	–9.5 (2 h)	7.5 (O)	(Marczak et al., 2003)
		RIY	–11.3 (4 h)	7.5 (O)	
		VW	–10.8 (2 h)	7.5 (O)	
		VWIS	–12.5 (2 h)	12.5 (O)	
	Alcalase	LY	–26 (2 h)	30 (O)	(He et al., 2013b)
		TF	–12 (4 h)	30 (O)	
		RALP	–16 (2 h)	30 (O)	
	Pepsin + Pancreatin	GHS	–17 (6 h)	30 (O)	
Hemp seed	Pepsin + Pancreatin	WYT	–13 (2 h)	30 (O)	(Girgih et al., 2014b)
		WVYY	–34 (2 h)	30 (O)	
		SVYT	–24 (6 h)	30 (O)	
		PSLPA	–40 (4 h)	30 (O)	
		IPAGV	–36 (4 h)	30 (O)	
Red Seaweed (*P. palmata*)	Papain	IRLIIVLMPILMA	–33 (2 h)	50 (O)	(Fitzgerald et al., 2014)
Microalgae (*C. ellipsoidea*)	Alcalase	VEGY	–25 (2 h)	10 (O)	(Ko et al., 2012)
Wakame	Hot water extract	YH	–50 (3 h)/–34 (1 w)	50 (O)/10 (O)	

(*U. pinnatifida*)		KY	–45 (3 h)/–26 (1 w)	50 (O)/10 (O)	(Suetsuna, Maekawa, & Chen, 2004)
		WY	–46 (3 h)/–34 (1 w)	50 (O)/10 (O)	
		IY	–33 (3 h)/–25 (1 w)	50 (O)/10 (O)	
Bamboo shoot	Aqueous extract	DY	–17 (3 h)	10 (O)	(Liu et al., 2013)
Spinach	Pepsin + Pancreatin	MRW	–20 (2 h)	30 (O)	(Yang et al., 2003)
		MRWRD	–14 (4 h)	30 (O)	
		IAYKPAG	–15 (4 h)	100 (O)	
		IAYKP	–10 (4 h)	80 (O)	
		KP	–9 (2 h)	60 (O)	
Mushroom (*T. giganteum)*	Aqueous extract	GQP	–33 (2 h)	1 (O)	(Lee, Kim, Park, Choi, & Lee, 2004)

BW, body weight; SBP, systolic blood pressure; values in the dose column indicate the dosage of oral (O) or intravenous (IV) administration, respectively; values in the SBP column indicate the maximum SBP reduction within the time period in the parentheses (within 24 h) or the endpoint SBP reduction for long term studies within days/weeks.

Table 2.5 Antihypertensive mechanisms of food-derived peptides in spontaneously hypertensive rats

Mechanism	Hydrolysate or peptide	Food matrix or protein source	Reference
Renin inhibition	Hydrolysate (by pepsin and pancreatin)	Hemp seed	(Girgih et al., 2014a)
	RVPSL	Egg white	(Yu et al., 2014)
	RALP, GHS	Rapeseed	(He et al., 2019)
ACE inhibition	Hydrolysate (by pepsin and pancreatin)	Hemp seed	(Girgih et al., 2014a)
	Hydrolysate (by thermolysin and pepsin)	Egg white	(Jahandideh et al., 2016)
	Protein hydrolysate (by alcalase)	Whey	(Wang et al., 2012b)
	DPYKLRP, LRP	Lactoferrin	(García-Tejedor et al., 2014)
	IPP, VPP	Casein	(Jäkälä et al., 2009)
	YAEERYPIL and RADHPFL	Ovalbumin	(Jakala, Pere, Lehtinen, Turpeinen, Korpela, & Vapaatalo, 2009)
	RVPSL	Egg white	(Yu et al., 2014)
	IQP, VEP, hydrolysate (by papain)	Blue algae (*Spirulina platensis*)	(Zheng et al., 2017)
	LY, RALP, GHS	Rapeseed	(He et al., 2019)
ACE2 activation/upregulation	IRW	Egg white	(Liao et al., 2019a; Majumder et al., 2015b)
	IQP, VEP, hydrolysate (by papain)	Blue algae (*Spirulina platensis*)	(Zheng et al., 2017)
	LY, RALP, GHS	Rapeseed	(He et al., 2019)
AT_1R blockage	RVPSL	Egg white	(Yu et al., 2014)
	IQP, VEP, hydrolysate (by papain)	Blue algae (*Spirulina platensis*)	(Zheng et al., 2017)
	Hydrolysate (by thermolysin and pepsin)	Egg white	(Jahandideh et al., 2016)
MasR upregulation	IQP, VEP, hydrolysate (by papain)	Blue algae (*Spirulina platensis*)	(Zheng et al., 2017)
	LY, RALP, GHS	Rapeseed	(He et al., 2019)
Endothelium-dependent vasorelaxation (NO-mediated)	Hydrolysate (by thermolysin and pepsin)	Egg white	(Jahandideh et al., 2016)
	AVFQHNCQE	Chicken foot	(Mas-Capdevila et al., 2019)
	DPYKLRP, hydrolysate (pepsin)	Lactoferrin	(García-Tejedor et al., 2017)
	IRW, IQW, LKP	Egg white	(Majumder et al., 2013a; Majumder et al., 2015a)
	[a] IPP, VPP	Casein	(Nonaka et al., 2014)
	α-lactorphin (YGLF)	α-lactalbumin	(Sipola et al., 2002)
	β-lactorphin (YLLF)	β-lactoglobulin.	
Endothelium-dependent vasorelaxation (Prostanoids (*e.g.,* PGI_2)-mediated)	Rapakinin (RIY)	rapeseed protein	(Yamada et al., 2010)
	Hydrolysate (by pepsin)	Lactoferrin	(García-Tejedor et al., 2017)
Endothelium dependent vasorelaxation (EDHF-mediated)	Hydrolysate (by pepsin)	Lactoferrin	(García-Tejedor et al., 2017)

Endothelium independent vasorelaxation	Hydrolysate (by thermolysin and pepsin) DPYKLRP Hydrolysate (by pepsin) β-lactorphin (YLLF)	Egg white Lactoferrin a-lactalbumin β-lactoglobulin.	(Jahandideh et al., 2016) (García-Tejedor et al., 2017) (Sipola et al., 2002)
ET-1 reduction	[b] ALPMHIR	β-lactoglobulin	(Maes et al., 2004)
ECE inhibition	lactoferricin B-derived peptides	Lactoferrin	(Fernández-Musoles et al., 2010)
Antioxidative stress	AVFQHNCQE IRW, IQW Hydrolysate (by thermolysin and pepsin)	Chicken foot Egg white Egg white	(Mas-Capdevila et al., 2019) (Majumder et al., 2013a; Majumder et al., 2015a) (Jahandideh et al., 2016)
Opioid-like activity	AVFQHNCQE α-Lactorphin (YGLF) β-lactorphin (YLLF)	Chicken foot α-lactalbumin β-lactoglobulin.	(Mas-Capdevila et al., 2020) (Nurminen et al., 2000)
Ca^{2+} channel blocker	[c] VY [d] WH	Sardine Bonito muscle	(Matsui et al., 2005) (Wang et al., 2010)

Studies were performed in spontaneously hypertensive rat unless otherwise specified: [a] Wistar-Kyoto rats, [b] endothelial cells, [c] vascular smooth muscle cells, and [d] vascular smooth muscle cells and Wistar-Kyoto rats. ACE, angiotensin converting enzyme, ACE2, angiotensin-converting enzyme 2, AT_1R, angiotensin II type 1 receptor, ECE, endothelin converting enzyme; ET-1, endothlin-1; PGI_2, prostaglandin I_2; EDHF, endothelium-derived hyperpolarizing factor.

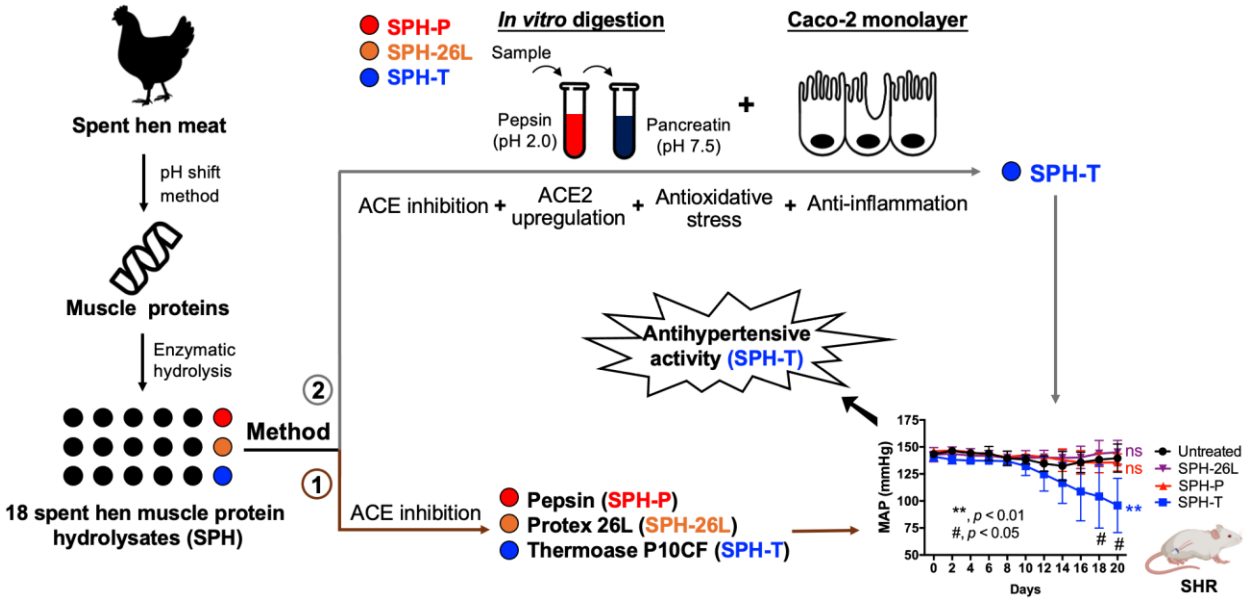
Table 2.6 Blood pressure-lowering effect of antihypertensive peptides in hypertensive subjects

Peptide	Product prototype	Description	SBP	Reference
IPP	Capsules with milk protein hydrolysate (enriched by IPP)	- A 4-week trial with 70 Caucasians with pre or stage 1 hypertension. - 2 capsules (totally 15 mg IPP) (per day)	- 3.8 mmHg ↓ at 4 weeks for stage 1 hypertensive subjects - No effect on prehypertension	(Boelsma & Kloek, 2010)
VPP and IPP	Liquid yogurt (containing VPP and IPP)	- A 12-week trial with 53 subjects - 150 g (per day)	- 5.5 mmHg ↓ at 6 week - 6.1 mmHg ↓ at 12 weeks	(Nakamura et al., 2004)
Milk peptides	Fermented milk (by *Lactobacillus helveticus* LBK-16H)	- A 21-week trial with 39 subjects - 150 mL (per day)	- 6.7 mmHg ↓ at 21 weeks	(Seppo, Jauhiainen, Poussa, & Korpela, 2003)
VY	Formulated drink	- A 4-week trial with 29 subjects - 6 mg (per day)	- 9.7 mmHg ↓ at 1 week - 9.3 mmHg ↓ at 4 weeks -	(Kawasaki et al., 2000)
VY	Vegetable drink with sardine muscle protein hydrolysate (containing VY)	- A 13-week trial with 63 subjects - 195 g drink (0.4 mg VY) (per day)	- 7.6 mmHg ↓ in the first week	(Kawasaki et al., 2002)
LKPNM	Tablets with thermolysin-digested bonito muscle protein hydrolysate (< 3 kDa, containing LKPNM)	- 61 borderline and mildly hypertensive subjects - First round for 5 weeks with treatment and placebo groups - Second round for 5 weeks with the above treatment group and placebo group reversed - 10 tablets (totally 1.5 g) (per day)	- 12 mmHg ↓ in the first round - 7 mmHg ↓ in the second round	(Fujita et al., 2001)
Pea peptides	Orange juice with thermolysin-digested pea protein hydrolysate (< 3 kDa)	- A 3-week trial with 7 subjects - 3 g (per day)	5-6 mmHg ↓ at 2-3 weeks	(Li et al., 2011)

SBP, systolic blood pressure.

CHAPTER 3 – Preparation of Spent Hen Muscle Protein Hydrolysate with Antihypertensive Activity [2]

Graphic abstract



[2] This chapter has been published in whole as: Fan, H., Yu, W., Liao, W., & Wu, J. (2020). Spent hen protein hydrolysate with good gastrointestinal stability and permeability in Caco-2 cells shows antihypertensive activity in SHR. *Foods, 9*(10), 1384.

3.1 Introduction

Numerous antihypertensive peptides are characterized from various food proteins with great efficacy in managing hypertension; however, research has consistently shown a discrepancy between their *in vivo* efficacies and *in vitro* activities; many protein hydrolysates or peptides possessing good *in vitro* activity failed to exert physiological effects in spontaneously hypertensive rat (SHR), including hydrolysates produced from salmon, cod, and haddock, and peptides such as FKGRYYP (from chicken creatine kinase), FFGRCVSP (from egg ovalbumin), and ERKIKVYL (from egg ovalbumin), among others (Fujita et al., 2000; Jensen et al., 2014). One possible reason is the presence of numerous proteases/peptidases in the gastrointestinal digestion and intestinal epithelium that may render peptides inactive. Despite being presented as major barriers, gastrointestinal digestion and transepithelial transport are also valuable strategies to assist the generation of peptides with enhanced activities (Ewart et al., 2009; Fan et al., 2018b; Fujita et al., 2000; Liang et al., 2018; Miguel et al., 2008). For example, two ovotransferrin-derived antihypertensive peptides IRW and IQW required pepsin to liberate from their respective parent peptides IRWCT and IQWCA (Majumder & Wu, 2010). A meat-derived peptide IWHHT liberated IWH and IW during gastrointestinal digestion while IWH further generated WH after transepithelial transport (Fan et al., 2018b; Gu et al., 2019); IWH and IW possess antihypertensive, antioxidant, and anti-inflammatory activities while WH is an anti-atherosclerotic peptide (Gu et al., 2019; Tanaka et al., 2015). Under these circumstances, the activity of the parent peptide is dictated by the fragments formed in the gastrointestinal tract.

Another deficiency lies in an incomplete understanding of the mechanisms of action. As depicted in Chapter 2, although lots of peptides are initially characterized as ACEi peptides, many of them have not been confirmed their *in vivo* ACE inhibitory effects (Aluko, 2015); further

investigations indicated that the antihypertensive actions of these peptides involved other mechanisms, including improvement of vascular inflammation, restoration of nitric oxide-dependent vasorelaxation, and upregulation of angiotensin-converting enzyme 2 (ACE2), among others (Wu, Liao, & Udenigwe, 2017). This is further supported by various studies in which some peptides, possessing much weaker *in vitro* ACEi activity than that of captopril, caused comparable BP reduction *in vivo* (Jensen et al., 2014; Pan et al., 2015). Hence, ACE inhibition is not always a reliable *in vitro* parameter to assess the antihypertensive potential of a hydrolysate or a peptide. For example, a previously-identified ACEi peptide IRW (IC_{50} value of 0.64 μM) reduced BP in SHR, but no *in vivo* ACE inhibition was detected; later, mitigated vascular inflammation and restored endothelium-dependent vasorelaxation mediated by upregulation of ACE2 was found to be the major mechanism (Liao et al., 2019). Studies have also reported the importance of antioxidant and anti-inflammatory activities in BP regulation (He et al., 2019; Jahandideh et al., 2016; Tsai et al., 2020). These findings prompted us to also consider other biomarkers, such as ACE2 upregulating (ACE2u), antioxidant, and anti-inflammatory activities, other than ACE inhibition solely, for assessing the antihypertensive potential of a hydrolysate or peptide *in vitro*.

As reviewed in Chapter 2, one of the major value-added use of spent hens is bioactive peptide-based functional food ingredients. Despite being a byproduct, spent hen is indeed a rich source of muscle proteins that have been proven as excellent sources of antihypertensive peptides (Toldrá et al., 2018; Udenigwe & Howard, 2013). Recently, Yu, Field, & Wu (2018a) prepared a spent hen muscle protein hydrolysate (SPH) with anti-inflammatory effects in young rodents; an ameliorated inflammation is beneficial to BP reduction. Therefore, we hypothesized that spent hen muscle proteins are a source of antihypertensive peptides. The current work aims to prepare an SPH with antihypertensive activity. To achieve this goal, nine enzymes were used in isolation or in

combination to produce SPH through enzymatic hydrolysis; two approaches were used to screen the SPH with the most potent antihypertensive effect, including 1) the conventional approach (based on ACEi activity only) and 2) a multiple evaluation approach (based on ACEi, ACE2u, antioxidant, and anti-inflammatory activities, coupled with the simulated gastrointestinal digestion and transepithelial permeability), before being administrated to SHR. ACEi activity was determined using an *in vitro* biochemical assay; ACE2u and antioxidant activities were assessed in vascular smooth muscle A7r5 cells (VSMCs), while anti-inflammatory activity was evaluated in vascular endothelial EA.hy926 cells (ECs).

3.2 Materials and Methods

3.2.1 Materials

Spent hen (~70–80 weeks old, ~1.12 kg) carcasses were purchased from a local supermarket (T&T) in Edmonton, Alberta, Canada. Thermoase PC10F (from *Bacillus thermoproteolyticus Var. Rokk*, 90,000 U/g protein) and Protease S (10,000 U/g) and Protease M (40,000 U/g) were obtained from Amano Enzyme Inc. (Nagoya, Japan). Alcalase 2.4L (2.4 U/g) was bought from Novozymes (Franklinton, NC); Protex 50FP (500,000 U/g), Protex 26L (2000 U/g), and Protex 6L (580,000 U/g)were purchased from Genencor International Inc. (Rochester, NY); pepsin (from porcine gastric mucosa, 1064 U/mg), trypsin (from porcine pancreas, 1983 U/mg), methane-sulfonic acid, 2,4,6-trinitrobenzenesulfonic acid (TNBS), acetonitrile (ACN), trifluoroacetic acid (TFA), ACE (from rabbit lung), N-hippuryl-His-Leu (HHL), cytochrome C, aprotinin, vitamin B_{12}, $(glycine)_3$, and glycine, were purchased from Sigma (Oakville, ON, Canada). VSMCs (A7r5, CRL-1444), ECs (EA.hy926, CRL-2922), and human colon carcinoma cells (Caco-2, HTB-37) cell lines were obtained from ATCC (Manassas, VA, USA). Dulbecco's modified Eagle's medium (DMEM),

0.25% (*w/v*) trypsin-0.53 mM ethylenediaminetetraacetic acid (EDTA), fetal bovine serum (FBS), Hanks balanced salt solution (HBSS with Ca and Mg), nonessential amino acids (NEAA), 4-(2−68 hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and penicillin−streptomycin were obtained from Gibco Invitrogen (Burlington, ON, Canada).

3.2.2 Preparation of spent hen muscle protein hydrolysate

Spent hen muscle proteins were extracted from spent hen carcasses using a pH-shift method (Wang & Wu, 2012). Briefly, spent hen muscle was collected after deboning, skinning, and removal of external fat, and was then homogenized (for 2.5 min) in deionized water (ddH_2O) using a Wearing heavy-duty blender (Wearing Commercial, Torrington, CT). The homogenate was left at pH 11.0 (using NaOH) for 30 min for protein dissolution under continuous stirring (500 rpm) before the first centrifugation at 10,000× g (20 min, 4 °C). Then, the supernatant was collected and adjusted to pH 5.0 (using HCl) for another 30-min stirring (500 rpm). After the second centrifugation, the precipitate (protein extract) was collected, washed three times (using ddH_2O), and freeze-dried. The extraction process was repeated three times.

The protein extract (~93% protein) was dissolved in ddH_2O (5%, *w/w*). After being heated at 90 °C for 10 min for protein denaturation, the slurry was hydrolyzed by nine enzymes in a jacket beaker, connected with a Titrando (Metrohm, Herisan, Switzerland) and a circulating water bath (Brinkman, Mississauga, ON, Canada) for a constant pH and temperature control, respectively. Protein hydrolysates were prepared by using either one (4% enzyme/substrate, E/S, *w/w* protein) or two enzymes (2% E/S for each, *w/w* protein) for 3 h, with hydrolysis parameters depicted in the *Appendix: Supplementary Table 3.1-3.2*. With regards to two-enzyme hydrolysis, enzymes with similar working pH were added together; while for those with different working pH, the first enzyme was added for 1.5 h followed by the second one for another 1.5 h (without enzyme

inactivation in between) under their respective working conditions. After the hydrolysis, the slurry was heated at 95 °C for 10 min to terminate the reaction and then centrifuged at 10,000 g for 15 min at 4 °C. The supernatant was freeze-dried and kept at −20 °C for further analysis. Each hydrolysate was produced in triplicate.

3.2.3 Desalting protocol, simulated gastrointestinal digestion, and ACE inhibitory assay

Prior to treatment with cells, SPHs were desalted according to the protocol described in Fan et al. (2019). Briefly, samples were dissolved in ddH_2O and loaded onto the Sep-Pak 35cc tC18 cartridge (WAT043350, Waters, Milford, MA, USA). The cartridge was first washed with two column volumes (CV) of ddH_2O for salt removal. Then 50% ACN (2 CV) and 100% ACN (1 CV) were added sequentially to wash the cartridge; the ACN eluent was collected, vacuum evaporated, and freeze-dried.

Simulated gastrointestinal digestion was performed as described in Fan et al. (2019). Briefly, SPHs (5% protein in ddH_2O, *w/v*) were digested by pepsin (1% E/S, *w/w* protein) for 1.5 h at 37 °C, pH 2.0 (adjusted with 3 M HCl). Then, the digest was adjusted to pH 7.5 with 1 M NaOH, with half of the digest collected as pepsin digest and another half further digested by pancreatin (1% E/S, *w/w* protein) for 1.5 h (at 37 °C). The reaction was terminated by heating the digests to 95 °C (maintained for 10 min).

Determination of ACE inhibition of SPHs followed the procedures described in Wu, Aluko, & Muir (2002). ACE, HHL, SPHs were dissolved in 100 mM potassium phosphate buffer containing 300 mM NaCl (pH 8.3). Firstly, an aliquot of sample (10 µL) and HHL (50 µL, 5 mM) was mixed and pre-warmed at 37 °C in a 2-mL polypropylene tube. Then, an aliquot of pre-warmed ACE (20 µL, 2 mU) was added and incubated for 30 min with agitation (450 rpm) on an Eppendorf

Thermomixer R (Brinkmann Instruments, NY, USA). The reaction was stopped by adding 125 µL of 1 M HCl solution and then analyzed on a reverse-phase ultra-performance liquid chromatography (RP-UPLC) system described in (Fan et al., 2019). The IC_{50} value was defined as the sample concentration inhibiting 50% of ACE activity.

3.2.4 Cell culture of vascular smooth muscle A7r5 cells, endothelial EA.hy926 cells, and Caco-2 cells

Cell culturing protocol of VSMCs (passages 4–11), ECs (passages 3–10), and Caco-2 (passages 22–28) cell lines referred to previous studies (Fan et al., 2018b; Liang et al., 2018; Liao, Fan, & Wu, 2018b; Xu et al., 2017). Cells were grown in DMEM supplemented with 10% FBS, 25 mM HEPES, and 1% antibiotics (penicillin-streptomycin) at 37 °C in a 100% humidified atmosphere with 5% CO2; nonessential amino acids (1%) were supplemented for ECs and Caco-2 cell lines. The growth media were changed every three days for VSMCs and ECs and every 2 days for Caco-2 cells.

3.2.5 Western blotting

The confluent VSMCs were placed in a quiescing medium (the same as that of the growth medium but with 1% FBS). VSMCs were treated with 2.5 mg/mL of SPHs for 24 h for ACE2 detection. ECs were treated with 2.5 mg/mL of SPHs for 18 h before the addition of 10 ng/mL of tumor necrosis factor alpha (TNFα) for a 6 h co-treatment for detection of intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). The dose of hydrolysate was selected based on our previous studies (Liang et al., 2018). Cells were lysed in boiling Laemmle's buffer containing 50 mM Dithiothreitol (DTT) and 0.2% Triton-X-100.

Cell lysates were loaded onto a 9% separating gel and transferred to a nitrocellulose membrane (diameter 0.45 µm, 1620115, Bio-Rad, Montreal, QC, Canada) for incubation with antibodies. Bands of ACE2 (Abcam, Toronto, ON, Canada), ICAM-1 (Santa Cruz, Dallas, TX, USA), and VCAM-1 (Santa Cruz) were normalized to α-tubulin (Abcam). Donkey-anti-rabbit 800 CW or donkey-anti-mouse IRDye 680 RD secondary antibodies (Licor Biosciences, Lincoln, NE, USA) were used to visualize the fluorescent bands in a Licor Odyssey BioImager, which were quantified using Image Studio Lite 5.2 (Licor Biosciences).

3.2.6 Superoxide detection

Superoxide generation in VSMCs was detected by dihydroethidium (DHE) staining, as described by Wang et al. (2020) with slight modifications. Cells were treated with 2.5 mg/mL of SPHs for 1 h before the addition of 1 µM of Ang II for 0.5 h. Then, DHE (20 µM) was added and incubated with cells for 30 min (protected from light). Later, the cells were washed three times with a non-phenol-red DMEM (21063029, Thermo Fisher Scientific, Burlington, ON, Canada). The fluorescence signal was detected by an Olympus IX81 fluorescent microscope (Olympus, Tokyo, Japan). Each data point was taken from three random fields. The total fluorescence intensity was quantified using ImageJ software (https://imagej.net/Welcome) and the mean fluorescence intensity per cell (MFI/cell) was determined. Results were expressed as fold change of the Ang II-treated group; the untreated group was without any hydrolysates or Ang II treatment.

3.2.7 Caco-2 transport study of spent hen muscle protein hydrolysate

Preparation of trans-well plates and transport experiments followed the procedures described in Fan et al. (2018b) and Liang et al. (2018). After cells were seeded for a week, transepithelial electrical resistance (TEER) was monitored every two days using an ohmmeter (World Precision

Instruments, Sarasota, FL, USA), and, on day 21, only wells with TEER values >400 Ω/cm^2 were used. The gastrointestinal digest of SPH-T (20 mg/mL) was dissolved in HBSS, pre-warmed, and added to the apical chambers (0.5 mL); the permeates in the basolateral chambers (1.5 mL) were collected for up to 4 h. Only samples in wells with TEER values >400 Ω/cm^2 after transport study were used for later analysis. Peptide concentrations were determined by the Modified Lowry Protein Assay Kit (Thermo Fisher Scientific, Burlington, ON, Canada). The permeability of transport was expressed as the % (*w/w*) of peptides transported. Chromatograms of the samples before and after transport were analyzed using an Acquity BEH C18 column (1.7 μm, 2.1 × 100 mm) on an RP-UPLC system. Samples (10 μL) were eluted using a gradient of chromatographic grade H_2O and ACN (both containing 0.05% TFA) at 0.3 mL/min as follows: 1% B (0–3 min) and 1–23% B (3–28 min); absorbance was monitored at 220 nm.

3.2.8 Cytotoxicity

Cytotoxicity of SPHs against VSMCs, ECs, and Caco-2 cells followed the alamarBlue fluorescence assay provided by Thermo Fisher Scientific (Burlington, ON, Canada). Cells were seeded on a 96-well plate at 1.0 × 10^4 cells/well. After reaching 80% of confluency, cells were treated with SPHs (dissolved in culture medium) for 24 h. Then, culture media were replaced with 200 μL of 10% alamarBlue solution (dissolved in culture medium) for 4 h of incubation (protected from direct light), after which 150 μL was transferred into an opaque 96 well plate for detection of fluorescence signal; emission and excitation wavelengths were at 590 nm and 560 nm, respectively. The control was without any treatment. The concentration used was 2.5 mg/mL for VSMCs and ECs and 20 mg/mL for Caco-2 cells.

3.2.9 Ethics statement, animal model, and telemetry recording

The animal protocol (#AUP 00001571) was approved by the Animal Welfare Committee at the University of Alberta following the guidelines issued by the Canadian Council on Animal Care and adhered to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health. Twelve- to fourteen-week-old SHRs (290 ± 10 g) were obtained from the Charles River (Senneville, QC, Canada). Upon arrival, rats were acclimatized in the university animal core facility, fed with standard rat chow and water ad libitum and exposed to a 12:12 h of light: dark cycle under controlled humidity and temperature. After one week, they were surgically implanted with telemetry transmitters (HD-S10, Data Sciences International, St. Paul, MN, USA) as previously described by Fan et al. (2019) with slight modifications. To reduce stress in animals, the transmitter was placed in the left groin area instead of being placed on the left hip area, with the catheter inserted into the common femoral artery and advanced into the abdominal aorta; the catheter was secured to the vessel using a vicryl 5-0 suture. After surgery, the rats were caged individually and allowed a 7-day postoperative recovery.

Rats were randomly assigned into four groups (n = 3): untreated and three hydrolysate groups (SPHs digested by thermoase, pepsin, and Protex 26L). Hydrolysates were dissolved in 10 mL of Ensure (Abbott Nutrition, QC, Canada) and administrated orally to rats at a daily dose of 1,000 mg/kg body weight (BW) once per day from day 1; the untreated group was given Ensure only. The dose was selected based on our previously-reported studies (Fan et al., 2019; Jahandideh et al., 2016). Mean arterial pressure (MAP) was recorded over a continuous 24 h (10 s of every 1 min) every two days until day 20 (BP on day 0 was recorded as the baseline). At the end of the experiment, animals were sacrificed by cardiac blood collection under anesthesia.

3.2.10 Statistical analysis

Data of ACEi activity of SPHs were performed in triplicate and were analyzed using one-way analysis of variance (ANOVA) by IBM SPSS Statistics Version 23 (Chicago, IL, USA) followed by Duncan's multiple range tests. Data from the cell study were expressed as mean ± standard error (SEM) of four independent experiments (except for cytotoxicity, replicated six times) and were analyzed by one-way ANOVA followed by Dunnett's multiple test using GraphPad Prism version 6 (San Diego, CA, USA). BP (mean ± SEM) was analyzed by two-way ANOVA followed by Tukey's test using GraphPad. A value of $p < 0.05$ was considered significant.

3.3 Results

3.3.1 ACE inhibitory activity of spent hen muscle protein hydrolysates

Table 3.1 shows *in vitro* ACEi activity of 18 SPHs prepared by 9 enzymes, either individually or in combination. ACEi activities of the one-enzyme digested SPHs were expressed as ACEi IC_{50} values. Among them, SPHs digested by pepsin (SPH-P) and Protex 26L (SPH-26L) showed the highest activity, with their respective IC_{50} values of 23 and 24 µg/mL, followed by SPH digested by thermoase (SPH-T, 30 µg/mL). To study whether or not ACEi activity could be further improved by a combination of enzymes, two enzymes with similar hydrolysis temperature and pH were added together to prepare two-enzyme digested SPHs; trypsin and Protease M were excluded due to their low abilities in producing ACEi peptides. As shown in Table 3.1, ACEi activity was not further improved by enzyme combinations, being much lower than those of SPH-P, SPH-26L, and SPH-T (as determined by the ACEi percentage at a fixed sample concentration, 0.05 mg/mL). Therefore, pepsin, Protex 26L, and thermoase exhibited the highest ability in producing ACEi peptides from spent hen muscle proteins.

3.3.2 ACE2 upregulating, antioxidant, and anti-inflammatory activities of spent hen muscle protein hydrolysate prepared by pepsin, Protex 26L or thermoase

Since SPH-P, SPH-26L, and SPH-T possessed the highest ACEi activity (they also contained a high proportion of low-molecular-weight (LMW) peptides as reported in Yu (2016)), their ACE2u, antioxidant, and anti-inflammatory activities in VSMCs or ECs were further studied. None of the three SPHs affected the viability of both cells (Figure 3.1A-B). As seen in Table 3.2, only SPH-T upregulated ACE2 expression ($p < 0.05$) while all three SPHs diminished Ang II-induced oxidative stress in VSMCs. The three SPHs also reduced expressions of VCAM-1 ($p < 0.05$) but not ICAM-1 in TNFα-stimulated ECs. ACEi, ACE2u, antioxidant, and an-inflammatory activities of the three SPHs were further assessed against gastrointestinal digestion.

3.3.3 Effect of gastrointestinal digestion on ACE inhibitory, ACE2 upregulating, antioxidant, and anti-inflammatory activities of spent hen muscle protein hydrolysate prepared by pepsin, Protex 26L or thermoase

ACEi activities of SPH-26L and SPH-P, not SPH-T, were reduced markedly ($p < 0.001$) after simulated gastrointestinal digestion (Figure 3.2A). ACE2 expression induced by all three SPHs was not affected after the digestion (Figure 3.2B). Antioxidant activity of SPH-T was maintained while those of SPH-26L and SPH-P were reduced after the digestion (Figure 3.2C-D). All three SPHs mitigated TNFα-induced upregulation of VCAM-1 in ECs but not ICAM-1 throughout the digestion (Figure 3.2E-F). SPH-T digested by pepsin and pancreatin (SPH-TPP) was selected to further determine its stability and permeability across Caco-2 monolayers.

3.3.4 Transport of thermoase-pepsin-pancreatin digested spent hen muscle protein hydrolysate across Caco-2 cell monolayer and its effects on ACE inhibitory, ACE2 upregulating, antioxidant, and anti-inflammatory activities

SPH-TPP did not show any cytotoxicity against Caco-2 cells (Figure 3.1C). Figure 3.3 presents the chromatographic profiles of SPH-TPP before and after the transport. The permeability at 4 h was 3.87% based on the peptide transported. The transport process did not affect ACEi activity of SPH-TPP (Figure 3.4A), whereas ACE2u and antioxidant activities in VSMCs by SPH-TPP were further improved after transport (Figure 3.4B-D). Besides, its ability in attenuating VCAM-1 expression in TNFα-induced ECs was improved ($p < 0.05$); surprisingly, inhibition of ICAM-1 expression was enhanced after the transport, despite not being significantly compared to the TNFα-treated group (Figure 3.4E-F).

3.3.5 Antihypertensive effect of spent hen muscle protein hydrolysate prepared by pepsin, Protex 26L or thermoase in spontaneously hypertensive rats

SPH-T was orally administrated to SHR (at 1,000 mg/kg BW per day) to explore its *in vivo* activity; SPH-P and SPH-26L were also administrated to SHR due to their high *in vitro* ACEi activities. None of the three SPHs affected body weight gain and organ weights (kidney, liver, and heart) over the treatment period (Figure 3.5). Only SPH-T reduced BP after a period of 20 days, with MAP lowered from 141.1 mmHg to 95.9 mmHg ($p < 0.01$) (Figure 3.6). SPH-P and SPH-26L did not affect BP throughout the treatment period. We also noted a relatively larger SEM in the SPH-T group than those in the other two groups of animals, which was due to the more rapid BP reduction of one animal over the treatment period.

3.4 Discussion

This study reported the preparation of bioactive peptides from spent hen muscle proteins. ACEi activity of SPHs varied among different enzymes, but a combined use of enzymes appeared to diminish this variation (Table 3.1); however, compared to one-enzyme hydrolysis, the use of two enzymes did not further enhance ACEi activity of SPHs. Among SPHs obtained, SPH-P, SPH-26L, and SPH-T possessed the highest ACEi activity, with IC_{50} values of 23–30 μg/mL (Table 3.1). Their ACEi activities were higher than many hydrolysates of other sources such as casein, albumin, ovalbumin, ovotransferrin, and egg white (Fan et al., 2019; Hyun & Shin, 2000; Liao et al., 2018c; Majumder et al., 2010). ACEi activity of a protein hydrolysate is dictated by peptide sequences, depending largely on proteases, protein sequences, and the conditions applied to release the peptides (Aluko, 2015). Indeed, the variation in ACEi activity of various SPHs among different enzymes was due to their different cleavage specificities (Table 3.1). Muscle proteins have been reported to be particularly rich in antihypertensive peptides (Toldrá et al., 2018; Udenigwe et al., 2013). A previous study demonstrated that chicken muscle proteins are excellent sources of ACEi peptides, superior to those of many others such as milk, egg, soybean, and fish (Gu, Majumder, & Wu, 2011); this might, to some extent, explain the potent ACEi activity of SPHs. Our results further supported a great potency of chicken muscle proteins as a raw material for the production of antihypertensive peptides. Compared with protein hydrolysates from other meat byproducts such as skin, bone, viscera, blood, and sarcoplasmic proteins, SPHs appeared to have a more potent ACEi activity (Cheng et al., 2008; Gallego et al., 2019; Lee et al., 2012; Ren et al., 2011; Saeed et al., 2020). By comparing the ACEi activity, three SPHs, SPH-P, SPH-26L, and SPH-T, were selected for evaluation of ACE2u, antioxidant, and anti-inflammatory activities in vascular cells.

VSMCs and ECs are two important components of the blood vessel wall that collaboratively maintain vascular homeostasis and regulate BP (Lilly, 2014; Münzel et al., 2008). Hypertension is

associated with vascular remodeling that, at the cellular level, can be caused by oxidative stress, inflammation, and migration (Harvey, Montezano, & Touyz, 2015; Hernanz et al., 2015; Münzel et al., 2008). VSMCs (A7r5) and ECs (EA.hy926) are two well-established models of evaluating cellular antioxidant and anti-inflammatory activities of bioactive peptides (Fan et al., 2018a); ACE2 upregulation in VSMCs has recently been used to evaluate the antihypertensive potential of bioactive peptides (Liao et al., 2019; Wang et al., 2020; Wu et al., 2017). For example, IRW, derived from egg white, and LRW, derived from pea, both upregulated ACE2 in VSMCs by approximately two times (Liao et al., 2018a; Wang et al., 2020). BP reduction is accompanied with ACE2 upregulation in various tissues in SHRs including heart, kidney, aorta, and mesenteric arteries (He et al., 2019; Liao et al., 2018a; Majumder et al., 2015; Pan et al., 2015; Zheng et al., 2017). SPH-T enhanced ACE2 expression in VSMCs, indicating its potential antihypertensive ability *in vivo*. SPH-T, SPH-26L, and SPH-P all diminished oxidative stress in Ang II-induced VSMCs (Table 3.2), consistent with the fact that muscle proteins are rich in antioxidant peptides (Liu et al., 2016). All three SPHs inhibited expression of VCAM-1 ($p < 0.05$) to a similar extent but did not affect that of ICAM-1 in TNFα-stimulated ECs. Hydrolysates or peptides with anti-inflammatory effects have been prepared from various food commodities such as meat, zein, beans, and egg white, some of which have been validated their *in vivo* efficacies (Chen et al., 2019; Liang et al., 2018; Majumder et al., 2013a; Majumder et al., 2013b; Yu, Field, & Wu, 2018a, 2018b). Next, we studied the fate of these activities of SPH-T, SPH-26L, and SPH-P during simulated gastrointestinal digestion and transepithelial transport.

Gastrointestinal digestion did not affect ACEi, ACE2u, antioxidant, and anti-inflammatory activities of SPH-T, but reduced those of SPH-26L and SPH-P particularly ACEi and antioxidant activities (Figure 3.2). These results suggested different susceptibility of the three SPHs to

gastrointestinal digestion. Since many potent chicken meat-derived ACEi peptides contain lysine or arginine residues, they are good substrates of trypsin (Gu et al., 2011). This explained why the decline in ACEi activity occurred mainly during pancreatic digestion (containing trypsin). Previous research indicated that peptides exert cellular antioxidant activity through either acting as direct free radical scavengers or activating cellular signaling such as the nuclear factor erythroid 2−related factor 2 pathway, demonstrating that antioxidant activity of peptides depends both on their amino acid compositions and sequences (Du et al., 2016; Majumder et al., 2013a; Tsai et al., 2020; Wang et al., 2020; Yi et al., 2020). A higher resistance to gastrointestinal digestion of SPH-T than SPH-26L and SPH-P might be responsible for its more retained antioxidant activity (Figure 3.1C-D). We also observed that ACEi activity of SPH-T was retained to a greater extent than those of SPH-26L and SPH-P. Indeed, many ACEi peptides have also been reported with antioxidant activity (Gu et al., 2019; Gu & Wu, 2016; Majumder et al., 2013a; Wang et al., 2020), which suggested that these two types of peptides might share more common sequences in SPH-T. Nevertheless, SPH-T showed good resistance to gastrointestinal digestion and was further subjected to permeability study in Caco-2 monolayers. The permeability of SPH-TPP was 3.87 % in 4 h (Figure 3.3), higher than that of a zein hydrolysate (1.2%), which was prepared by a sequential hydrolysis using thermolysin, pepsin, and pancreatin (Liang et al., 2018). Bioactive peptides have a permeability of generally less than 1% (Xu et al., 2019). A relatively higher permeability in this study might be due to a high proportion of LMW peptides in the SPH-TPP, resulting from a triple digestion process by thermoase, pepsin, and pancreatin. The transport process did not affect ACEi but enhanced ACE2u, antioxidant, and anti-inflammatory activities, indicating a possible further degradation during transport. For example, the ability in inhibiting VCAM-1 and ICAM-1 expressions were significantly ($p < 0.05$) strengthened after transport,

similar to findings reported by Liang et al. (2018). There are numerous peptidases in Caco-2 cells that may contribute to the formation of new peptides during the transport process. The high permeability of SPH-TPP with enhanced bioactivity indicated a likely high bioavailability and *in vivo* efficacy of SPH-T. Indeed, SPH-T, not SPH-26L and SPH-P, significantly reduced BP of SHRs (Figure 3.6), despite a higher *in vitro* ACEi activity of SPH-26L and SPH-P than SPH-T (Table 3.1). This implied that *in vitro* ACEi activity was not always a reliable indicator for screening a hydrolysate for *in vivo* study; a combination of *in vitro* activity measurement with gastrointestinal stability and transepithelial permeability is likely a more reliable approach.

In this study, spent hen muscle proteins were hydrolyzed by 9 enzymes in isolation or in combination and 18 SPHs were produced. Although SPH-26L and SPH-P showed higher ACEi activity than that of SPH-T, only SPH-T significantly reduced BP in SHR after oral administration. This study suggested that stability during gastrointestinal digestion and transepithelial transport of protein hydrolysates (peptides) is important for its *in vivo* activity, rather than its *in vitro* activity solely. Our results advocated the use of this multiple evaluation approach in evaluating the antihypertensive potential of a hydrolysate or a peptide. More work is needed to explore the antihypertensive mechanisms of SPH-T and understand the contribution of the above-mentioned activities to BP reduction.

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Table 3.1 ACEi activity of one-enzyme and two-enzyme digested SPHs

One-enzyme	ACE inhibition (%) * (IC_{50}, μg/mL)	Two-enzymes	ACE inhibition (%)
Alcalase (A)	52.5 ± 1.1 (57 ± 2.4) [d]	A + 6L	38.0±1.3 [d]
Protex 6L (6L)	42.8 ± 2.2 (79 ± 1.7) [e]	A + S	43.7±1.0 [c]
Protease S (S)	59.8 ± 1.0 (39 ± 0.5) [c]	A + T	55.2±0.2 [a]
Thermoase (T)	**64.8 ± 0.9 (30 ± 1.4)** [b]	6L + S	45.1±0.3 [c]
Trypsin	18.6 ± 2.7 (189 ± 4.7) [g]	6L + T	54.4±2.4 [a]
Protease M	39.1 ± 0.2 (118 ± 5.0) [f]	S + T	52.7±3.4 [a]
Protex 50FP (50FP)	52.7 ± 0.2 (50 ± 1.6) [d]	50FP + 26L	52.6±1.1 [a]
Protex 26L (26L)	**69.5 ± 0.7 (24 ± 1.5)** [a]	50FP + P	50.3±3.3 [b]
Pepsin (P)	**70.8 ± 0.2 (23 ± 0.9)** [a]	26L + P	54.3±0.8 [a]

*ACEi activity was expressed as inhibitory percentage (%) of ACE activity, being tested at 0.05 mg/mL of spent hen muscle protein hydrolysates (SPHs) (IC_{50} values of one-enzyme digested SPHs are shown in the parenthesis). Values do not share a common superscript lowercase letter within a column differ significantly ($p < 0.05$) (n = 3). Working parameters of hydrolysis are presented in *Appendix: Supplementary Table 3.1 and 3.2*.

Table 3.2 ACE2u, antioxidant, and anti-inflammatory activities of SPH-T, SPH-P, and SPH-26L

Samples	VSMCs		ECs	
	ACE2	Oxidative stress	ICAM-1	VCAM-1
	Untreated	Ang II (+)	TNFα (+)	TNFα (+)
SPH-T	1.82 ± 0.36 *	0.73 ± 0.06 *	1.02 ± 0.08	0.80 ± 0.03 *
SPH-P	1.01 ± 0.17	0.77 ± 0.07 *	1.05 ± 0.13	0.73 ± 0.04 *
SPH-26L	0.98 ± 0.08	0.67 ± 0.13 *	1.10 ± 0.04	0.74 ± 0.07 *

SPH-T, SPH-P, and SPH-26L refer to spent hen muscle protein hydrolysates (SPHs) prepared respectively by thermoase, pepsin, and Protex 26L. ACE2u activity was expressed as fold change as that of untreated group; cells were treated with SPHs for 24 h (n = 4). Antioxidant activity was expressed as fold change as that of Ang II-treated group; cells were treated with SPHs for 1 h before Ang II treatment (1 μM) for 0.5 h, followed by the addition of 20 μM of DHE (n = 4). ICAM-1 and VCAM-1 levels were determined after cells were treated with SPHs for 18 h before TNFα treatment (10 ng/mL) for 6 h; anti-inflammatory activity was expressed as fold change as that of TNFα-treated group (n = 4). *, $p < 0.05$, indicating a difference compared with the untreated (ACE2), Ang II-treated (oxidative stress), or TNFα-treated (ICAM-1/VCAM-1) group, respectively. Cells were treated with SPHs at 2.5 mg/mL.

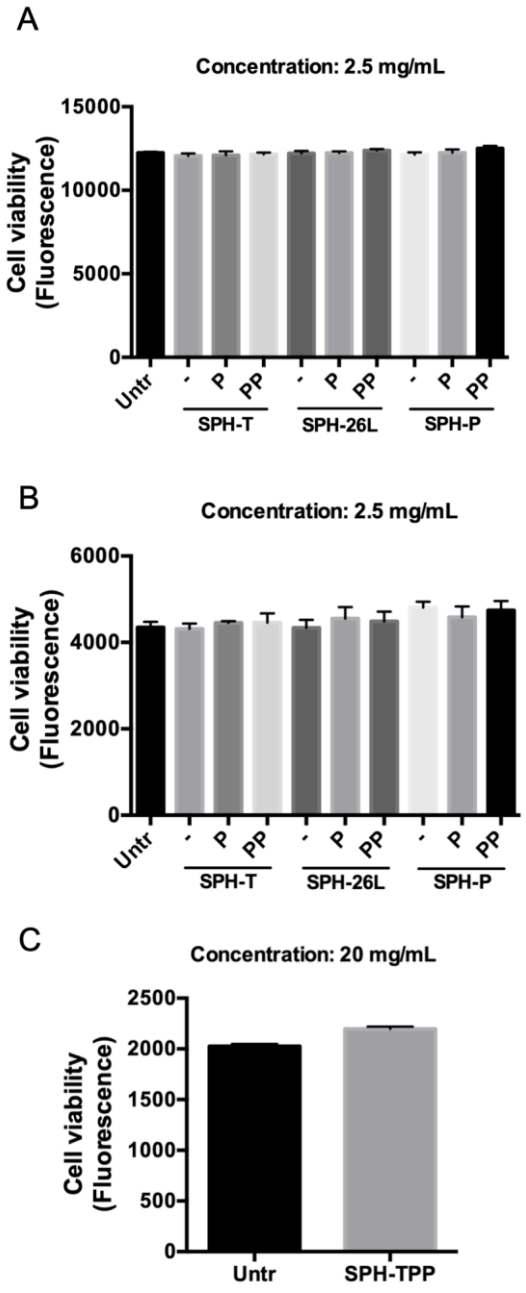


Figure 3.1 Cytotoxicity of SPHs in (**A**) VSMCs, (**B**) ECs, and (**C**) Caco-2 cells. SPH-T, SPH-26L, and SPH-P refer to spent hen muscle protein hydrolysates (SPH) prepared by thermoase, Protex 26L, and pepsin, respectively. -, P, and PP indicates non-, pepsin-, and (pepsin + pancreatin)-digestion, respectively. SPHs were tested at 2.5 mg/mL in VSMCs and ECs and 20 mg/mL in Caco-2 cells. All samples were treated for 24 h, followed by alamarBlue® cell viability assay. Data were expressed as means ± SEM (n = 6).

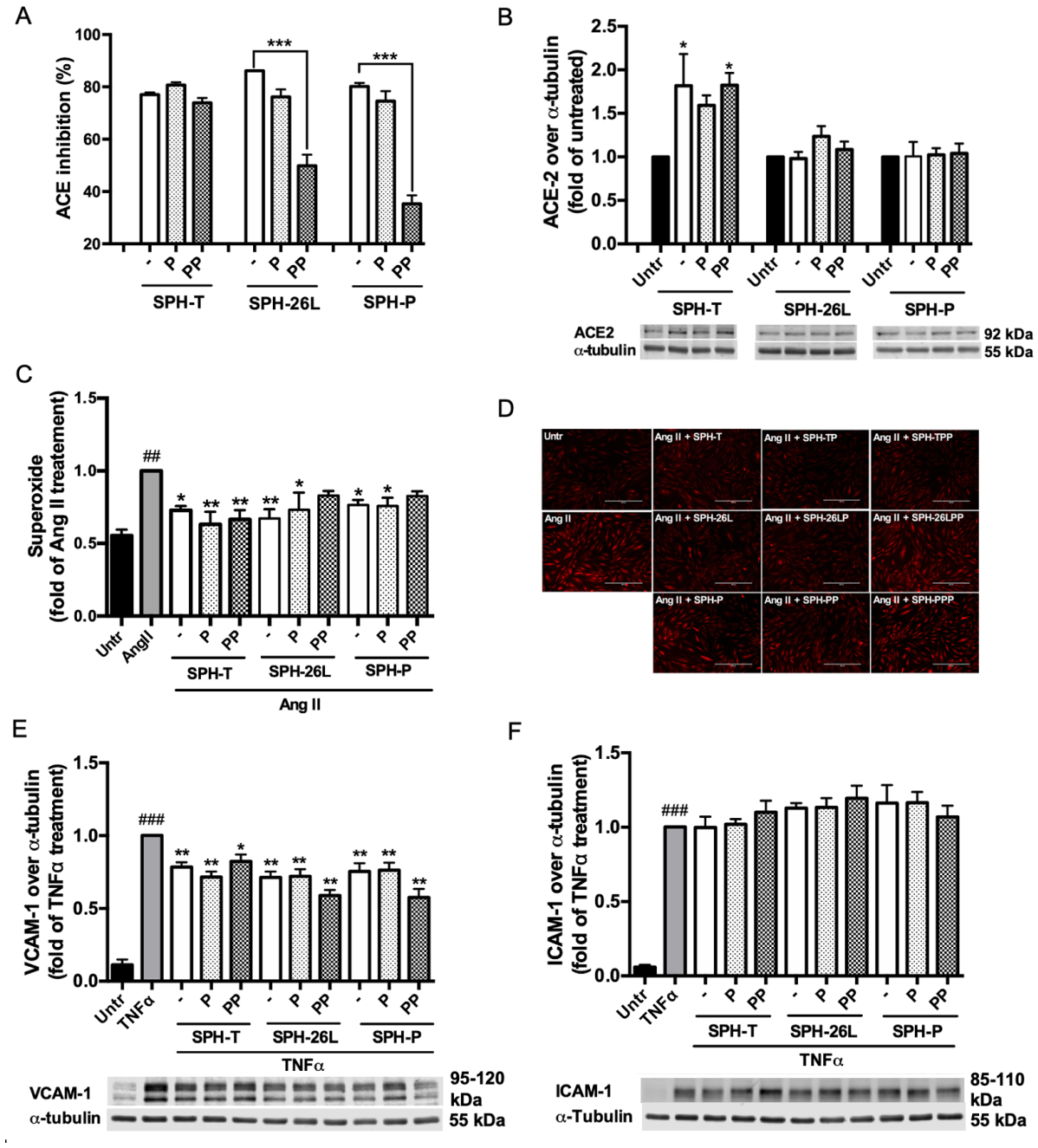


Figure 3.2 Effect of gastrointestinal digestion on (**A**) ACEi, (**B**) ACE2u, (**C-D**) antioxidant, and (**E-F**) anti-inflammatory activities of SPHs. SPH-T, SPH-P, and SPH-26L refer to spent hen muscle protein hydrolysates (SPHs) prepared respectively by thermoase, pepsin, and Protex 26L. -, P, and PP indicates non-, pepsin-, and (pepsin + pancreatin)-digestion, respectively (n = 4). (**A**) ACEi activity was determined using an *in vitro* biochemical assay (***, $p < 0.001$). (**B**) ACE2u activity in VSMCs was expressed as fold change as that of untreated group; cells were treated with SPHs for 24 h (*, $p < 0.05$, compared to untreated group). (**C-D**) Antioxidant activity in VSMCs

was expressed as fold change as that of Ang II-treated group; cells were treated with SPHs for 1 h before Ang II treatment (1 μM) for 0.5 h, followed by the addition of 20 μM of DHE (##, $p < 0.01$, compared to untreated group; *, $p < 0.05$, **, $p < 0.01$, compared to Ang II-treated group). (**E-F**) ICAM-1 and VCAM-1 levels in ECs were determined after cells were treated with SPHs for 18 h before TNFα treatment (10 ng/mL) for 6 h; anti-inflammatory activity was expressed as fold change as that of TNFα-treated group (###, $p < 0.001$ compared to untreated group; *, $p < 0.05$, **, $p < 0.01$, compared to TNFα-treated group). ACEi activity was determined at 0.125 mg/mL of SPHs; cells were treated with 2.5 mg/mL of SPHs.

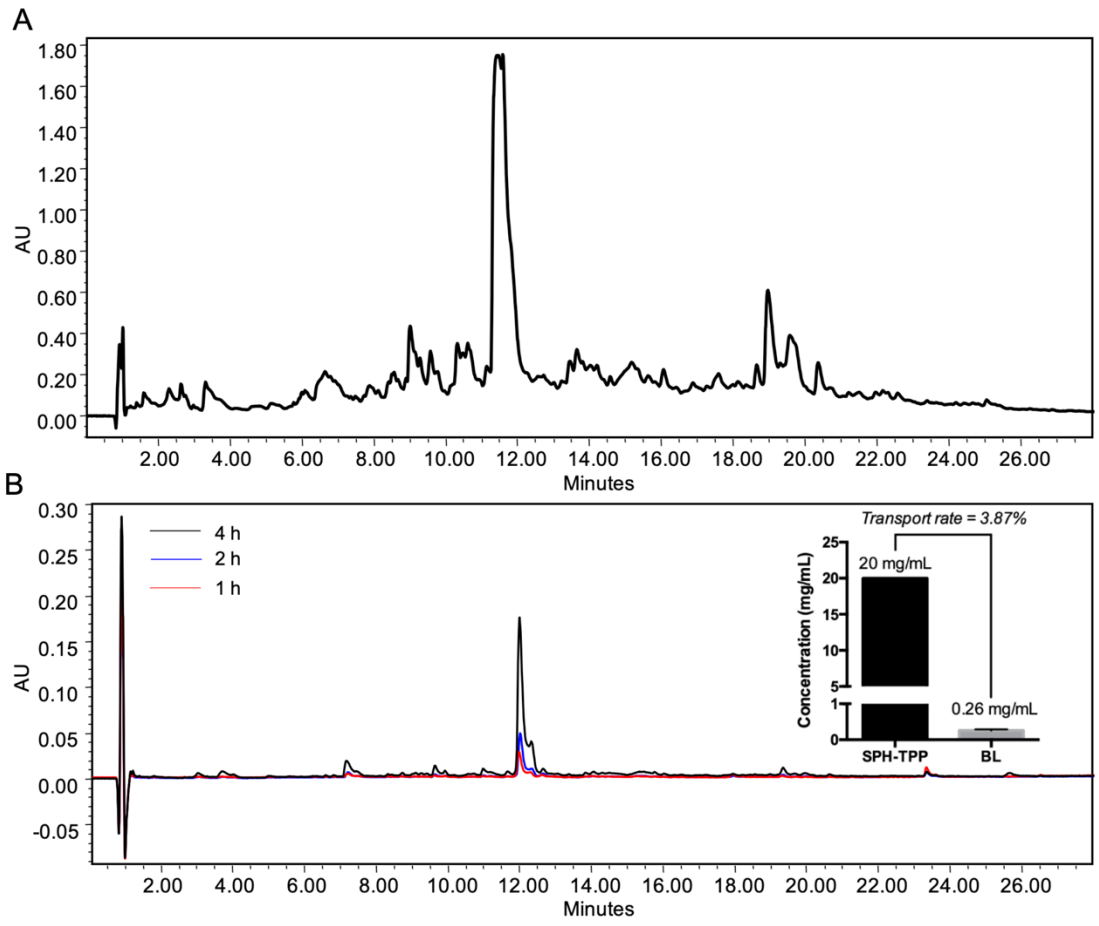


Figure 3.3 Chromatograms of SPH-TPP (**A**) and its permeate (**B**) after 4 h of transport across Caco-2 cell monolayers. Transport permeability (at 4 h) was calculated based on the % of peptides transported to the basolateral (BL) side (n = 4). SPH-TPP, spent hen muscle protein hydrolysate digested by thermoase, pepsin, and pancreatin.

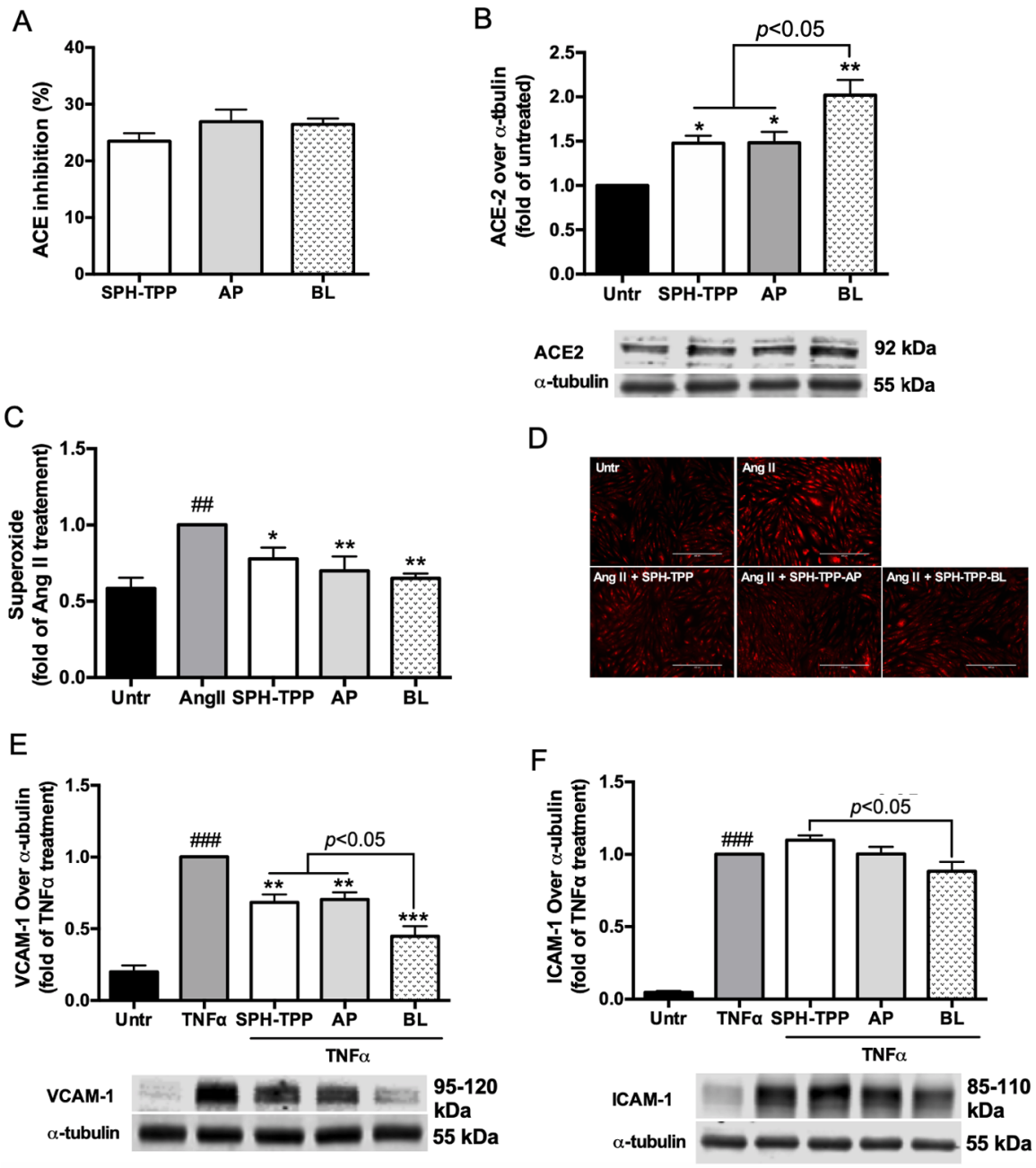


Figure 3.4 Effects of transport across Caco-2 cell monolayers on (**A**) ACEi, (**B**) ACE2u, (**C**-**D**) antioxidant, and **(E-F)** anti-inflammatory activities of SPH-TPP. SPH-TPP, spent hen muscle protein hydrolysate digested by thermoase, pepsin, and pancreatin; AP, apical chamber; BL, basolateral chamber. (**A**) ACEi activity was determined using an *in vitro* biochemical assay (n = 3). (**B**) ACE2u activity in VSMCs was expressed as fold change as that of untreated group; cells were treated with SPHs for 24 h (n = 4) (*, $p < 0.05$, **, $p < 0.01$, compared to untreated group). (**C**-**D**) Antioxidant activity in VSMCs was expressed as fold change as that of Ang II-treated

group; cells were treated with SPHs for 1 h before Ang II treatment (1 μM) for 0.5 h, followed by the addition of 20 μM of DHE (n = 4) (##, $p < 0.01$ compared to untreated group; *, $p < 0.05$, **, $p < 0.01$ compared to Ang II-treated group). **(E-F)** ICAM-1 and VCAM-1 levels in ECs were determined after cells were treated with SPHs for 18 h before TNFα treatment (10 ng/mL) for 6 h; anti-inflammatory activity was expressed as fold change as that of TNFα-treated group (n = 4) (###, $p < 0.001$ compared to untreated group; **, $p < 0.01$, ***, $p < 0.001$ compared to TNFα-treated group). ACEi activity was determined at 16 μg/mL of SPHs; cells were treated with 2.5 mg/mL of SPHs.

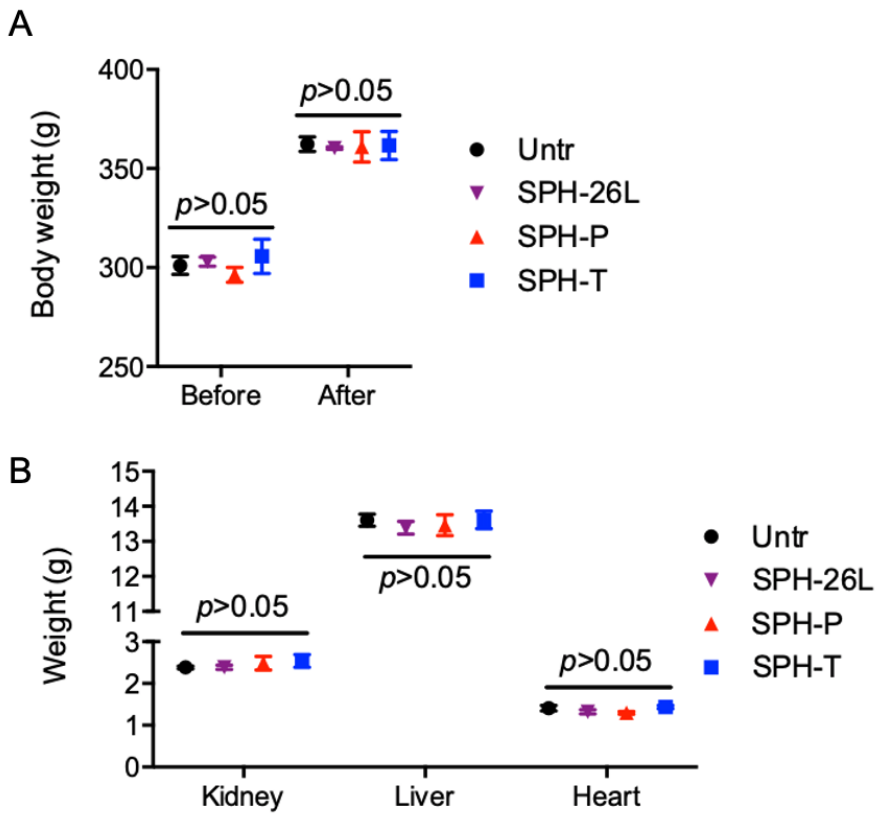


Figure 3.5 Effect of oral administration of SPHs on body and organ weights in SHRs. **(A)** Body weight was obtained before and after (at the end point of the experiment) 20 days of oral administration of SPHs to SHRs. **(B)** Organ weight was obtained after euthanizing the animals. SPH-T, SPH-P, and SPH-26L refer to spent hen muscle protein hydrolysates (SPHs) prepared by thermoase, pepsin, and Protex 26L, respectively. Untr, untreated group.

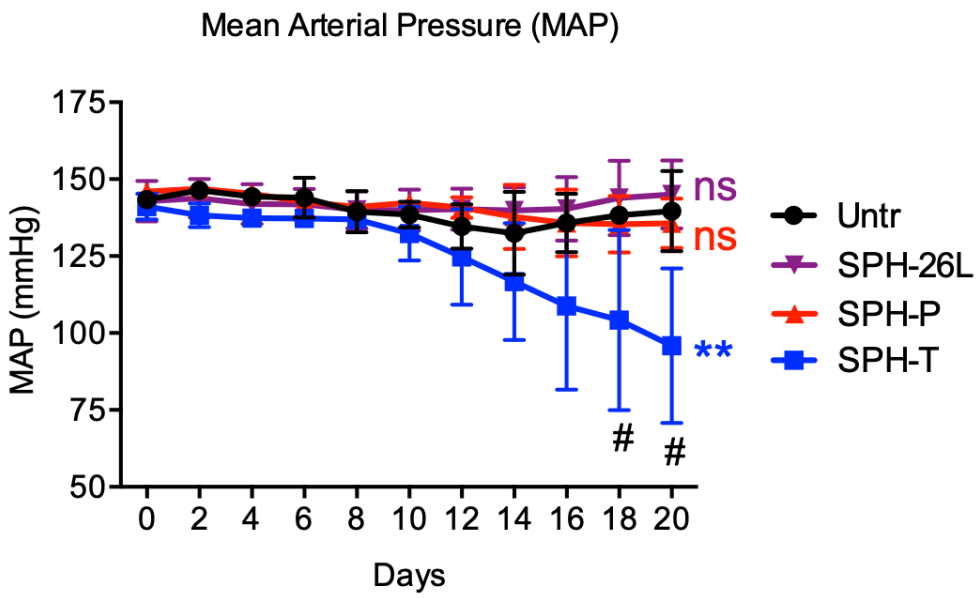
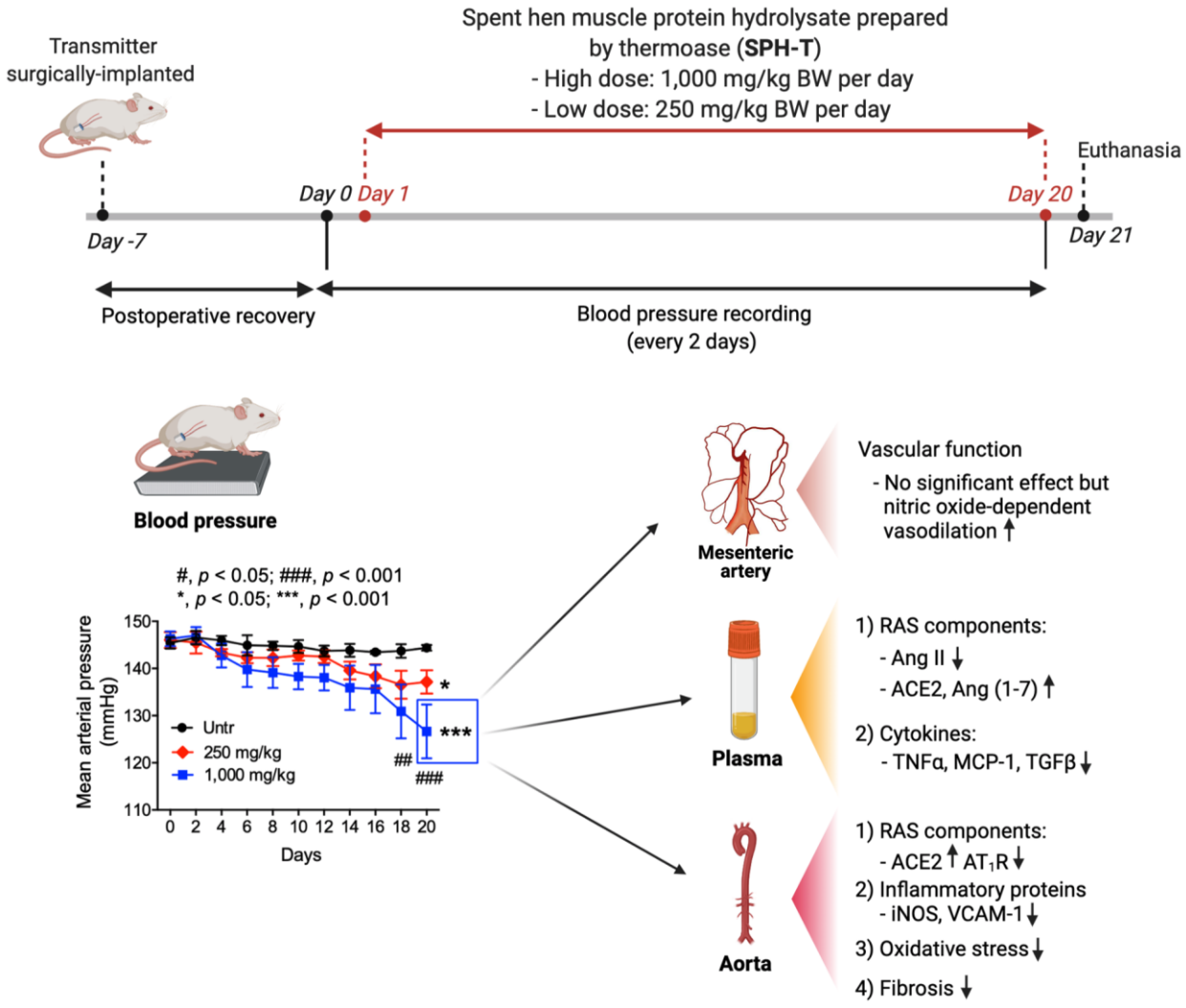


Figure 3.6 Effects of SPH-T, SPH-26L, and SPH-P on blood pressure in spontaneously hypertensive rats. SPH-T, SPH-P, and SPH-26L refer to spent hen muscle protein hydrolysates (SPHs) prepared by thermoase, pepsin, and Protex 26L, respectively. SPHs were orally administrated at 1,000 mg/kg body weight per day for a continuous 20 days. Each data point was represented as the mean arterial pressure (MAP) recorded over a 24-h period and was expressed as means ± SEM (n = 3). #, $p < 0.05$, compared to untreated group (Untr, without SPH treatment) at the time point; **, $p < 0.01$, 'ns', not significant, compared to the untreated group over the entire treatment period.

CHAPTER 4 – Antihypertensive Effect of Spent Pen Muscle Protein Hydrolysate Prepared by Thermoase in Spontaneously Hypertensive Rats [3]

Graphical abstract



[3] The chapter will be submitted to *Food Research International* for consideration of publication.

4.1 Introduction

Many peptides derived from various food proteins have shown antihypertensive effects in spontaneously hypertensive rat (SHR). For example, two chicken meat protein hydrolysates produced by gastrointestinal proteases reduced blood pressure (BP) of SHR significantly within 2 h (Udenigwe et al., 2017). A bovine casein hydrolysate exerted antihypertensive effect in SHR in both short- (2 h) and long-term (28 d) trials (Yamada et al., 2013). Yet many antihypertensive peptides are initially characterized as angiotensin-converting enzyme (ACE) inhibitory (ACEi) peptides, more recent studies demonstrated that their *in vivo* mechanisms are beyond ACE inhibition (Wu, Liao, & Udenigwe, 2017). The renin-angiotensin system (RAS) is the major regulator of hypertension; within the RAS, ACE cleaves angiotensin (Ang) I to Ang II that binds with Ang II type 1 receptor (AT_1R) and elevates BP; however, ACE2 converts Ang II into Ang (1-7) that binds with the Mas receptor (MasR) and reduces BP. Antihypertensive peptides are reported to reduce BP through targeting both the ACE-Ang II-AT_1R and the ACE2-Ang (1-7)-MasR axes, such as LY, RALP, GHS, IQP, VEP and IRW, among others (He et al., 2019b; Liao et al., 2019; Yu et al., 2014; Zheng et al., 2017). Besides, an increasing number of antihypertensive peptides have been reported to reduce BP through other mechanisms, such as improving vascular function, ameliorating aberrant inflammation, and reducing oxidative stress (Jahandideh et al., 2016; Liao et al., 2019; Mas-Capdevila et al., 2019; Tsai et al., 2020; Yamada et al., 2010).

In Chapter 3, we have prepared a spent hen muscle protein hydrolysate using thermoase PC10F (SPH-T), with great *in vitro* ACEi, ACE2 upregulating (ACE2u), antioxidant, and anti-inflammatory activities, as well as good gastrointestinal stability and transepithelial permeability; oral administration of SPH-T to SHR reduced BP at a daily dose of 1,000 mg/kg body weight (BW) in a preliminary trial (n=3). The current work aims to further study the antihypertensive effect of

SPH-T and understand the contribution of the above-mentioned activities to BP reduction in SHR. SPH-T was orally administrated to the animal at both high (1,000 mg/kg body weight [BW]) and low (250 mg/kg BW) doses; the high-dose group was used to studythe mechanisms of action underlying its BP reduction.

4.2 Materials and Methods

4.2.1 Materials

Spent hen carcasses (Lohmann, age of 70-80 weeks old; ~1.1 kg, with feather, beak, and internal organs removed) were purchased from a local supermarket (T&T) in Edmonton, Alberta, Canada. Thermoase PC10F (from *Bacillus thermoproteolyticus Var. Rokk*) was obtained from Amano Enzyme Inc. (Nagoya, Japan).

4.2.2 Preparation of spent hen muscle protein hydrolysate using thermoase (SPH-T)

Spent hen muscle protein extraction and preparation of SPH-T were described in Chapter 3.

4.2.3 Ethics statement, animal model, telemetry recording, and experimental design

Ethics statement, animal surgery, and telemetry recording of BP according to the description in Chapter 3. Animals were randomly assigned into 3 groups: untreated (control, n = 7), low dose (250 mg/kg/day BW, n = 6), and high dose (1,000 mg/kg/day BW, n = 7). The doses were selected based on Chapter 3 and previous studies (Fan et al., 2019; Jahandideh et al., 2016). BP was recorded for a continuous 24 h (10 seconds in every 1 min) every 2 days until day 20 (BP on day 0 was recorded as the baseline). SPH-T was dissolved in 10 mL of Ensure (Abbott Nutrition, QC, Canada) and orally administered to rats once per day from day 1; the untreated group was given Ensure only. Systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure

(MAP), and heart rate (HR) were recorded. At the end of the experiment, animals were sacrificed by cardiac blood collection under anesthesia, with mesenteric arteries (MA) being isolated immediately for the vascular function study. Tissues and plasma (separated immediately from blood using EDTA-coated tubes (BD Vacutainer, NJ, USA) at 1,000 × g for 20 min at 4 °C) were frozen in liquid nitrogen and then stored at -80 °C.

4.2.4 Vascular function

Second-order branches of MA were used to assess the *ex vivo* vascular function (Jahandideh et al., 2014). After normalization and equilibration, arteries were exposed to 10^{-5} M of phenylephrine (PE, Sigma, Oakville, ON, Canada) twice, followed by a single dose of 3 µM of methacholine (MCh, Sigma) to evaluate the functional integrity of the endothelium and smooth muscle. A cumulative concentration-response curve to PE (10^{-8} to 10^{-4} M) was performed. Then, all vessels were pre-constricted (80% of maximum) using PE. To study the endothelium-dependent vasorelaxation, cumulative concentration-response curves to MCh (10^{-10} to 10^{-4} M) were performed to assess the role of (nitric oxide, NO) in the presence or absence of a NO synthase (NOS) inhibitor (N(ω)-nitro-L-arginine methyl ester, L-NAME, 10^{-4} M). To study the endothelium-independent vasorelaxation, cumulative concentration-response curves to sodium nitroprusside (SNP, an exogenous NO donor, 10^{-10} to 10^{-5} M, Sigma) were performed.

4.2.5 Plasma biomarker analysis

Concentrations of ACE2 (CSB-E14308r), Ang II (CSB-E04494r), and Ang (1-7) (CSB-E14241r) were evaluated by ELISA kits obtained from CUSABIO TECHNOLOGY (Houston, TX, USA). ACE activity was assessed using a fluorometric kit (CS0002, Sigma) and its result was expressed as relevant fluorescent unit per min (RFU min^{-1}). Circulatory cytokine levels were

quantified using rat cytokine strips obtained from Signosis (EA-1501, Santa Clara, CA, USA). All determinations were performed as per the manufacturers' manuals.

4.2.6 Immunostaining

The aortas were embedded in Tissue-Tek® O.C.T Compound (Sakura Finetek USA, Torrance, CA, USA) and frozen immediately at –80 °C. Ten-micrometer tissue sections were prepared and mounted on glass slides at –20 °C using Leica CM1860 (Leica Biosystems, Concord, ON, CA, USA). Tissue sections were fixed in cold acetone (–20 °C), washed with phosphate buffer saline (PBS, pH 7.5), before being blocked in 1% bovine serum albumin (BSA) with 0.1% Triton-X-100 in PBS for 60 min at room temperature in a humid chamber. The sections were immunostained overnight at room temperature with nitrotyrosine primary antibody (Dilution 1:20 in 2% BSA in PBS; Novus biologicals, Oakville, ON, Canada). Upon washing with PBS, the sections were incubated with secondary antibody (Dilution 1: 250; Alexa Fluor 546 (red), Invitrogen, Burlington, ON, Canada) in 2% BSA in PBS for 60 min (protect from light). After PBS washing, the sections were covered with glass coverslips with a VECTASHIELD mounting medium containing DAPI (Vector Laboratories, Burlingame, CA, USA), and immediately visualized under Olympus IX81 fluorescence microscope (Olympus, Tokyo, Japan). Immunofluorescence of the obtained images was quantified using Image Studio Lite 5.2 (Licor Biosciences, Lincoln, NE, USA).

4.2.7 Western blotting

Vascular proteins were extracted from aortas using a protein extraction buffer (5 mM EDTA, 10 mM $Na_4P_2O_7$, 20 mm Tris, 100 mM sodium fluoride, and 1% NP-40) containing a 1% (v/v) protease inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA, USA). The homogenate was centrifuged at 15,000 × g for 15 min at 4 °C. Protein concentrations in the supernatant were

determined by bicinchoninic acid (BCA) assay (Thermo Fisher Scientific). Samples were stored at –80 °C until western blotting. Samples (calibrated to the same protein mass) were loaded on a 9% separating gel and transferred to a nitrocellulose membrane (diameter 0.45 μm, Bio-Rad, Montreal, QC, Canada) for incubation with antibodies. Bands of ACE (Abcam, Toronto, ON, Canada), ACE 2 (Abcam), AT_1R (Novus biologicals, Oakville, ON, Canada), angiotensin II type 2 receptor (AT_2R; Abcam), Mas receptor (MasR; Novus biologicals), inducible NOS (iNOS; BD Biosciences, San Jose, CA, USA), cyclooxygenase 2 (COX2; Abcam), intracellular adhesion molecule-1 (ICAM-1; Santa Cruz, Dallas, TX, USA), vascular cell adhesion molecule-1 (VCAM-1; Abcam), and type I collagen (Abcam) were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Abcam); bands of phospho-endothelial NOS (p-eNOS; Abcam) was normalized to its "total" form (eNOS; BD Biosciences). Donkey-anti-mouse 800 CW or Goat-anti-rabbit IRDye 680 RD secondary antibodies (Licor Biosciences, Lincoln, NE, USA) were used to visualize the bands in a Licor Odyssey BioImager; the fluorescence signal was obtained using Image Studio Lite 5.2 (Licor Biosciences).

4.2.8 Statistical analysis

Data were expressed as mean values with standard errors of mean (SEM). BP and HR were analyzed by two-way ANOVA with Tukey's test; all other results including vascular function (MCh and SNP curves were firstly fitted using nonlinear regression), plasma biomarkers, and western blotting were analyzed by unpaired *t* test using GraphPad Prism Version 6 (San Diego, CA, USA). $P < 0.05$ was considered statistically different.

4.3 Results

4.3.1 Blood pressure reduction

Both treatments did not change body organ weights significantly over the experimental period (Figure 4.1). The initial (on day 0) SBP of SHRs was approximately 167.3 mmHg, and the untreated group (without any treatment) maintained SBP over the treatment period (Figure 4.2A). SBP was reduced to 156.8 and 143.0 mmHg after the low-dose (250 mg/kg BW) and high-dose (1,000 mg/kg BW) treatments with SPH-T for 20 days, respectively. Similar to that of SBP, MAP and DBP were also reduced significantly in both groups over the treatment period (Figure 4.2B-C). Both treatments did not significantly affect HR of the animals (Figure 4.2D). Only the high-dose group was selected for subsequent mechanistic studies.

4.3.2 Improved vasodilation towards nitric oxide (NO) dependency

Mch-induced vasorelaxation after a pre-constriction by PE is a commonly-used *ex vivo* method to study the mechanism of vasorelaxation, in the presence of various inhibitors, such as L-NAME, superoxide dismutase, and others (Hart, 2019). As illustrated in Figure 4.3A, Mch-induced vasorelaxation was not affected after the high-dose treatment. Interestingly, through blocking the NO generation by adding L-NAME, a significantly deferred relaxation was observed in the high-dose group, whereas only a slight effect was detected in the untreated group (Figure 4.3B-C). The high-dose SPH further enhanced NO-dependent vasodilation as reflected by a significant increase in the delta area under the curve (ΔAUC) compared with the untreated group (Figure 4.3D). Vascular expression of eNOS, a key enzyme for NO generation, was unaffected by the treatment (Figure 4.3E). Also, the addition of SNP, an exogenous NO donor, did not affect vasodilatory function (Figure 4.3F).

4.3.3 Modulation of the RAS components

For the circulating RAS, the high-dose treatment decreased the circulating Ang II level from 25.0 to 5.7 pg/mL ($p < 0.01$) while increased ACE2 and Ang (1-7) levels significantly ($p < 0.05$); ACE activity was not affected (Figure 4.4). In the vascular RAS, SPH-T treatment increased expression of ACE2 but reduced that of AT_1R significantly ($p < 0.05$) (Figure 4.5A-B); expressions of ACE, AT_2R, and MasR were not affected (Figure 4.5C-E).

4.3.4 Attenuation of vascular inflammation

Oral administration of SPH-T reduced cytokines level such as the circulating tumor necrosis factor alpha (TNFα) and monocyte chemoattractant protein-1 (MCP-1) levels (Figure 4.6A-B). In addition, expressions of vascular inflammatory proteins including iNOS and VCAM-1, not COX2 and ICAM-1, were significantly reduced over the treatment period (Figure 4.6C-F).

4.3.5 Amelioration of nitrosative stress and fibrosis

The high-dose SPH-T treatment reduced vascular oxidative/nitrosative stress, since it decreased the level of nitrotyrosine, a major indicator of peroxynitrite anion ($ONOO^-$) formation (Figure 4.7) (Escobales & Crespo, 2005). The treatment also reduced the circulating level of transforming growth factor beta (TGFß) and the vascular expression of type I collagen (Figure 4.8A-B).

4.4. Discussion

Orally administrating SPH-T at the high dose (1,000 mg/kg/day BW) reduced the MAP (146.3 to 126.7 mmHg), SBP (167.3 to 143.0 mmHg), and DBP (125.0 to 112.2 mmHg) of SHR over a period of 20 days; the low-dose group (250 mg/kg/day BW) also reduced BP significantly. Both

treatments did not impact HR of SHR, suggesting that cardiac function was not affected (Figure 4.2). It has been reported that lowered BP by 5 mmHg can significantly reduce morbidity and mortality caused by hypertension and its complications (McQueen et al., 2005; Wexler, 2007). Protein hydrolysates derived from various foodstuffs that reduce BP have continuously been reported (Fujita, Yokoyama, & Yoshikawa, 2000; Girgih et al., 2016; Jahandideh et al., 2016; Majumder et al., 2013; Yu, Zhao et al., 2017). SPH-T reduced BP in SHR, being comparable to hydrolysates prepared from egg and milk proteins over up to18 days of oral administration at the low dose of 240-250 mg/kg BW and the high dose of 1,000-1,200 mg/kg BW (Jahandideh et al., 2016; Jahandideh et al., 2014; Wang et al., 2012). Indeed, some chicken meat-derived peptides such as IWHHT and its gastrointestinal-digested fragments, IWH and IW, were reported to reduce BP in SHR through either oral or intravenous administration (Fujita et al., 2000). Both protein hydrolysates and pure peptides have shown great potency in BP regulation; however, protein hydrolysates outweigh pure peptides in the aspect of cost-effective, energy-efficient, and commercially scalable production. Other than that, protein hydrolysates may exert multiple physiological functions due to the presence of various bioactive peptides therein. To further look into the mechanisms underlying antihypertension of SPH-T, the plasma and aorta of SHR in the high-dose group were subsequently analyzed.

Dysregulation of the RAS leads to elevated BP and cardiovascular damage (Crowley & Coffman, 2012). With regards to the effect of the high dose treatment on the circulating RAS, a remarkable reduction in Ang II concentration was observed; there was no change in ACE activity, whereas ACE2 and Ang (1-7) levels were increased (Figure 4.4). This suggested that Ang II reduction was likely due to the conversion to Ang (1-7) by the upregulated ACE2. Ang II is a major vasoconstrictor, its reduction indicated a big potential of SPH-T in reducing BP. Besides,

the increased circulating levels of Ang (1-7) and ACE2 could further contribute to BP reduction (Benter et al., 1995). Asides from the circulating RAS, the local RAS such as in the vasculature also contributes to BP reduction (Danser, 2003). The action of local RAS is mainly mediated by Ang II and its receptors such as AT_1R, as well as the counterbalancing ACE2-Ang (1-7)-MasR axis which offset pathological responses caused by the Ang II-AT_1R axis (e Silva & Teixeira, 2016; Liao et al., 2019). The vascular expression of AT_1R was downregulated by the high-dose treatment while that of ACE2 was upregulated, despite not affecting ACE, AT_2R, and MasR expressions, after the high-dose treatment (Figure 4.5). Therefore, SPH-T might lower BP of SHR by modulating both the circulating and vascular RAS, through reduced circulating level of Ang II and increased Ang (1-7) and ACE2 levels, as well as upregulated vascular ACE2 expression and downregulated that of AT_1R.

Endothelial dysfunction precedes hypertension by altering regulatory functions of the endothelium, resulting in imbalanced production of contracting (e.g. Ang II) and relaxing factors (e.g. NO, prostacyclin) (Cau, Evora, & Tostes, 2018). A reduced NO production or bioavailability plays a central role in endothelial dysfunction (Cau et al., 2018). To understand the contribution of NO, L-NAME, a NOS inhibitor, was used to pre-treat the vessels (Figure 4.3A-D). The high-dose SPH-T treatment demonstrated a tendency in enhancing Mch-induced vasodilation despite being not significant ($p = 0.17$) (Figure 4.3A). Intriguingly, compared with the untreated group, the high-dose SPH-T significantly retarded Mch-mediated vasodilation in the presence of L-NAME and shifted it towards NO-dependent vasodilatory mechanism (Figure 4.3B-D). We further analyzed the phosphorylation of eNOS, a major enzyme for NO production in the vasculature (Zhao, Vanhoutte, & Leung, 2015); however, no significant change in eNOS phosphorylation was detected ($p = 0.34$) (Figure 4.3E). Thus, the high-dose SPH-T enhanced endothelial NO-dependent

vasodilation without causing a direct vasodilatory effect. Previous studies involving NO-dependent vasodilatory mechanism usually contributed to vasodilation, whereas our study provided a sort of new finding to the mechanisms of antihypertensive peptides on vascular function (García-Tejedor et al., 2017; Jahandideh et al., 2016; Majumder et al., 2013; Miguel et al., 2007). Despite an insignificant improvement in vasodilation in young SHRs, it is interesting to study the vasodilatory effect of SPH-T in aged animals, which are accompanied with a progressive decline of NO production and bioavailability (Cau, Carneiro, & Tostes, 2012; Smith et al., 2006). Besides, vasodilatory responses to SNP were not changed in the treated group, indicating no effect of SPH-T on endothelium-independent vasodilation.

SPH-T ameliorated vascular inflammation and oxidative stress over the treatment period (van der Velde, et al., 2015). The high-dose treatment decreased the circulating levels of inflammatory cytokines/chemokines, TNFα and MCP-1, as well as vascular expression of VCAM-1 and iNOS, without affecting that of COX2 and ICAM-1 (Figure 4.6). There are many anti-inflammatory peptides, which attenuated inflammation in endothelial cells (downregulation of MCP-1, ICAM-1, VCAM-1, etc.) and in vascular smooth muscle cells (downregulation of iNOS, COX2, etc.) (Gu & Wu, 2016; Huang et al., 2010; Liao et al., 2016; Lin et al., 2017; Majumder et al., 2013; Song et al., 2020; Wang et al., 2020). Peptides are natural free radical scavengers and are reported to ameliorate oxidative stress in various cells and animals (He et al., 2019a; Liao et al., 2016; Tsai et al., 2020; Wang et al., 2018; Wang et al., 2020; Yi et al., 2020). Within the vasculature, reactive oxygen species such as superoxide anion can react with overproduced NO (of inflammatory origin) and form $ONOO^-$, damaging various cell components and causing nitrosative stress. The 3-nitrotyrosine is a major marker of $ONOO^-$ formation (Escobales et al., 2005). Our study showed that the high-dose SPH-T reduced nitrotyrosine level in the vasculature (Figure 4.7), suggesting

an antioxidant effect being exerted in the vasculature. Vascular inflammation and oxidative stress can exacerbate fibrosis and lead to pathological vascular remodeling, contributing to the development of hypertension and many other cardiovascular diseases (Escobales et al., 2005; Intengan & Schiffrin, 2001; Lan, Huang, & Tan, 2013). The high-dose treatment reduced the circulating level of TGFß that stimulates extracellular matrix deposition including various types of collagens, which contributes to fibrosis and increased arterial stiffness (Grainger, 2007; Harvey et al., 2016). Notably, vascular expression of type I collagen was significantly inhibited, which indicated the protective role of SPH-T against vascular fibrosis and remodeling (Figure 4.8). Therefore, SPH-T reduced vascular inflammation, oxidative stress, and fibrosis in SHR, at least at the high-dose level.

In summary, SPH-T reduced high BP in SHRs, being possibly mediated by modulation of the RAS (both circulating and vascular RAS) as well as improvement of vascular inflammation, oxidative stress, and fibrosis. Future research is needed to ascertain whether the anti-inflammatory, antioxidant, and anti-fibrotic effects of SPH-T are mediated by the reduced circulating Ang II or increased ACE2/Ang (1-7) level. Besides, the role of SPH-T in shifting vasodilation towards a NO-dependent manner and its contribution to BP reduction in SHR remains to be explored in aged animals, with a progressive decline in NO generation and bioavailability. These questions are yet to be answered to achieve a better understanding of *in vivo* mechanisms of action of SPH-T, which underlies its functional food application for the prevention and treatment of hypertension.

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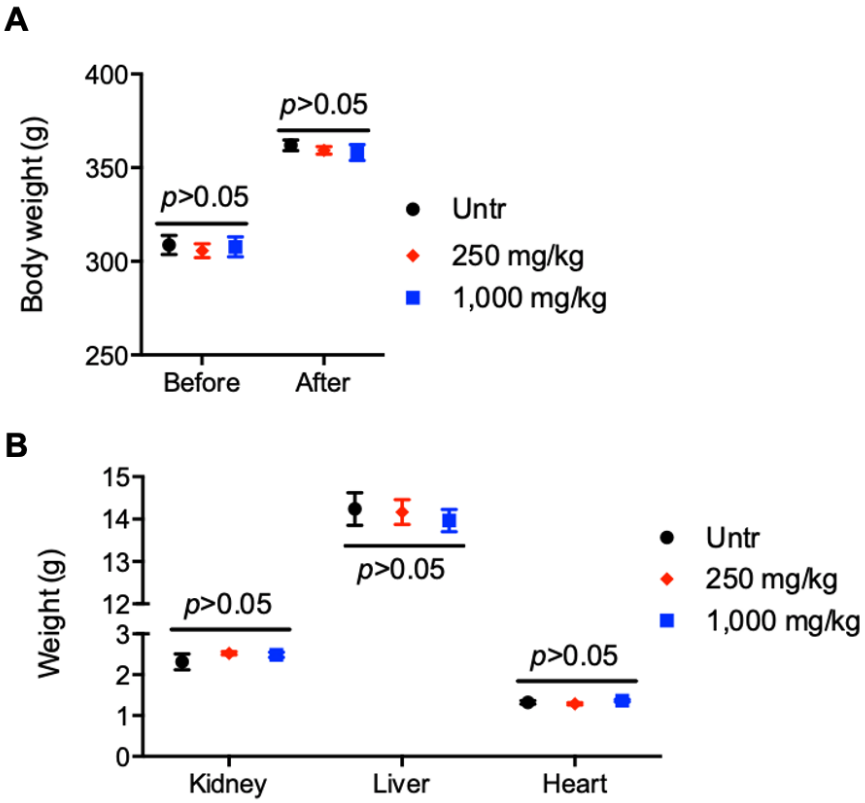


Figure 4.1 Effect of oral administration of SPH-T on body and organ weights in SHRs. **(A)** Body weight was obtained before and after (at the end point of the experiment) 20 days of oral administration of SPH-T to SHRs. **(B)** Organ weight was obtained after euthanizing the animals. Untr, untreated group.

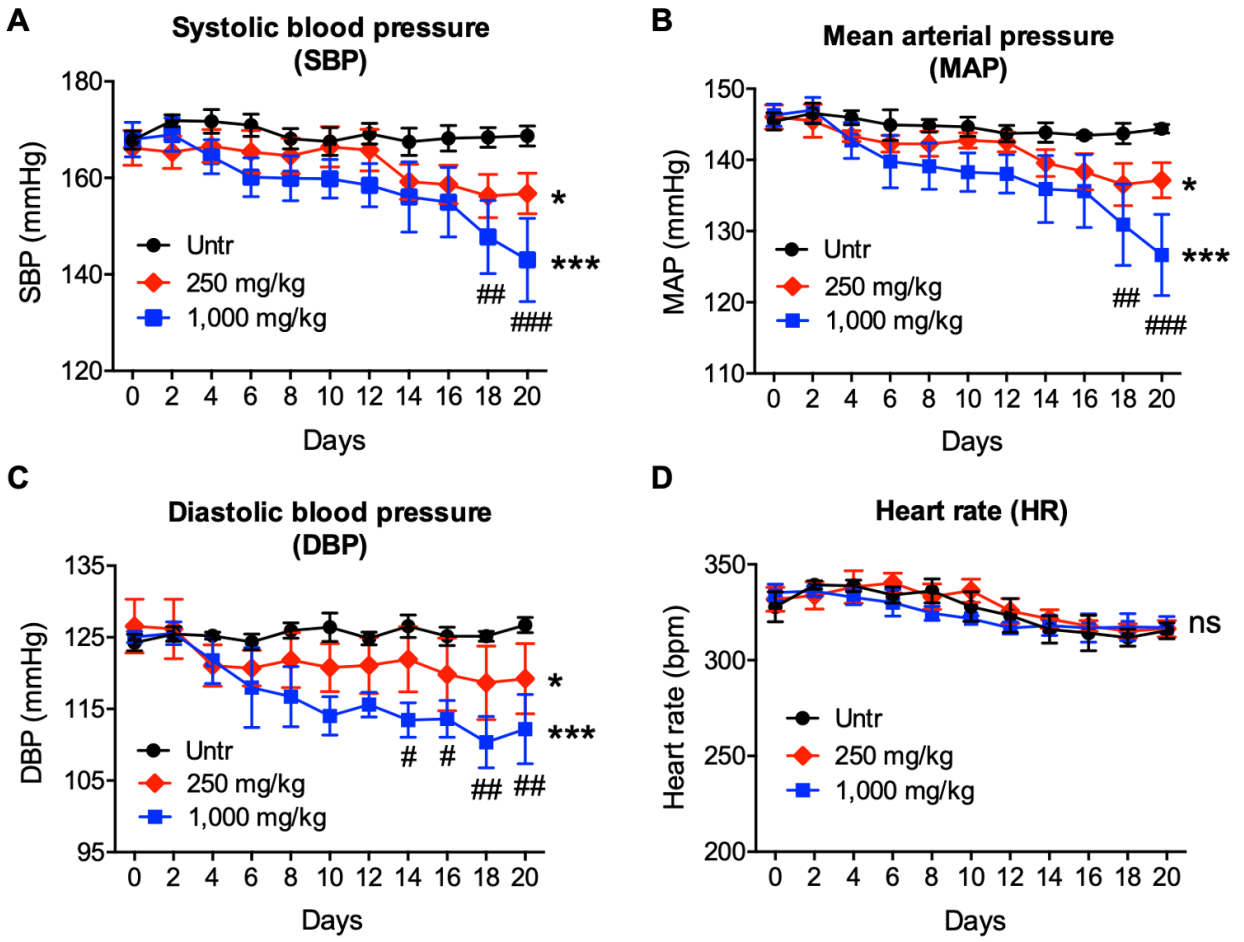


Figure 4.2 Effect of oral administrated of SPH-T on blood pressure and heart rate in SHRs. Blood pressure and heart rate of each time point represented the mean values recorded over a 24 h period. Data represented as mean ± SEM from n=6 animals per group. *, $p < 0.5$, ***, $p < 0.001$, ns, not significant ($p > 0.05$), as compared to the untreated group (Untr, without treatment) over the whole period; #, $p < 0.5$, ##, $p < 0.01$, ###, $p < 0.001$, as compared to the untreated group at a specific time point. SBP, MAP, DBP, and HR represent systolic blood pressure, mean arterial pressure, diastolic blood pressure, and heart rate, respectively.

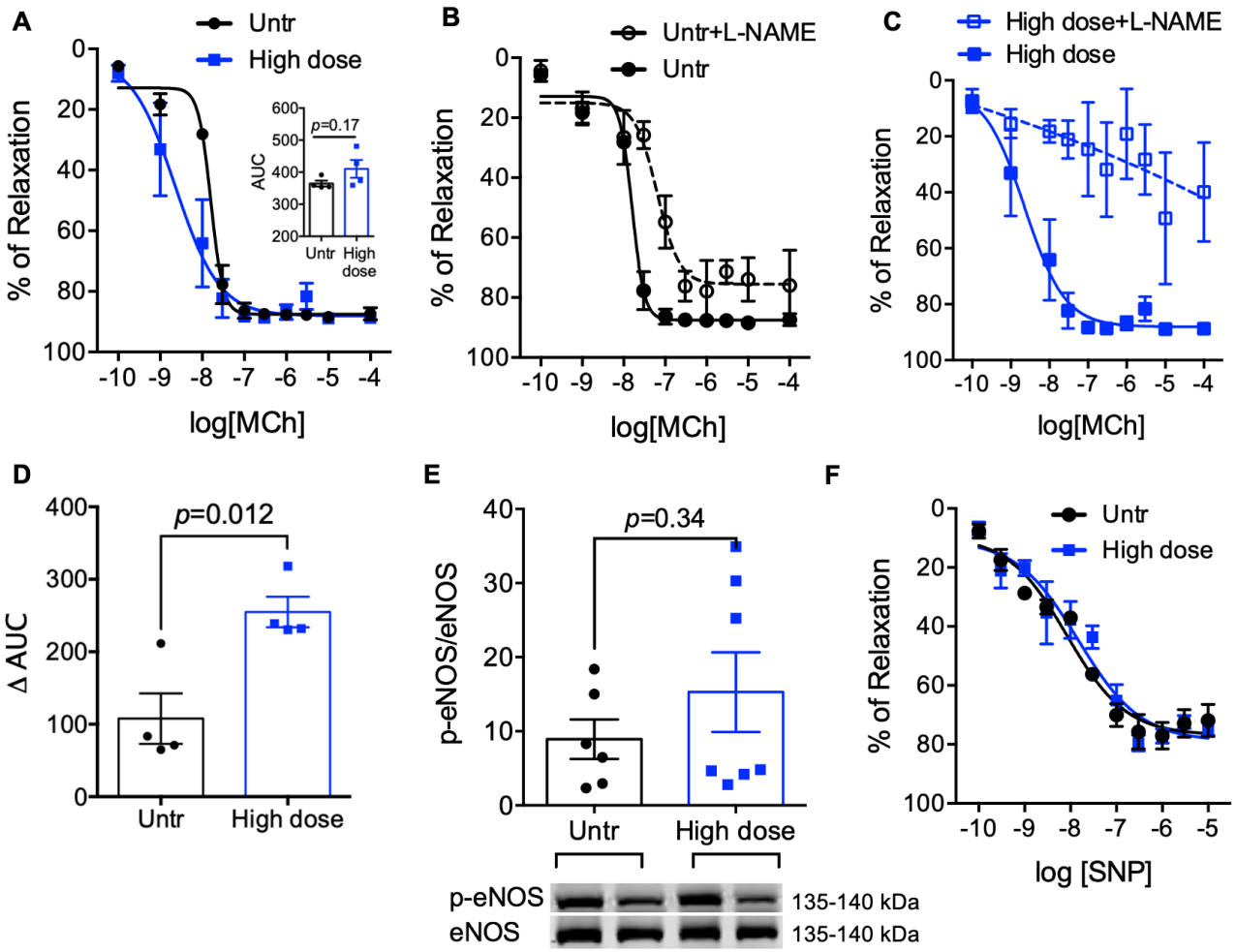


Figure 4.3 Effect of high-dose SPH-T treatment on vasorelaxation of isolated MA and eNOS expression in aorta. (**A**) Cumulative concentration response curves to methacholine (Mch) in MA (n=4). (**B-D**) Effect of addition of L-NAME (100 μM) prior to Mch-induced vasorelaxation in the untreated and high dose group (ΔAUC, delta area under the curve) (n=4). (**E**) Change in p-eNOS expression (normalized to total eNOS) in aorta (n=6-7). (**F**) Effect of SNP on vasorelaxation of MA (n=4).

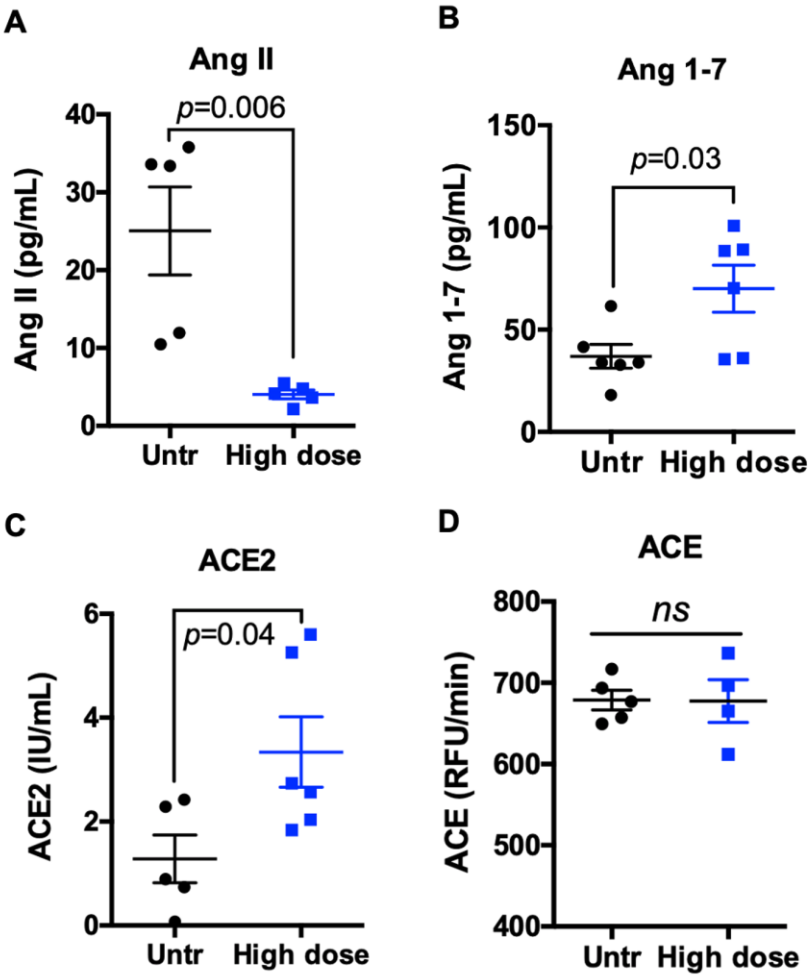


Figure 4.4 Effect of high-dose SPH-T treatment on the circulating RAS components. Plasmas were collected at the end point for evaluating the circulating level of (**A**) Ang II concentration, (**B**) Ang (1-7) concentration, (**C**) ACE2 concentration, and (**D**) ACE activity. Data were represented as mean ± SEM from 5-6 animals per group.

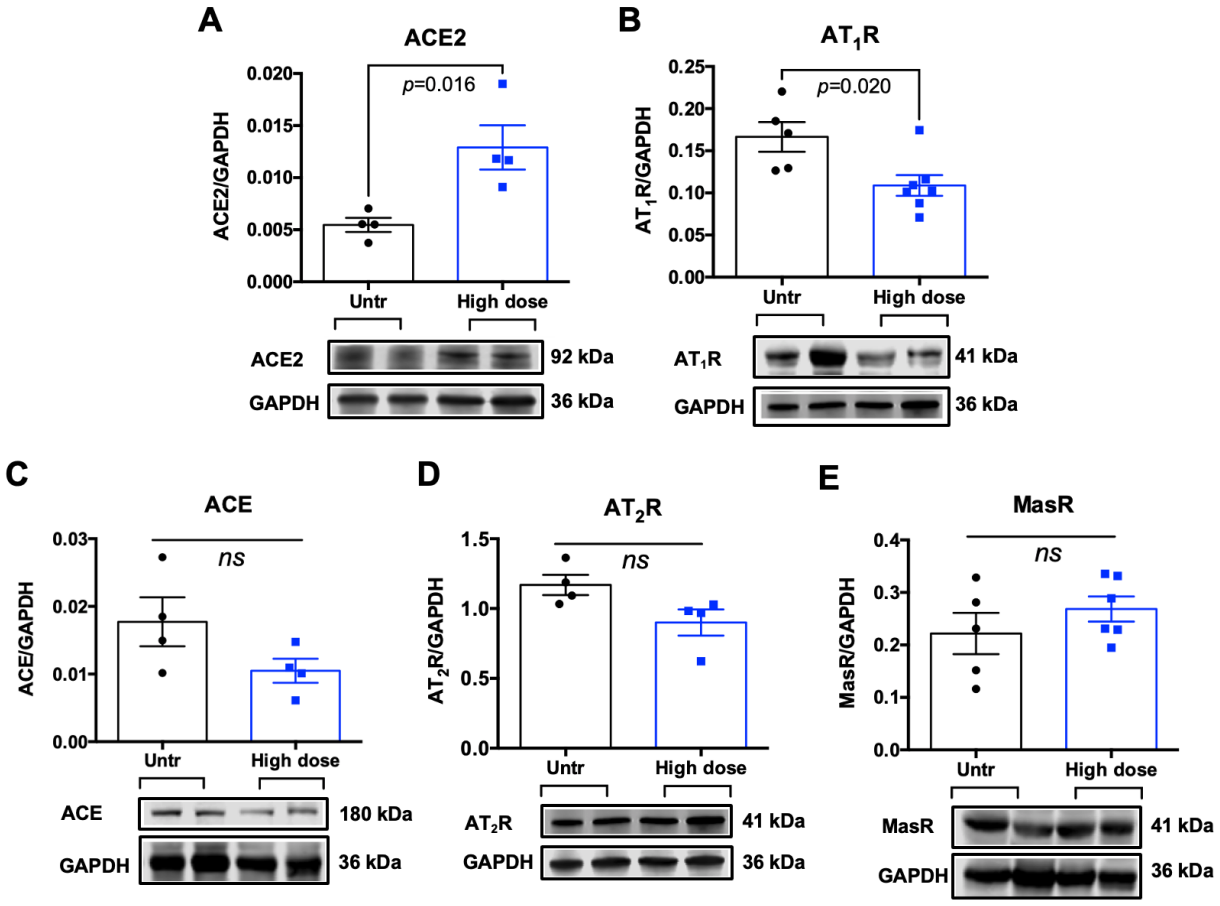


Figure 4.5 Effect of high-dose SPH-T treatment on aortic expression of RAS components. Vascular expressions of (**A**) ACE2, (**B**) AT_1R, (**C**) ACE, (**D**) AT_2R, and (**E**) MasR were normalized to GAPDH. Data were represented as mean ± SEM from 4-6 animals per group.

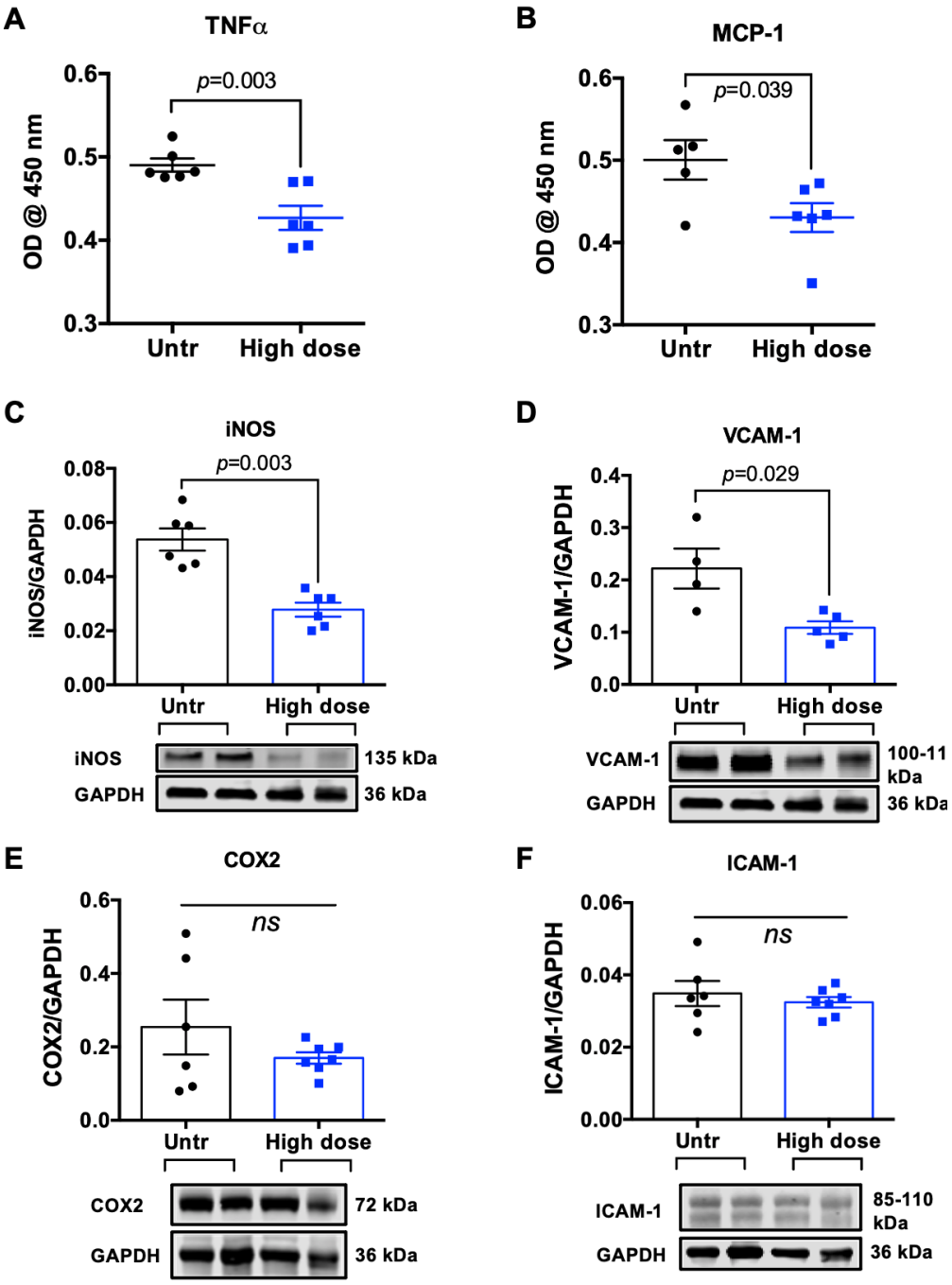


Figure 4.6 Effect of high-dose SPH-T treatment on circulating inflammatory cytokines and inflammatory markers in aorta. Plasmas were collected at the end point for evaluating the circulating level of (**A**) TNFα and (**B**) MCP-1. Vascular expressions of (**C**) iNOS, (**D**) VCAM-1, (**E**) COX2, and (**F**) ICAM-1 were normalized to GAPDH. Data were represented as mean ± SEM from 4-6 animals per group.

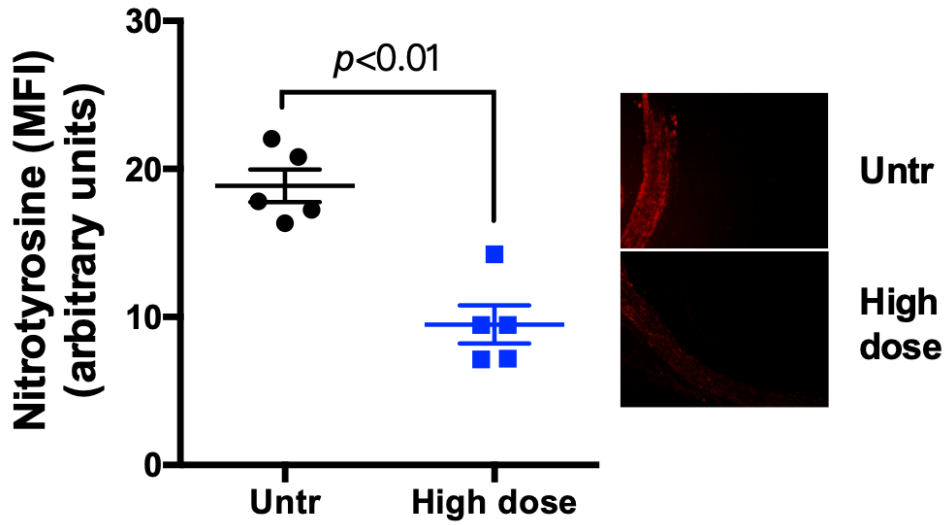


Figure 4.7 Effect of high-dose SPH-T treatment on nitrotyrosine level in aorta. Data were represented as mean ± SEM from 5 animals per group.

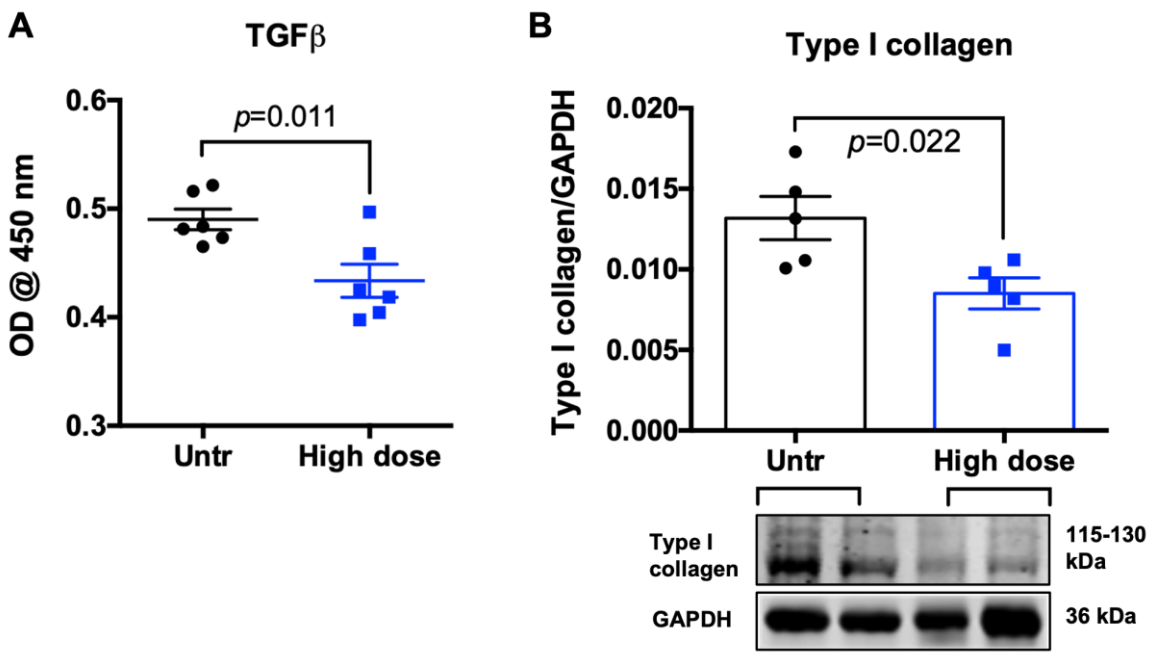
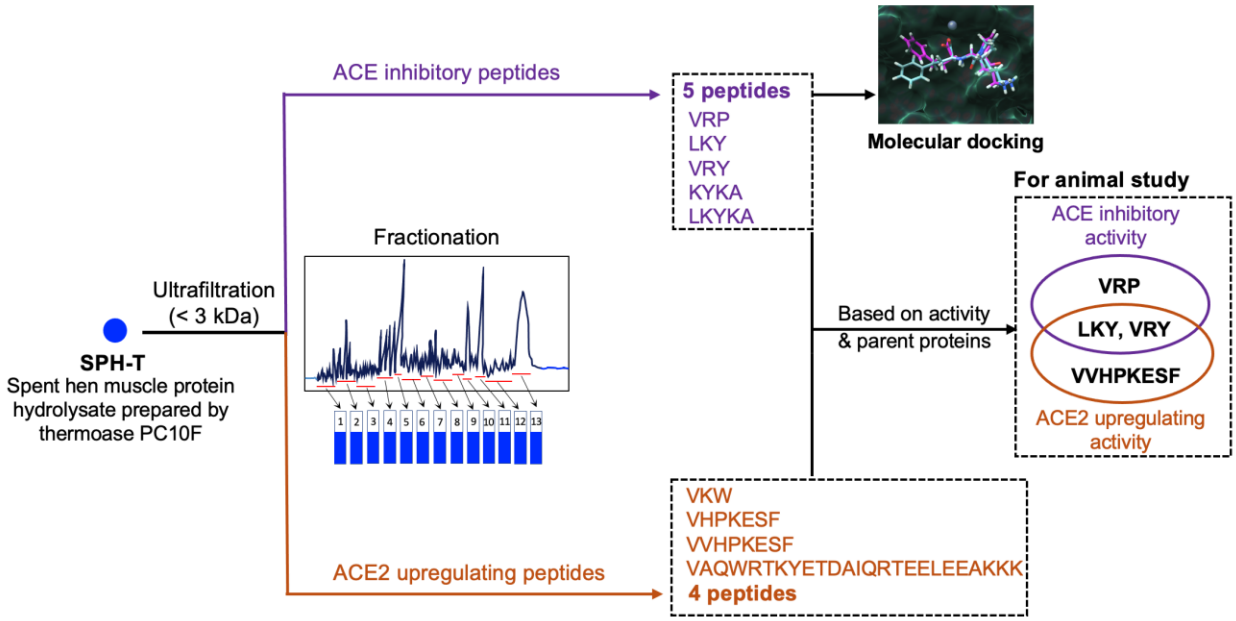


Figure 4.8 Effect of high-dose SPH-T treatment on vascular fibrosis. Expression of type I collagen was normalized to GAPDH. Data were represented as mean ± SEM from 5-6 animals per group.

CHAPTER 5 – Purification and Identification of Novel ACE Inhibitory and ACE2 Upregulating Peptides from Spent Hen Muscle Protein Hydrolysate Prepared by Thermoase [4]

Graphical abstract



[4] The whole chapter has been published: Fan, H., & Wu, J. (2020). Purification and identification of novel ACE inhibitory and ACE2 upregulating peptides from spent hen muscle proteins. *Food Chemistry, 345*, 128867.

5.1 Introduction

Angiotensin-converting enzyme (ACE) and angiotensin-converting enzyme 2 (ACE2) maintain a delicate balance in regulating the renin-angiotensin system (RAS) and thus blood pressure (BP). The former converts angiotensin (Ang) I to Ang II that increases BP via Ang II type 1 receptor (AT_1R), while the latter degrades Ang II into Ang (1-7) that reduces BP via Mas receptor (MasR) (Patel et al., 2014; Unger, 2002). Within the RAS, inhibition of ACE activity has been the primary target for treating hypertension and developing antihypertensive therapy (Udenigwe & Mohan, 2014). Until recently, upregulation of ACE2 level leading to activation of the ACE2-Ang (1-7)-MasR axis has become an emerging direction for developing novel antihypertensive agents (Patel, et al., 2014). In ACE2-knockout animals, ACE2 deficiency exacerbated Ang II-induced cardiac and vascular pathological responses including hypertension, oxidative stress, fibrosis, and remodeling (Alghamri et al., 2013; Zhong et al., 2010), but the trend can be blunted by supplementing recombinant ACE2 (Zhong et al., 2010). In spontaneously hypertensive rat (SHR), the most-widely used animal model of hypertension, ACE2 level is significantly lower than that in normotensive Wistar-Kyoto rats (Yang et al., 2013), while, upregulating ACE2 attenuated hypertension and hypertension-linked pathological responses (Díez-Freire et al., 2006). ACE2 has shown considerable effect in managing cardiovascular diseases.

Bioactive peptides are reported to augment ACE2 and Ang (1-7) levels both in cellular and animal studies. For example, Ile-Pro-Pro (IPP), derived from casein, improved Ang (1–7)-induced mesenteric arterial relaxation in SHR (Ehlers et al., 2011). An egg peptide, Ile-Arg-Trp (IRW), enhanced ACE2 expression in rat vascular smooth muscle A7r5 cells (VSMCs); its ability to reduce BP in SHR was proved to be primarily via upregulation of the ACE2-Ang (1-7)-MasR axis (Liao et al., 2018; Liao et al., 2019a). A pea-derived peptide, Leu-Arg-Trp (LRW), an isomer of

IRW, exerted its antioxidant, anti-proliferative, and anti-inflammatory effects possibly by upregulating ACE2 in VSMCs (Wang et al., 2020). These findings suggested that upregulation of ACE2 is a promising target for developing antihypertensive peptides. Indeed, several ACE2 upregulating (ACE2u) peptides such as LSDRFS and SDRFSY were successfully isolated from pea proteins, manifesting the feasibility of identifying this novel type of peptides from food proteins (Liao et al., 2019b).

In Chapters 3 and 4, we prepared a spent hen muscle protein hydrolysate using thermoase PC10F (SPH-T) that possessed antihypertensive effect in SHR. SPH-T possessed both excellent *in vitro* ACEi and ACE2u activities and the latter has also been confirmed in SHR; despite being not validated *in vivo*, ACEi activity remains the primary target for developing antihypertensive peptides. Thus, the current work aims to isolate and identify both ACEi peptides and ACE2u peptides from SPH-T.

5.2 Materials and Methods

5.2.1 Materials

Spent hen carcasses (age of 70-80 weeks old; weight of ~1.1 kg, with feather, beak, and internal organs removed) were purchased from a local supermarket (T&T) in Edmonton, Canada. Thermoase PC10F (from *Bacillus thermoproteolyticus Var. Rokk*, 90,000 U/g protein) was obtained from Amano Enzyme Inc. (Nagoya, Japan). Pepsin (from porcine gastric mucosa), pancreatin (from the porcine pancreas), acetonitrile, trifluoroacetic acid (TFA), ACE (from rabbit lung), and hippuryl-His-Leu (HHL) were purchased from Sigma-Aldrich (Oakville, ON, Canada). Ammonium acetate and ammonium carbonate were obtained from Fisher Scientific (Ottawa, ON, Canada). Peptides (purity > 99%) were synthesized by Genscript Corp (Piscataway, NJ, USA).

Dulbecco's modified Eagle's medium (DMEM), 0.25% (w/v) trypsin-0.53 mM EDTA, fetal bovine serum (FBS), Hanks balanced salt solution (HBSS with Ca and Mg), 4-(2−68 hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), nonessential amino acids (NEAA), and penicillin−streptomycin were obtained from Gibco Invitrogen (Burlington, ON, Canada). VSMCs A7r5 (CRL-1444) cell line was purchased from ATCC (Manassas, VA, USA).

5.2.2 Preparation of spent hen muscle protein hydrolysate using thermoase (SPH-T)

Spent hen muscle protein extraction and preparation of the hydrolysate followed protocols as described in Chapter 3.

5.2.3 Peptide purification

SPH-T was ultra-filtrated through a 3-kDa molecular weight cut-off membrane (Amicon Division, W. R. Grace & Co., Beverly, USA). The permeate was then purified through cation exchange chromatography (CEC), with a High-Prep 16/10 SP FF column (16 mm × 100 mm, GE Healthcare, Sweden) on an AKTA explorer 10XT system (GE Healthcare, Uppsala, Sweden). Samples (1.5 mL, 50 mg/mL) were linearly eluted (3 mL/min) using ammonium acetate (10 mM, pH 4.0, solvent A) and ammonium carbonate (0.5 M, pH 8.8, solvent B): 0%-0%-3%-8%-15%-100%-100%-0%-0%B (0-2-3-13-16-16.5-17-18-19 column volume). Absorbance was monitored at 220 and 280 nm. Ten fractions were collected and determined their ACEi and ACE2u activities. Later, Fraction 10 (F10) and F7, which showed the highest ACEi or ACE2u activity, respectively, were further fractionated by a two-step purification using reverse-phase high-performance liquid chromatography (RP-HPLC), with a Waters 600 system and a 2998 photodiode array (PDA) detector monitoring the absorbance at 220 nm (Waters, Milford, MA, USA). Fractions (F10 and F7) were first eluted using an X-bridge preparative C_{18} column (10 × 150 mm, 5 µm, Waters,

Milford, MA, USA); three resultant fractions (F10-4, F7-6, and F7-4) were subjected to the second fractionation using an X-bridge Analytical C_{18} column (3.0 × 250 mm, 5 µm, Waters, Milford, MA, USA). Mobile phases were ddH_2O (solvent A) and ACN (solvent B) both containing 0.1% TFA. Gradients of the RP-HPLC fractionation were: F10 (0% →5%→35%B, 0→5→65 min), F10-4 (0%→10%→10%→0%B, 0→5→30→40 min), F7 (0% →5%→35%B, 0→5→65 min), F7-6 (21%→21%B, 0→24 min), F7-4 (15%→17%B, 0→45 min). Samples were all dissolved in 100% solvent A and filtered through 0.22-µm membrane filters (Millipore Corp. Bedford, MA). Finally, the most active fractions in ACEi or ACE2u activity were analyzed by liquid chromatography with tandem mass spectrometry (LC-MS/MS). The fractionation process is shown in Figure 5.1.

5.2.4 Peptide sequencing

Peptide sequences were analyzed using an Atlantis dC_{18} column (75 µm × 150 mm, 3 µm, Waters, Milford, MA, USA) on a nanoAcquity reverse-phase ultra-performance liquid chromatography (RP-UPLC) system, coupled with and a Micromass Quadrupole Time-of-Flight (Q-TOF) premier mass spectrometer, as previously described in Fan et al. (2019b). Solvents were LC/MS grade H_2O (solvent A) and ACN (solvent B) containing 0.1% formic acid. Data were interpreted by MassLynx software version 4.1 (Waters Milford, MA, USA) by *de novo* sequencing.

5.2.5 ACE inhibitory activity assay

ACEi activity was measured referred to protocol illustrated in Chapter 3.

5.2.6 Cell culture of vascular smooth muscle A7r5 cells (VSMCs) and western blotting

Cell culturing protocol of VSMCs (passages 4-11) followed the procedures performed in Chapter 3. VSMCs were treated with SPH-T (fractions) (2.5 mg/mL) or peptides (50 µM) for 24

h. SPH-T and its fractions were desalted before being added to treat with VSMCs (Fan et al., 2019b). Doses were selected based on our previous study (Liao et al., 2019b) as well as Figure 5.2. Cells were lysed in a boiling Laemmle's buffer containing 50 mM Dithiothreitol (DTT) and 0.2% Triton-X-100. The lysates were run on a 9% separating gel and transferred to a nitrocellulose membrane (diameter 0.45 μm, 1620115, Bio-Rad, Montreal, QC, Canada) for antibody incubation. ACE2 (Abcam, Cambridge, UK) was normalized to α-tubulin (Abcam). Donkey-anti-rabbit 800 CW or goat-anti-rabbit IRDye 680 RD secondary antibodies (Licor Biosciences, Lincoln, NE, USA) were used to visualize the fluorescent bands in a Licor Odyssey BioImager, which were quantified using Image Studio Lite 5.2 (Licor Biosciences).

5.2.7 Molecular docking

The crystal structure of human testicular ACE (tACE) was retrieved from the Protein Data Bank (PDB: 1O86, tACE-lisinopril complex). Peptide ligands and tACE were prepared using Chimera (version 1.14, University of California, San Francisco, CA, USA) (Pettersen et al., 2004). Docking was performed using Dock 6.8 (http://dock.compbio.ucsf.edu/) (University of California, San Francisco, CA, USA); each peptide was docked as a flexible body in 100,000 different orientations. A grid box of 10 $Å^3$ surrounding lisinopril in 1O86 was selected as the binding site. The best binding mode was selected based on the lowest binding energy and score including van der Waals and electrostatic interaction implemented in DOCK 6.8. Stereo representations of peptides and tACE were prepared using Chimera.

5.2.8 Statistical analysis

Data were subjected to one-way analysis of variance (ANOVA) followed by Dunnett's multiple test using GraphPad Prism Version 6 (San Diego, CA, USA). A value of $p < 0.05$ was considered statistically different.

5.3 Results

5.3.1 Purification of ACE inhibitory peptides and ACE2 upregulating peptides

SPH-T had both high ACEi and ACE2u activities; there was no cytotoxicity observed in VSMCs at its concentration up to 2.5 mg/mL (Chapter 3: Figure 1A). After ultrafiltration, SPH-T (< 3 kDa) possessed an ACEi IC_{50} value of 0.014 mg/mL while also significantly upregulated ACE2 expression ($p < 0.05$) in VSMCs in a dose-dependent manner (0 – 2.5 mg/mL), which suggested the presence of both ACEi peptides and ACE2u peptides (Figure 5.2). Then, SPH-T (< 3 kDa) was fractionated by CEC and a two-step RP-HPLC as shown in a simplified flow diagram in Figure 5.1. Ten fractions were collected after CEC (Figure 5.3); F10 showed the highest ACEi activity whereas F7 possessed the highest ACE2u activity. Next, F10 and F7 were further subjected to RP-HPLC fractionation.

5.3.1.1 Fractionation of ACE inhibitory peptides

F10 was separated into 9 fractions and ACEi peptides were mostly enriched in the fourth fraction (F10-4) (Figure 5.4A-B). The second-step RP-HPLC separation generated 9 sub-fractions from F10-4 (Figure 5.4C-D), in which F10-4-7 possessed the highest ACEi activity, with an IC_{50} value of 0.14 µg/mL.

5.3.1.2 Fractionation of ACE2 upregulating peptides

As shown in Figure 5.4E-F, the first-step RP-HPLC purification of F7 generated 9 fractions, in which the sixth (F7-6) and fourth (F7-4) factions upregulated ACE2 expression significantly ($p < 0.05$) in VSMCs. These two fractions were further fractioned by a second-step RP-HPLC, and three sub-fractions, F7-6-3, F7-4-3, and F7-4-5 retained the highest ACE2u activity (Figure 5.4G-J).

5.3.2 Peptide sequencing

Four fractions, F10-4-7, F7-6-3, F7-4-3, and F7-4-5, purified above were analyzed by LC-MS/MS. There were 25 peptide sequences identified from F10-4-7 after *de novo* sequencing; they were then synthesized for validation of their ACEi activities. Meanwhile, 11 peptide sequences were identified from F7-6-3 (1), F7-4-3 (4), and F7-4-5 (6). Their sequences are listed in Table 5.1 and Figure 5.5, along with the molecular weight and parent proteins; the MS spectra of these peptides are presented in Figures 5.6 and 5.7.

5.3.3 Activity validation of synthetic peptides

Table 5.1 shows ACEi activity of the identified peptide sequences; most of them contained 3-4 amino acid residues. The very low IC_{50} value (0.14 μg/mL) of F10-4-7 suggested the presence of very potent ACEi peptides therein. Five most potent peptides, VRP, LKY, VRY, KYKA, and LKYKA, were identified, with IC_{50} values of 0.64, 0.81, 5.77, 2.87, and 0.034 μg/mg, respectively. VRP and LKY have been reported in bonito and sesame, respectively (Matsumura et al., 1993; Nakano et al., 2006); the other peptides have not been reported yet.

The peptides identified from fractions F7-6-3, F7-4-3, and F7-4-5 were determined for their ACE2u activities in VSMCs. Results showed that only VKW, VHPKESF, VVHPKESF, and

VAQWRTKYETDAIQRTEELEEAKKK significantly ($p < 0.05$) increased ACE2 expression (Figure 5.5). These peptides had a relatively larger size than ACEi peptides, indicating a possibly different structural feature of ACE2u peptides from that of ACEi peptides, since the latter are generally very short peptides. To date, there is very limited information on ACE2u peptides from the literature.

5.3.4 Inhibitory mechanism of ACE inhibitory peptides

Molecular interactions between tACE and the identified ACEi peptides are presented in Figure 5.8; their stereo interactions are shown in Figure 5.9. Lisinopril was first used to validate the accuracy and efficiency of this model, which were supported by a substantial overlap of the docked lisinopril with its counterpart in the crystal structure of lisinopril-tACE complex (PDB: 1O86) (Figure 5.10). The binding scores corresponded with ACEi activities of these peptides, which interacted with different residues of tACE and formed varying numbers of H-bonds.

5.3.5 Selection of spent hen peptides for animal study

We aimed to select the peptide candidates that reflect, to the largest extent, the antihypertensive profile of SPH-T for validation of their *in vivo* efficacies in SHR. Firstly, among the identified 5 ACEi peptides and 4 ACE2u peptides, only peptides from major muscle proteins including myosin and actin were considered. Based on this criterion, KYKA, LKYKA, VKW were excluded. The longest peptide containing 25 amino acids, VAQWRTKYETDAIQRTEELEEAKKK, was not considered either since there are no reports on antihypertensive peptides with such a long sequence in literature to the best of our knowledge. Thus, the potential candidates were VRP, LKY, VRY, VVHPKESF, and VHPKESF (Table 5.2). Given ACE/ACE2 balance plays an important role in

regulating BP, we cross-evaluated ACEi or ACE2u activity of the identified peptides, in which LKY and VRY showed both potent ACEi and ACE2u activities.

5.4 Discussion

Over the past decades, a substantial number of antihypertensive peptides have been identified from various food proteins (Aluko, 2015), and a great deal of knowledge on peptide discovery, structure-function relationship, bioavailability, efficacy, and mechanisms has been reported (Fujita, Yokoyama, & Yoshikawa, 2000; Sánchez-Rivera et al., 2014; Wu, Aluko, & Nakai, 2006; Wu, Liao, & Udenigwe, 2017). Our understanding of the mechanisms of antihypertensive peptides has evolved from ACE inhibition to AT_1R downregulation and blocking, restoration of nitric oxide-mediated vasorelaxation, improvement of antioxidant and anti-inflammatory status, and more recently on upregulation of ACE2 (Wu et al., 2017). The present study was proposed to identify novel peptides that upregulate ACE2 in VSMCs, the major component that contracts or relaxes blood vessels and controls BP (Lilly, 2014). The rationale was based on the recent characterization of the protective roles of ACE2 in the RAS and in BP regulation (Patel et al., 2014). In addition, a few bioactive peptides have recently shown ACE2u activity both *in vitro* and *in vivo*. For example, IPP, LY, RALP, GHS, IQP, VEP, and a *Spirulina platensis* hydrolysate reduced BP in SHR, associated with upregulated ACE2 level in plasma or various tissues including vessel, kidney, and heart (Ehlers et al., 2011; He et al., 2019; Majumder et al., 2015; Pan et al., 2015; Zheng et al., 2017). IRW, initially identified as an ACEi peptide from egg white, reduced BP independent of ACE inhibition after an 18-day oral administration. Later, it was found to upregulate ACE2 in VSMCs and in aorta and kidney of SHRs, and, moreover, to exert antihypertensive effect via mainly upregulating the ACE2-Ang (1-7)-MasR pathway in SHRs (Liao et al., 2018; Liao et al., 2019a). These findings suggested a new mechanism of BP reduction by bioactive peptides, and

ACE2 can be a new target for identifying novel antihypertensive peptides. However, there is scanty information on ACE2u peptides in the literature.

SPH-T (< 3 kDa) had an ACEi IC_{50} value of 0.014 mg/mL; SPH-T had an IC_{50} value of 0.030 mg/mL, similar to that of a previously-reported chicken muscle protein hydrolysate produced by thermolysin (0.045 mg/mL) (Fujita et al., 2000). SPH-T (< 3 kDa) treatment at 2.5 mg/mL upregulated ACE2 expression in VSMCs by ~ 0.8 folds, comparable to that of a pea protein hydrolysate, produced also using thermoase, as well as its derived peptide LRW (Liao et al., 2019b; Wang et al., 2020). Through fractionation, ACEi activities of the enriched fractions increased strikingly while ACE2u activities did not show a similar trend. After cation exchange fractionation, two different fractions, F10 and F7, retained the respective highest ACEi or ACE2u activity, indicating that ACEi peptides and ACE2u peptides were enriched in different fractions (Figure 5.3). This variation indicated different structural features of ACE2u peptides and ACEi peptides. After HPLC fractionation and *de novo* sequencing (Figure 5.4, 5.6, and 5.7), 25 peptides were identified from the most potent ACEi fraction (F10-4-7) while 11 peptides were from the most potent ACE2u fractions (F7-6-3, F7-4-3, and F7-4-5) (Table 5.1 and Figure 5.5). Five potent ACEi peptides (VRP, LKY, VRY, KYKA, and LKYKA) and four ACE2u peptides (VKW, VHPKESF, VVHPKESF, and VAQWRTKYETDAIQRTEELEEAKKK) were identified. Previous research has shown the antihypertensive, anti-inflammatory, and immunomodulatory activities of spent hen muscle protein hydrolysates using other proteases (Udenigwe et al., 2017; Yu, Field, & Wu, 2018a, 2018b); identification of ACEi and ACE2u peptides in this study further confirmed that spent hen muscle proteins are a reservoir of multifunctional bioactive peptides and are a suitable starting material that can be processed as functional food ingredients.

The structure-activity relationship of ACEi tripeptides have been extensively studied, and the three tripeptides identified in this study, VRP, LKY, and VRY, met the structural requirements, with a hydrophobic, a positively-charged, and an aromatic or cyclic residue in the N-terminal, middle, and C-terminal positions, respectively (Wu et al., 2006). Other tripeptides failing to fulfill these requirements did not exhibit high ACEi activities, even for those differing in only one amino acid residue, such as GKY, ERP, and VRL (Table 5.1). For peptides longer than 3 amino acids, their ACEi activities are likely to decrease since increased peptide length hampers their access to the active site of ACE (Fan, Liao, & Wu, 2019a). Unexpectedly, LKYKA showed the greatest ACEi activity, with an IC_{50} value of 0.034 μg/mL. The potent ACEi activity of LKYKA appeared to support a previous assumption for structural requirements of ACEi pentapeptides, which prefer a hydrophobic N-terminus and a hydrophobic tetrapeptide C-terminus with the positive charges at the second and fourth positions from the C-terminus (Fan et al., 2019b). Loss of the N-terminal leucine of LKYKA significantly reduced its ACEi activity (IC_{50} value increased from 0.034 to 2.87 μg/mL); a further loss of the N-terminal lysine of KYKA resulted in further reduction in ACEi activity (IC_{50} value increased from 2.87 μg/mL to be > 50 μg/mL). Besides, a significant reduction in ACE inhibition was observed in DLRP (IC_{50} value > 50 μg/mL) than that of LRP (0.46 μg/mL) reported previously (Gu & Wu, 2016). Peptides longer than 5 amino acid residues did not show high ACEi activities (Table 5.1). Next, we studied the molecular interactions between ACEi peptides and ACE. The C domain of somatic ACE, tACE, was selected for docking study since the active site converting Ang I to Ang II is located in this domain (Fan et al., 2019a). As seen in Figures 5.8 and 5.9, LKYKY, VRP, LKY, KYKA, and VRY formed interactions with tACE through varying numbers of H-bonds, and the order of their binding energies corresponded well with that of their IC_{50} values.

In comparison, there is no study on the structural features of ACE2u peptides, since the number of reported ACE2u peptides is too small to propose any structure-activity relationship. However, ACE2u peptides appeared to partially share structural features with that of ACEi peptides, such as a hydrophobic N-terminus and an aromatic or cyclic residue in the C-terminus, reflected in IRW, LRW, IQP, VEP, LY, VKW, VHPKESF, and VVHPKESF (Liao et al., 2019a; Wang et al., 2020; Zheng et al., 2017). Interestingly, IRW, LRW, IQP, VEP, and LY are all ACEi peptides. Moreover, some ACEi drugs such as lisinopril and enalapril also exert protective roles in cardiovascular function by enhancing the ACE2 level (Ferrario et al., 2005; Yang et al., 2013). Indeed, two ACEi peptides identified in this study, LKY and VRY, were also ACE2u peptides (Table 5.2). However, two ACE2u peptides, VHPKESF and VVHPKESF, were not ACEi peptides. Therefore, it is likely that ACEi peptides and ACE2u peptides have different structural requirements but may share some commonalities. Therefore, isolation of ACE2u peptides from food proteins using the conventional fractionation method remains necessary. Discovering more ACE2u peptides would enable us to study their structure-function relationships, which, in reverse, could contribute to the identification and design of new ACE2u peptides.

The ACE/ACE2 balance plays an important role in BP regulation; several ACEi drugs such as lisinopril and enalapril, and also ACEi peptides such as IRW, were reported to reduce BP and improve cardiovascular functions by upregulating ACE2 *in vivo* (Ferrario et al., 2005; Yang et al., 2013). Therefore, we further cross-determined both ACEi and ACE2u activities of peptides derived from the major muscle proteins (VRP, LKY, VRY, VVHPKESF, and VHPKESF) since these peptides are the most possible ones that could reflect the antihypertensive profile of SPH-T. Indeed, these peptides were classified into three groups: 1) ACEi peptide (VRP), ACE2u peptide (VVHPKESF and VHPKESF), as well as ACEi and ACE2u peptide (LKY and VRY). VHPKESF

is a fragment of VVHPKESF but possessed a similar ACEi activity but a lower ACE2u activity, as compared with those of VVHPKESF, therefore only VVHPKESF was further selected. Taken together, VRP, LKY, VRY, and VVHPKESF were selected for validating *in vivo* efficacy in SHR.

In summary, five potent ACEi peptides and four ACE2u peptides were identified from SPH-T using the conventional activity-guided fractionation method. The structural requirements of the identified ACEi peptides corresponded well with the structure-activity relationship reported in the literature; the molecular docking study confirmed that these peptides formed H-bonds with ACE. A lack of a sufficient number of ACE2u peptides makes it impossible to draw any structure-activity relationship, which relies heavily on a continuous discovery of new peptides. Furthermore, four peptides (VRP, LKY, VRY, and VVHPKESF) have been selected as the most possible candidates (reflecting the antihypertensive profile of SPH-T) for validating their antihypertensive effects in SHR.

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Table 5.1 Activity validation, molecular weight, and protein sources of ACEi peptides

Peptide sequence	IC_{50} (μg/mL)					Observed *m/z*	Calculated mass	Protein source
	> 250	> 50	> 25	> 10	< 10			
GKY	*					367.23	366.19	Titin
RPP				*		369.24	368.22	Myosin
VRP					0.64 ± 0.02	371.23	370.23	Myosin
YKA		*				381.23	380.21	Tropomyosin
TKH		*				385.23	384.21	Myosin
VRL		*				387.25	386.26	Myosin
KTF	*					395.21	394.22	Connectin
ERP		*				401.27	400.21	Myosin
KDF	*					409.25	408.20	Myosin
LKY					0.81 ± 0.04	423.24	422.25	Myosin
VRY					5.77 ± 0.17	437.24	436.24	Myosin
FRH		*				459.26	458.24	Myosin
WRN		*				475.32	474.23	Myosin
HALL	*					453.35	452.27	Myosin
DLRP		*				500.30	499.28	Myosin
TVRK	*					503.28	502.32	Myosin
KYKA					2.87 ± 0.53	509.32	508.30	Tropomyosin
KPLY		*				520.31	519.31	Myosin
YLLR		*				564.33	563.34	Myosin
LKYKA					0.034 ± 0.002	622.39	621.39	Tropomyosin
TRILL	*					615.42	614.41	Myosin
GQPGYS	*					608.35	607.26	Collagen
VPAMYV		*				679.51	678.34	Actin
GPVGFPGA				*		701.50	700.35	Collagen
FTKKPHPVQT			*			591.34	1181.66	Actin

Data were presented as means ± SEM (n=3).

Table 5.2 Selecting ACEi/ACE2u peptides for *in vivo* antihypertensive effect in SHR

Peptides	ACEi activity (IC_{50}, μg/mL)	ACE2u activity	ACEi peptide	ACE2u peptide	Parent proteins
ACEi peptides					
VRP	0.64 ± 0.02	1.14 ± 0.20	✓	×	Myosin
LKY	0.81 ± 0.04	1.79 ± 0.32	✓	✓	Myosin
VRY	5.77 ± 0.17	1.78 ± 0.44	✓	✓	Myosin
KYKA	2.87 ± 0.53	–	✓	–	Tropomyosin
LKYKA	0.034 ± 0.002	–	✓	–	Tropomyosin
ACE2u peptides					
VKW	–	1.60 ± 0.12	–	✓	Titin
VVHPKESF	>250	1.84 ± 0.19	×	✓	Myosin
VHPKESF	>250	1.75 ± 0.35	×	✓	Myosin
VAQWRTKYETDA-IQRTEELEEAKKK	–	1.52 ± 0.12	–	✓	Myosin

Peptides derived from the major muscle proteins were underlined and were cross-evaluated for their ACEi or ACE2u activity (excluding KYKA, LKYKA, and VKW that are from minor proteins in spent hen muscle as well as VAQWRTKYETDAIQRTEELEEAKKK with a long peptide chain that has not yet been reported as antihypertensive peptide in literature). VHPKESF was a fragment of VVHPKESF and possessed a lower ACE2u activity and a similar ACEi activity compared with those of VVHPKESF and was therefore excluded. Peptides underlined and bolded were selected for animal study and cross-evaluated for both ACEi and ACE2u activities. Data were expressed as means ± SEM of 3-4 independent experiments.

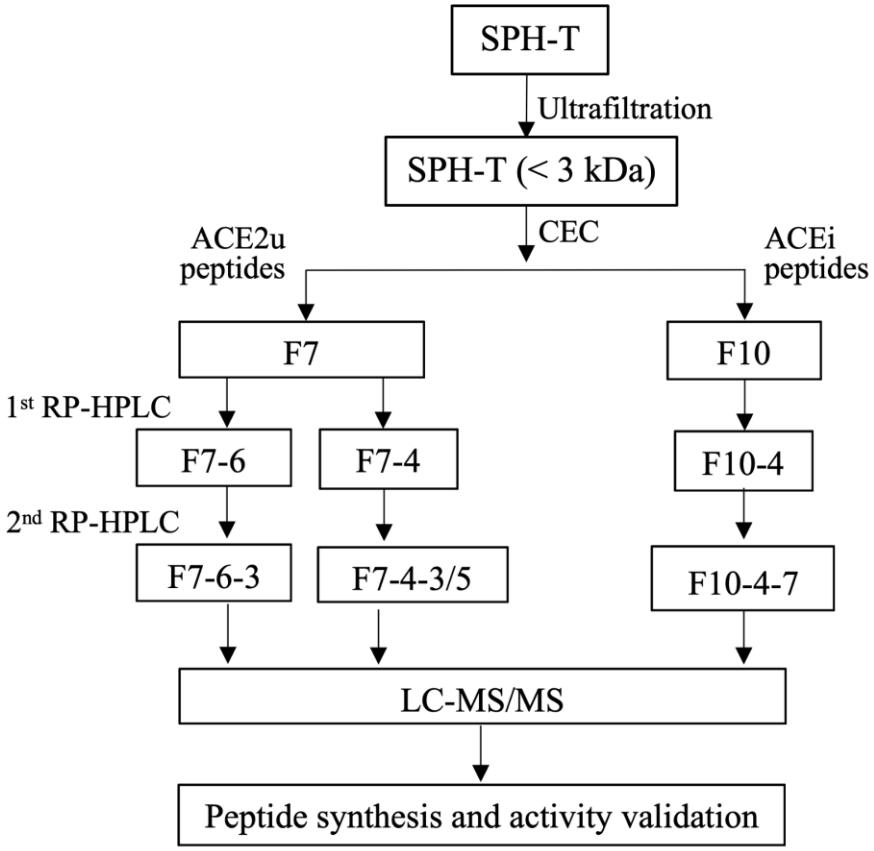


Figure 5.1 Schematic diagram of purification of ACEi and ACE2u peptides from SPH-T. CEC, cation exchange chromatography; LC-MS/MS, liquid chromatography with tandem mass spectrometry; RP-HPLC, reverse-phase high performance liquid chromatography; SPH-T, spent hen muscle protein hydrolysate prepared by thermoase.

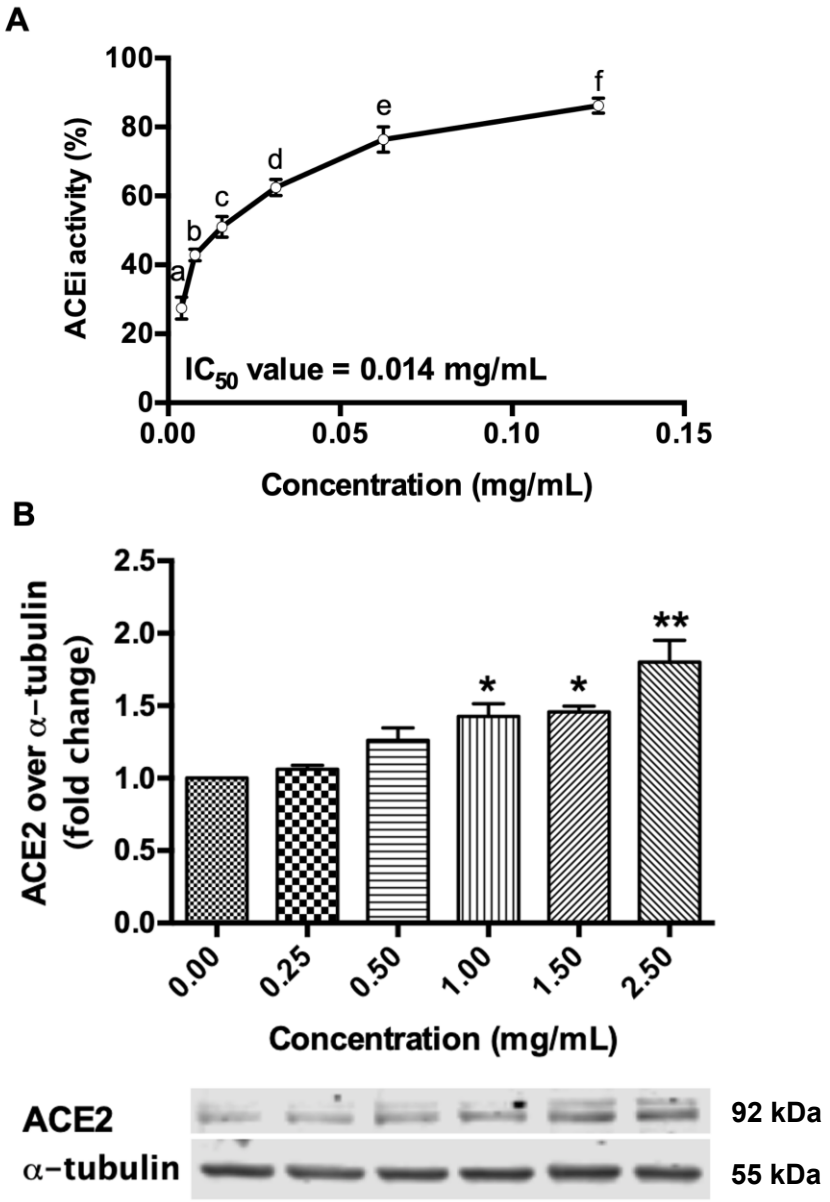


Figure 5.2 ACEi (A) or ACE2u (B) activity of SPH-T (< 3 kDa). ACE2 levels in VSMCs treated with spent hen muscle protein hydrolysate prepared by thermoase (SPH-T) were normalized to that of untreated group (without any treatment). Data were expressed as mean ± SEM of 3-4 independent experiments. Different letters (a-f) indicated a significant difference at $p < 0.05$. *, $p < 0.05$; **, $p < 0.01$, compared with the untreated group.

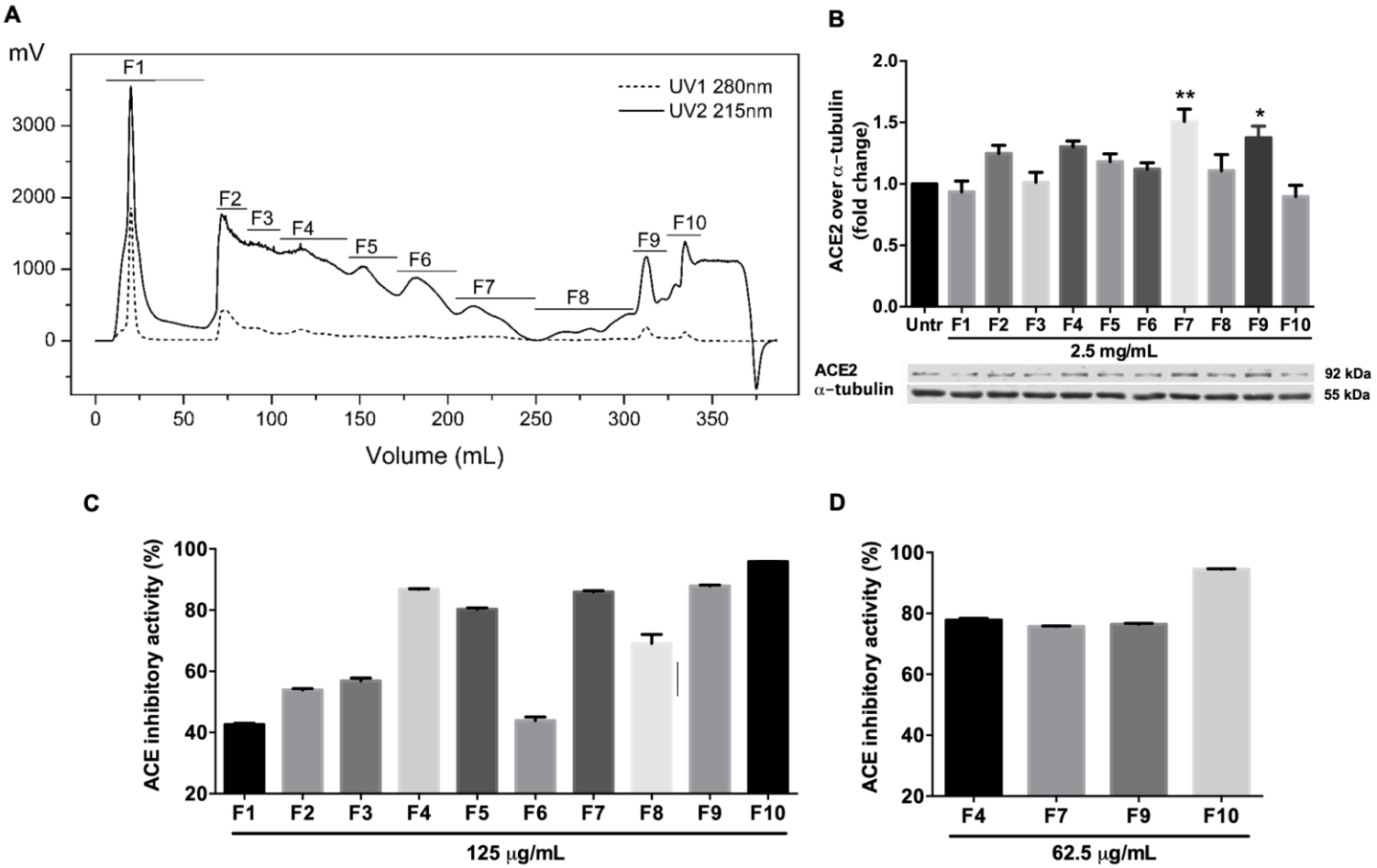


Figure 5.3 Cation exchange chromatogram of SPH-T (**A**) and ACE2u (**B**) or ACEi (**C-D**) activity of the derived fractions. (**B**) ACE2 protein levels (treated with 2.5 mg/mL of SPH-T or its fractions for 24 h) were expressed as fold change over the untreated group (Untr) (n=4-5). *, $p < 0.05$; **, $p < 0.01$, compared with the untreated group. (**C**) ACEi activity was first determined at a concentration of 125 μg/mL (n=3). The most active fractions, F4, F7, F9 and F10 were further determined at a concentration of 62.5 μg/mL (**D**). SPH-T, spent hen muscle protein hydrolysate prepared by thermoase.

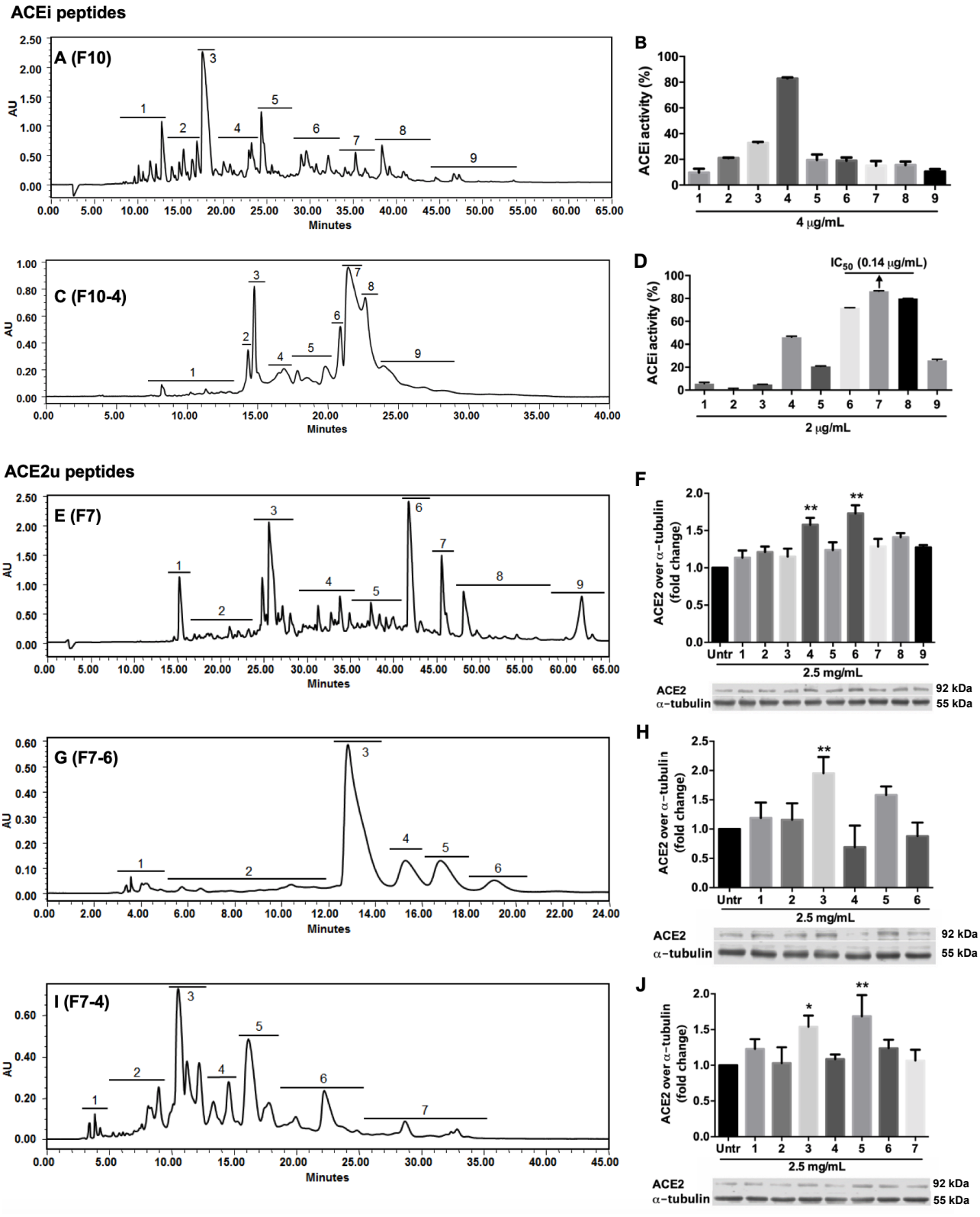
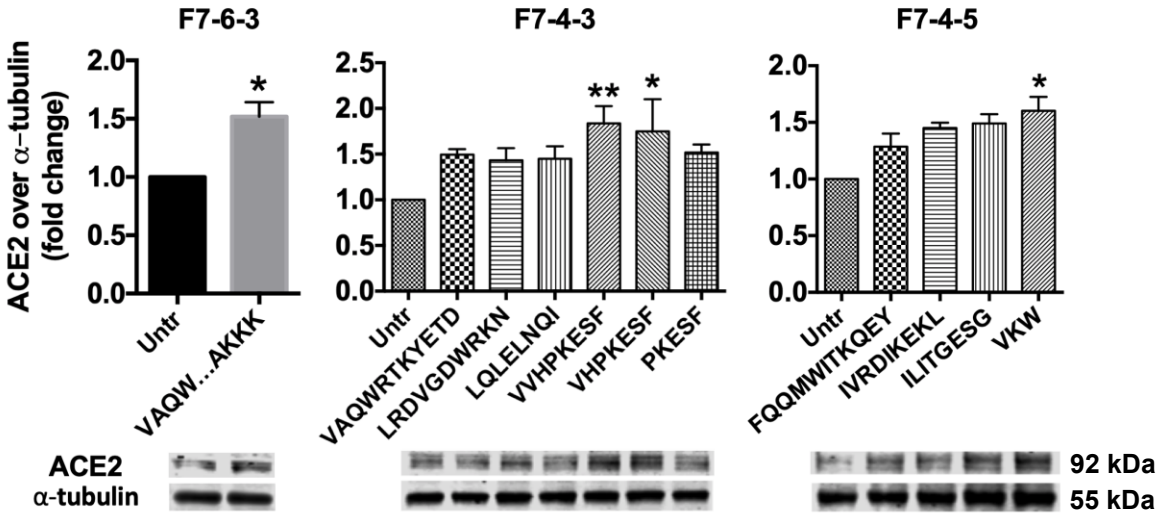

ACEi peptides
A (F10)
AU
Minutes
B
ACEi activity (%)
4 μg/mL
C (F10-4)
D
IC50 (0.14 μg/mL)
ACEi activity (%)
2 μg/mL
ACE2u peptides
E (F7)
F
ACE2 over α-tubulin (fold change)
Untr
2.5 mg/mL
ACE2
α-tubulin
92 kDa
55 kDa
G (F7-6)
H
I (F7-4)
J

Figure 5.4 RP-HPLC fractionation. The active fraction (F10) with ACEi activity was first fractionated on X-bridge preparative C_{18} column (**A**) and ACEi activity of each fraction was determined (**B**); the most active fraction (F10-4) was further fractionated on an X-bridge Analytical C_{18} column (**C**), and ACEi activity of each fraction was determined (**D**) (n=3). Similarly, the active fraction (F7) with ACE2u activity was first fractionated on X-bridge preparative C_{18} column (**E**) and ACE2u activity of each fraction was determined (**F**); the most active fractions (F7-4) and (F7-6) were further fractionated, respectively, on an X-bridge Analytical C_{18} column (**G** and **I**), and the ACE2u activity of each fraction was determined (**H** and **J**). ACE2 levels in VSMCs cells were expressed as fold change over the untreated group (Untr). $*$, $p < 0.05$; $**$, $p < 0.01$, compared to untreated alone. Data were presented as means ± SEM (n=4-5).



Activity validation, molecular weight and protein sources of ACE2u peptides

Fraction	Sequences	ACE2u activity (fold change)	Molecular weight	Parent proteins
		Untreated (1.00)		
F7-6-3	**VAQWRTKYETDA-IQRTEELEEAKKK**	**1.52 ± 0.12**	3051.4	Myosin
F7-4-3	VAQWRTKYETD	1.49 ± 0.06	1396.5	Myosin
	LRDVGDWRKN	1.43 ± 0.13	1258.4	Troponin
	LQLELNQI	1.45 ± 0.14	970.1	Myosin
	VVHPKESF	**1.84 ± 0.19**	941.5	Myosin
	VHPKESF	**1.75 ± 0.35**	842.5	Myosin
	PKESF	1.47 ± 0.10	606.3	Myosin
F7-4-5	FQQMWITKQEY	1.28 ± 0.12	1501.7	Actin
	IVRDIKEKL	1.45 ±0.05	1113.4	Actin
	ILITGESG	1.49 ±0.08	788.9	Myosin
	VKW	**1.60 ±0.12**	431.3	Titin

Figure 5.5 ACE2 level in VSMCs treated with peptides identified from F7-6-3, F7-4-3, and F7-4-5. Cells were treated with peptides (50 μM) for 24 h and ACE2 expressions were normalized to the untreated group (Untr). Data were expressed as means ± SEMs (n=3-4). *, $p < 0.05$; **, $p < 0.01$, compared with the untreated group. The inserted table summarized ACE2u activity, molecular weight, and protein source of the validated peptides (those significantly upregulating ACE2 expression were marked in bold).

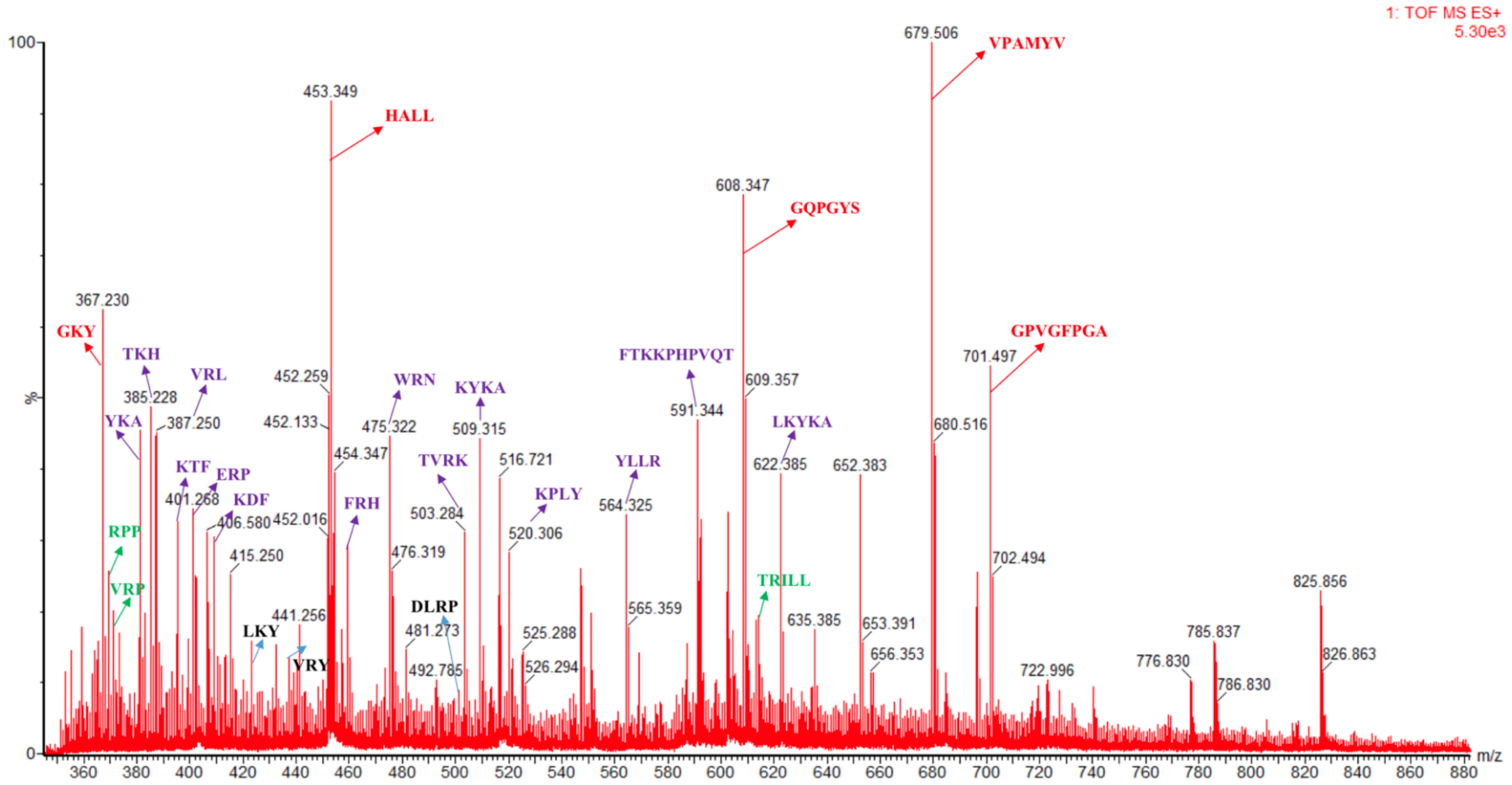


Figure 5.6 Mass spectrum of F10-4-7 from SPH-T (ACEi peptides).

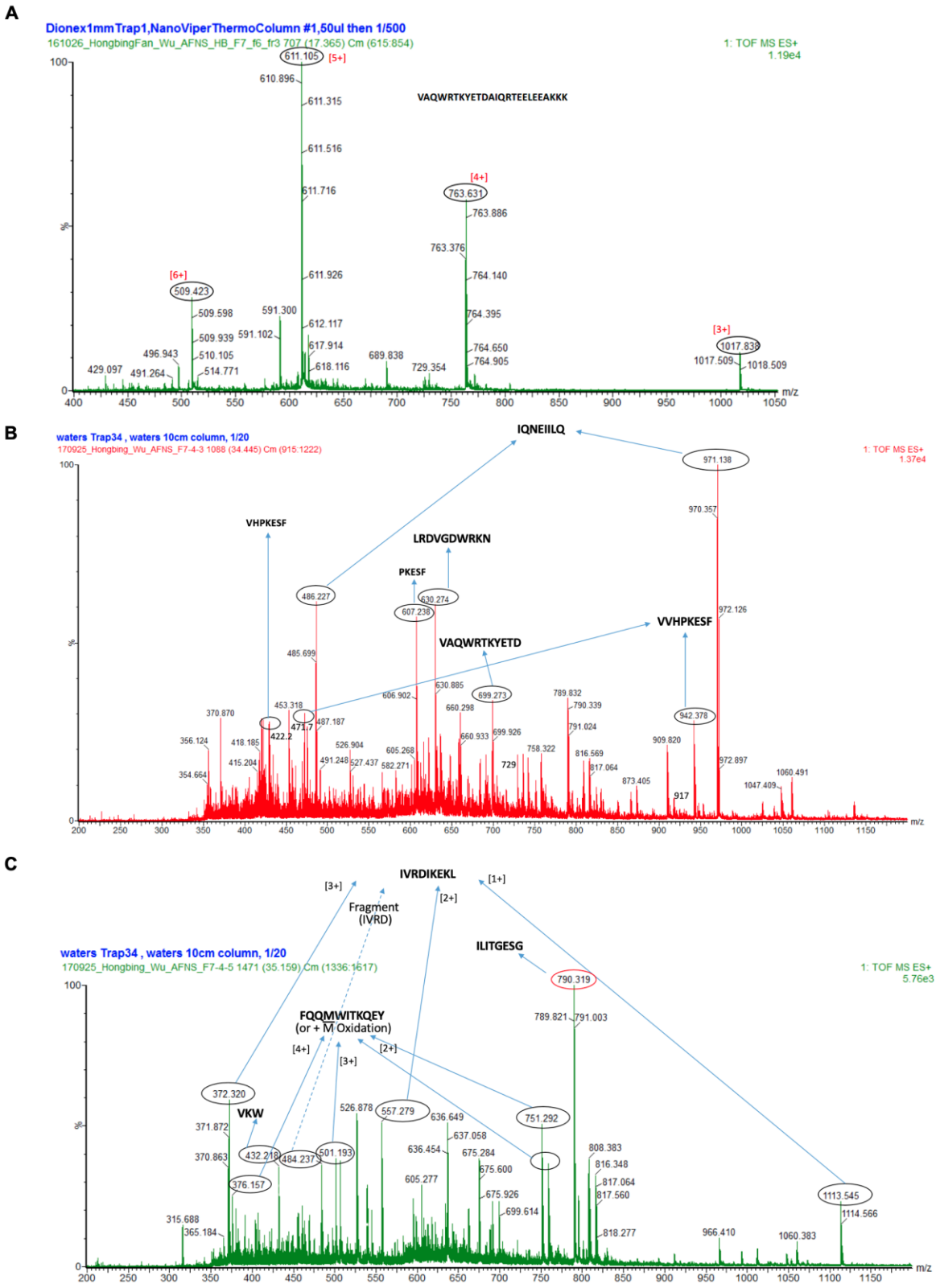
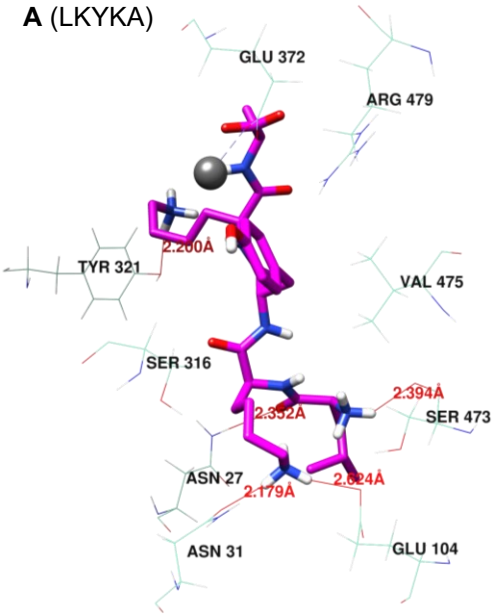
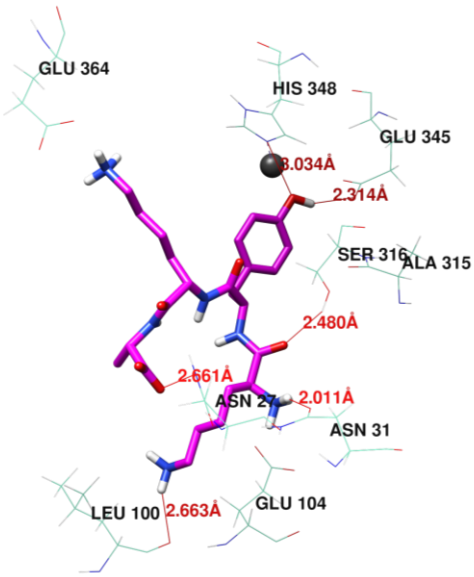


Figure 5.7 Mass spectra of F7-6-3 (**A**), F7-4-3 (**B**), and F7-4-5 (**C**) from SPH-T (ACE2u peptides).

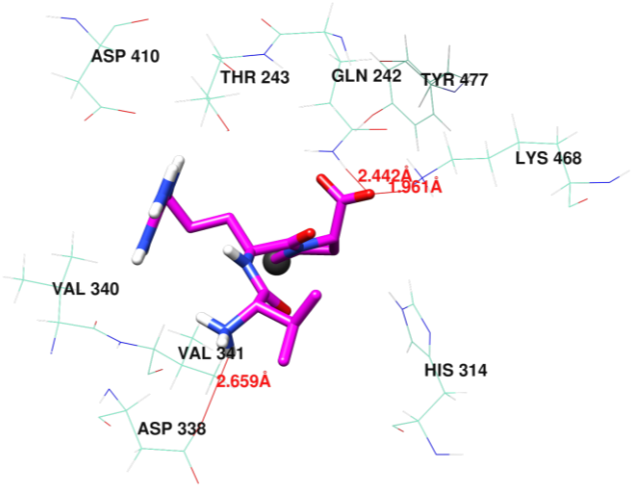
A (LKYKA)



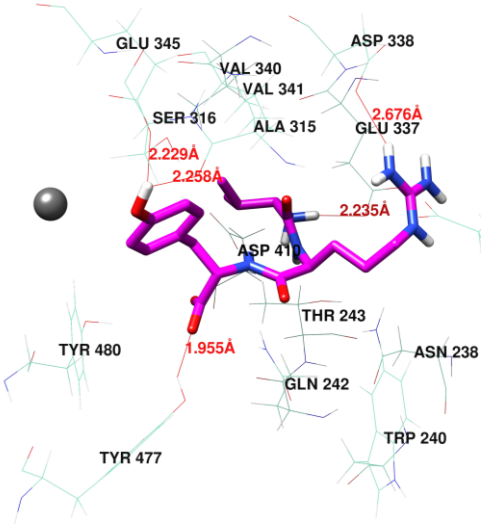
D (KYKA)



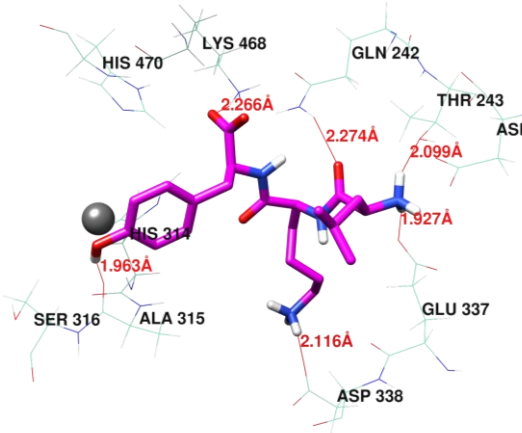
B (VRP)



E (VRY)



C (LKY)



ACEi Peptides	IC_{50} (μg/mL)	Grid Score	H-bonds	
			number	tACE residues
LKYKA	0.034	-88.43	5	N27, N31, E104, Y321, S473
VRP	0.64	-82.50	3	Q242, D338, K468
LKY	0.81	-82.26	6	Q242, T243, S316, E337, D338, K468
KYKA	2.87	-80.64	6	N27, N31, L100, S316, H348, E345,
VRY	5.77	-78.75	5	A315, S316, E337, D338, Y477

Figure 5.8 Predicted binding modes of the identified ACEi peptides with tACE (PDB: 1O86). The highest ranked docking pose of each peptide is presented. Peptides were docked at the active site of tACE. Peptides are shown in stick model, with backbone, nitrogen, oxygen, and hydrogen shown in magenta, blue, red, and white, respectively, while tACE is showed in wire model, with backbone, nitrogen, oxygen, and hydrogen shown in aquamarine, blue, red, and white, respectively. H-bonds between peptides and tACE are shown in red lines with distances. Zinc was presented in gray. The inserted table summarized the bind affinity (grid score), number of H-bonds formed, and tACE residues that were interacted with peptides.

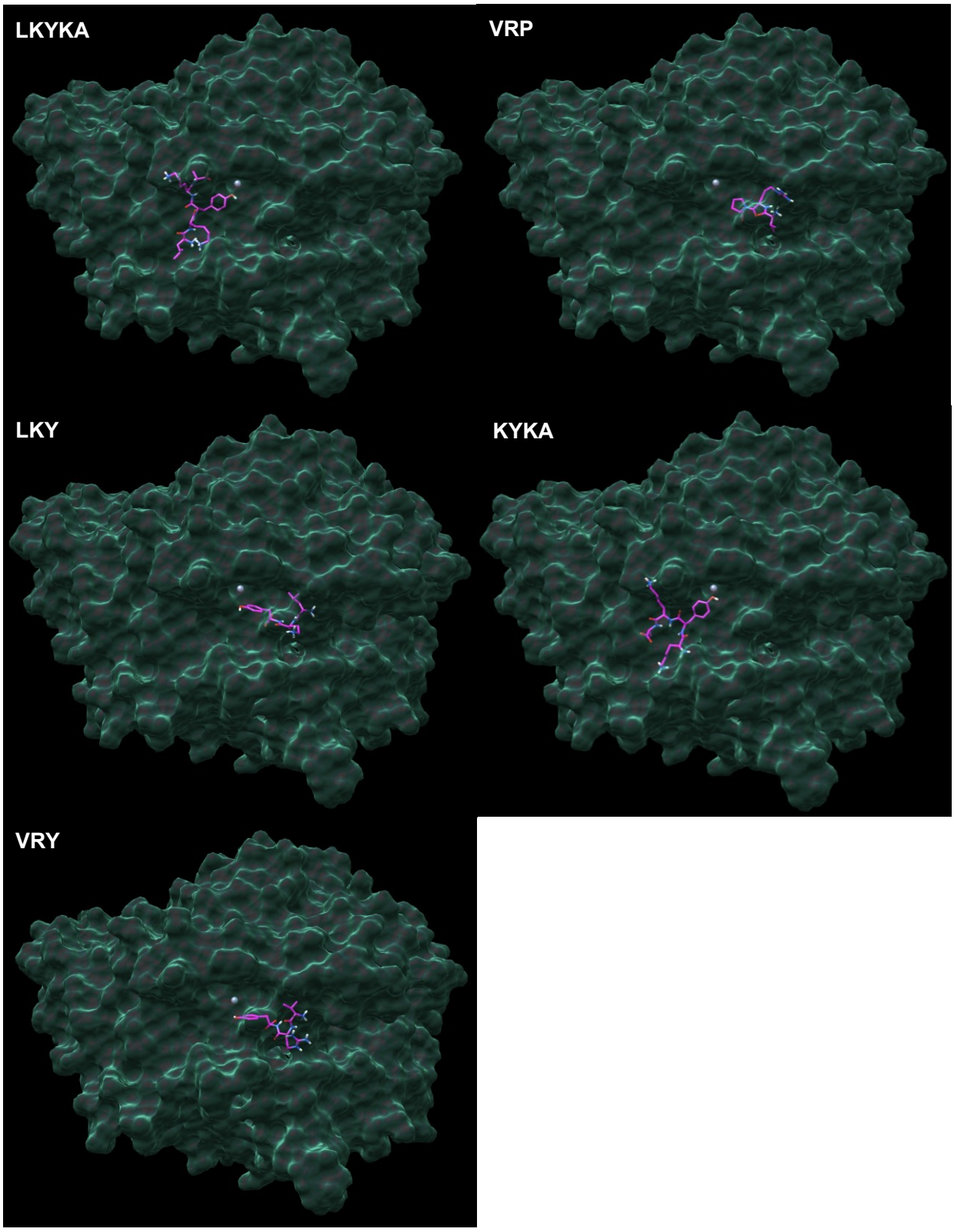

LKYKA
VRP
LKY
KYKA
VRY

Figure 5.9 Stereo positions of ACEi peptides interacted with tACE (PDB: 1086). tACE was shown in surface mode; peptides were shown in stick mode, with C-C backbone, oxygen, nitrogen, hydrogen shown in magenta, red, blue, and white, respectively; zinc was shown in gray.

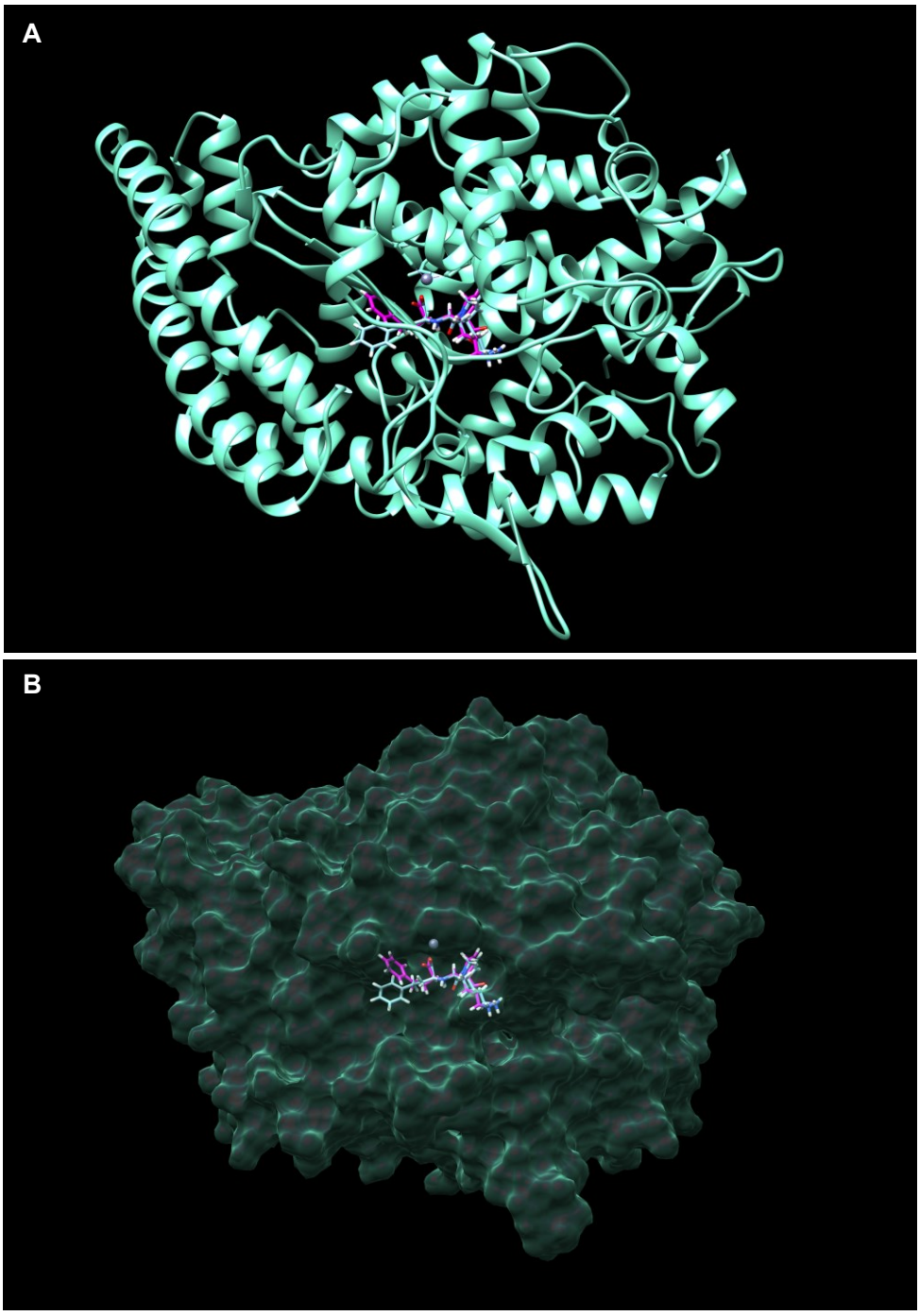
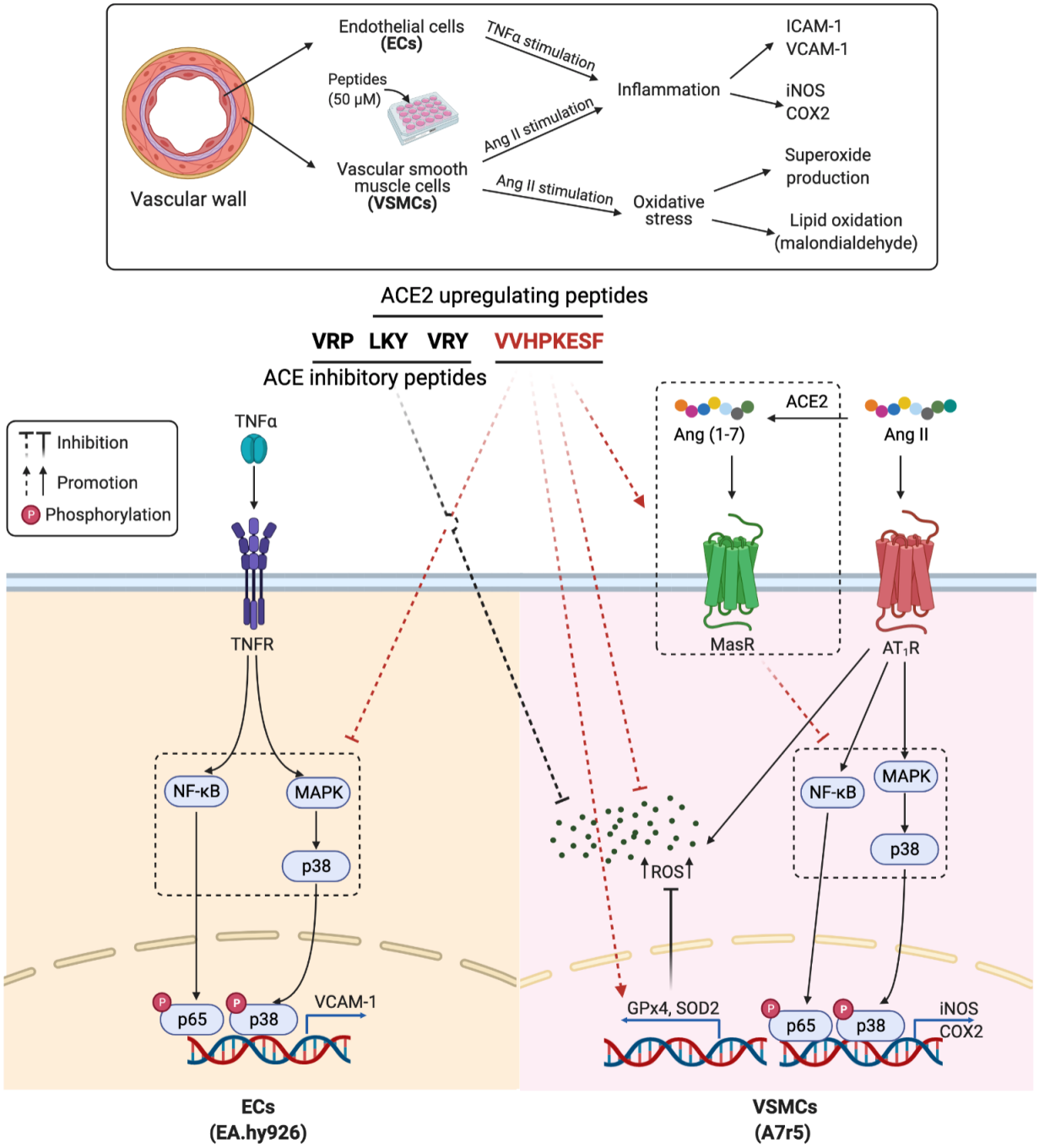


Figure 5.10 Molecular docking between lisinopril and tACE (PDB: 1O86). tACE was either presented in ribbon (**A**) or surface mode (**B**). Lisinopril obtained from PDB (1O86): backbone, aquamarine; oxygen, red; nitrogen, blue; white hydrogen. Lisinopril obtained from docking: backbone, magenta; oxygen, red; nitrogen, blue; white hydrogen. The grid score between lisinopril and tACE is –87.25 from Dock 6.8.

CHAPTER 6 – Antioxidant and Anti-inflammatory Effects of Spent Hen Muscle Protein-Derived ACE Inhibitory Peptides and ACE2 Upregulating Peptides in Vascular Cells [5]

Graphical abstract



[5] Part of this chapter (antioxidant study) has been published in Fan, H., Bhullar, Khushwant S., & Wu, J. Spent hen muscle protein-derived RAS regulating peptides show antioxidant activity in vascular cells. *Antioxidants*, 2021, *10*(2), 290. The rest will be submitted to the *Journal of Agricultural and Food Chemistry* for consideration of publication.

6.1 Introduction

Despite the complicated pathogenesis of hypertension, the hyperactive renin-angiotensin system (RAS), endothelial dysfunction, inflammation, and oxidative stress are identified as important pathological contributors (Dinh et al., 2014; Savoia et al., 2011; Touyz, 2000; Unger, 2002). Within the vasculature, excessive oxidative stress and inflammation result in many cardiovascular diseases including hypertension (Touyz & Schiffrin, 2004). Anatomically, vascular smooth muscle cells (VSMCs) and endothelial cells (ECs) are two major components of the vascular wall, both with the ability to develop inflammation and oxidative stress upon stimulation by various cytokines such as angiotensin (Ang) II and tumor necrosis factor alpha (TNFα) (Lilly, 2014; Touyz & Schiffrin, 2004). Ang II stimulates inflammation and oxidative stress via Ang II type 1 receptor (AT_1R), while TNFα stimulates both mainly via TNFα receptors (Blaser et al., 2016; Touyz & Schiffrin, 2000). Genetic models of hypertension such as spontaneously hypertensive rats (SHR) are reported to have elevated level of inflammation and oxidative stress, while supplementation with various agents such as α-tocopherol, ascorbic acid, and resveratrol significantly attenuate vascular inflammation and oxidative stress, improve vascular function, and prevent the development of hypertension (Chen et al., 2001; Fleenor et al., 2012; Zalba et al., 2000). Likewise, food-derived peptides have shown abilities in ameliorating inflammation and oxidative stress in various cell and animal models of hypertension (Yi et al., 2020; Tsai et al., 2020; Esfandi, Walters, & Tsopmo, 2019; Son, Chan, & Wu, 2018; Wang et al., 2020).

In Chapter 5, four spent hen peptides have been selected with great ACE inhibitory (ACEi) (VRP, LKY, and VRY) or ACE2 upregulating (ACE2u) activity (LKY, VRY, and VVHPKESF (V-F)). Before being assessed in SHR for their antihypertensive effects, we intended to first study their antioxidant and anti-inflammatory effects in two vascular cell lines, VSMCs (A7r5) and ECs

(EA.hy926), given the contribution of vascular inflammation and oxidative stress to the development of vascular dysfunction and hypertension (Steven et al., 2019). The antioxidant effect was assessed in VSMCs, while the anti-inflammatory effect was evaluated in both VSMCs and ECs.

6.2 Materials and Methods

6.2.1 Materials

Peptides including VRP, LKY, VRY, and V-F (purity > 98%) were synthesized by Genscript Corp (Piscataway, NJ, USA). Ang II, dithiothreitol (DTT), and Triton-X-100 were obtained from Sigma Aldrich (St Louis, MO, USA). TNFα and A779 were purchased from R&D Systems (Minneapolis, MN, USA). Losartan potassium and dihydroethidium (DHE) were obtained from Tocris (Oakville, ON, Canada) and Biotium (Fremont, CA, USA), respectively. Dulbecco's modified Eagle's medium (DMEM), 0.25% (w/v) trypsin-0.53 mM EDTA, fetal bovine serum (FBS), 4-(2−68 hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), nonessential amino acids (NEAA), and penicillin−streptomycin were obtained from Gibco Invitrogen (Burlington, ON, Canada). Human endothelial EA.hy926 cells (CRL-2922) and rat vascular smooth muscle A7r5 cells (CRL-1444) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA).

6.2.2 Cell culture of vascular smooth muscle A7r5 cells and endothelial EA.hy926 cells

Both VSMCs (passages 4-11) and ECs (passages 3-10) were grown in DMEM containing 10% FBS, 25 mM HEPES, and 1% penicillin-streptomycin at 37 °C in a 100% humidified atmosphere with 5% CO_2, but for ECs, NEAA (1%) was added as a supplement. The growth media were changed every three days for both cells.

6.2.3 Western blotting

The confluent cells were placed in a quiescing medium (the same recipe as that of the growth medium but with 1% FBS). VSMCs were treated with peptides (50 µM) for 1 h before adding Ang II (1 µM) for a co-treatment period of 23 h for detection of AT_1R, MasR, inducible nitric oxide synthase (iNOS), and cyclooxygenase 2 (COX2); AT_1R antagonist losartan potassium (50 µM) or MasR antagonist A779 (1 µM) might be added with peptides. ECs were treated with peptides (50 µM) for 18 h before adding 10 ng/mL of TNFα for a co-treatment of another 6 h for detection of intracellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1). For evaluating the involvement of intracellular signaling, VSMCs (or ECs) were treated with peptides for 1 h (or 18 h) before adding 1 µM of Ang II (or 10 ng/mL of TNFα) for a co-treatment period of 15 min for detection of nuclear factor kappa B (NF-κB) and mitogen-activated protein kinases (MAPK) including extracellular signal-regulated kinases 1/2 (ERK1/2), p38 MAPK, and c-Jun N-terminal kinases (JNK). Peptide concentrations and time of treatment were selected based on previous studies (Liao et al., 2016; Majumder et al., 2013a). Cells were lysed in a boiling Laemmle's buffer containing 50 mM DTT and 0.2% Triton-X-100.

Cell lysates were loaded onto a 9% separating gel and transferred to a nitrocellulose membrane (diameter 0.45 µm, Bio-Rad, Montreal, QC, Canada) for incubation with specific primary antibodies. Protein bands of superoxide dismutase 2 (SOD2; Abcam, Toronto, ON, Canada), glutathione peroxidase 4 (GPx4; Abcam), iNOS (BD Biosciences, San Jose, CA, USA), COX2 (Abcam), AT_1R (Invitrogen, Waltham, MA, USA), MasR (Novus Biologicals, Toronto, ON, CA), ICAM-1 (Santa Cruz, Dallas, TX, USA) and VCAM-1 (Santa Cruz, Dallas, TX, USA) were normalized to α-tubulin (Abcam) or glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Abcam). Protein bands of p65, ERK1/2, p38, and JNK were normalized to their total forms (Cell

Signaling Technology, Danvers, MA, USA). Donkey-anti-rabbit 800 CW or donkey-anti-mouse IRDye 680 RD secondary antibodies (Licor Biosciences, Lincoln, NE, USA) were used for visualization of the fluorescent bands using a Licor Odyssey BioImager; fluorescent signals were quantified using Image Studio Lite 5.2 (Licor Biosciences).

6.2.4 Superoxide detection

Superoxide generation in VSMCs was detected by DHE staining. VSMCs were treated with peptides (50 μM) for 1 h before the addition of 1 μM of Ang II for 30 min. Then, DHE (20 μM) was added and incubated with cells for 30 min (protected from light). After three times of washing with a non-phenol-red DMEM (21063029, Thermo Fisher Scientific, Burlington, ON, Canada), the fluorescence signal was detected by an Olympus IX81 fluorescent microscope (Olympus, Tokyo, Japan). Each data point was taken from three random fields. The total fluorescence intensity in each field was quantified using ImageJ software (https://imagej.net/Welcome) and the mean fluorescence intensity per cell (MFI/cell) was determined. The untreated group (control) was without any peptide or Ang II treatment.

6.2.5 Lipid peroxidation assay

Determination of malondialdehyde in cells was performed according to the manufacturer's instructions of the lipid peroxidation assay kit provided by Abcam. Briefly, cells were seeded at a concentration of $2x10^6$ cells in a 96-well plate and allowed to reach ~70% confluency before treatment. VSMCs were treated with peptides (50 μM) for 24 h followed by oxidative stress using 1 μM of Ang II for 30 min respectively. After the treatment, cells were washed twice with cold PBS, and homogenized in 300-μL lysis solution per well (with butylated hydroxytoluene). Samples were centrifuged at 13,000 × g for 10 min to collect the supernatant. The 200 μL of

supernatant was transferred to a fresh tube and 600 µL of thiobarbituric acid reagent was added. This mixture-containing tube was incubated at 95°C for 60 minutes and then immediately cooled to room temperature in an ice bath for 10 mins. After cooling, 200 µL of supernatant was added to a 96-well plate and the absorbance was detected immediately on a microplate reader at 532 nm. The final results were presented as malondialdehyde (nmol/mL).

6.2.6 Cytotoxicity

Cytotoxicity of peptides against VSMCs and ECs followed the alamarBlue assay depicted in Fan et al. (2018b). Cells were seeded on a 96-well plate at 1.0×10^4 cells/well. After reaching 80% of confluency, cells were treated with peptides (100 µM) dissolved in culture medium) for 24 h. Then, culture media were replaced with 200 µL of 10% alamarBlue solution (dissolved in culture medium) for 4 h of incubation (protected from light), after which 150 µL was transferred into an opaque 96 well plate for detection of the fluorescence signal, with emission and excitation wavelengths at 590 nm and 560 nm, respectively. The control was without any peptide treatment.

6.2.7 Statistical analysis

Data from the cell study were expressed as mean ± standard error (SEM) and were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple test using GraphPad Prism version 6 (San Diego, CA, USA). A value of $p < 0.05$ was considered significant.

6.3 Results

6.3.1 Cytotoxicity of VRP, LKY, VRY, and VVHPKESF (V-F) in vascular smooth muscle A7r5 cells and endothelial EA.hy926 cells

Prior to assessing antioxidant and anti-inflammatory activities of the four peptides in both cells, their effects on cell viability were determined (Figure 6.1). Incubation of peptides for 24 h at 100 μM, higher than 50 μM used in later experiments, indicated no cytotoxicity against VSMCs and ECs in this study.

6.3.2 Effect of VRP, LKY, VRY, and V-F on oxidative stress and inflammation in vascular smooth muscle A7r5 cells

Antioxidant effects of VRP, LKY, VRY, and V-F were evaluated in Ang II-stimulated VSMCs (Figure 6.2). All four peptides reduced oxidative stress with lower superoxide and malondialdehyde formations after peptide treatment (Figure 6.2A-B); only V-F activated expressions of antioxidant enzymes (SOD2 and GPx4) (Figure 6.2C-D). Among these peptides, only V-F mitigated Ang II-induced inflammation in VSMCs ($p < 0.05$), reflecting inhibition of iNOS and COX2 expressions (Figure 6.3).

6.3.3 Effect of VRP, LKY, VRY, and V-F on intracellular signaling pathways in vascular smooth muscle A7r5 cells

We further studied the effects of VRP, LKY, VRY, and V-F on modulating the intracellular signaling including NF-κB and MAPKs (p38 MAPK, ERK1/2, and JNK) pathways (Figure 6.4). Phosphorylation of NF-κB p65 and p38 MAPK, not of ERK1/2 and JNK, were inhibited by the treatment with V-F, indicating its regulatory roles in modulating intracellular signaling events and

thus contributing to its anti-inflammatory activity in Ang II-stimulated VSMCs. VRP, LKY, and VRY showed no effect on regulating the above signaling events.

6.3.4 Effect of VRP, LKY, VRY, and V-F on AT_1R and MasR expressions in vascular smooth muscle A7r5 cells

Ang II induces inflammation in VSMCs mainly via AT_1R, whereas ACE2 degrades Ang II to Ang (1-7) that binds with MasR and counterbalances the harmful effects of Ang II signaling. As shown in Figure 6.5, VRY and V-F decreased AT_1R expression while only V-F increased MasR expression in VSMCs ($p < 0.05$).

6.3.5 Involvement of AT_1R and MasR in the anti-inflammatory effect of V-F in vascular smooth muscle A7r5 cells

Only V-F attenuated inflammation in Ang II-stimulated VSMCs, therefore losartan potassium (AT_1R antagonist) and A779 (MasR antagonist) were added with V-F before treatment with Ang II to evaluate the involvement of AT_1R and MasR in the anti-inflammatory activity of V-F in VSMCs. Results showed that the addition of A779, not losartan potassium, significantly abolished Ang II-induced upregulation of iNOS and COX2 in VSMCs (Figure 6.6).

6.3.6 Effects of VRP, LKY, VRY, and V-F on inflammation and intracellular signaling pathways in endothelial EA.hy926 cells

Effects of VRP, LKY, VRY, and V-F on TNFα-stimulated inflammation in ECs were evaluated. TNFα treatment remarkably upregulated expressions of ICAM-1 and VCAM-1 in ECs ($p < 0.001$); only V-F reduced TNFα-stimulated upregulation of VCAM-1 ($p < 0.01$), and also appeared to reduce that of ICAM-1 but not being significantly ($p = 0.08$) (Figures 6.7). Similar to

the findings of V-F against Ang II-stimulated inflammation in VSMCs, it inhibited phosphorylation of NF-κB p65 and p38 MAPK, not ERK1/2 and JNK, indicating that its anti-inflammatory effect might be mediated by NF-κB and p38 MAPK pathways.

6.4 Discussion

VSMCs (A7r5) and ECs (EA.hy926) are two commonly-used cellular models for evaluating the antioxidant and anti-inflammatory effects of bioactive peptides (Fan et al., 2018a). The identified spent hen peptides (VRP, LKY, VRY, and V-F) were further assessed for their antioxidant and anti-inflammatory activities in Ang II-stimulated VSMCs and TNFα-induced ECs, as well as the possible intracellular signaling pathways involved. Prior to the treatment, cytotoxicity of these peptides in both cells was studied and none of them showed negative effects on cell viability at a concentration of 100 μM, which was higher than that used in this study (50 μM) (Figure 6.1).

Not surprisingly, all four peptides diminished Ang II-induced oxidative stress in VSMCs (Figure 6.2A-B). As reported, the presence of H and aromatic or cyclic amino acid residues, such as F, Y, and P, as well as N-terminal hydrophobic amino acid residues, such as V and L, contribute to a peptide's antioxidant activity (Chen et al., 1998; You et al., 2010). The imidazole ring of H and the aromatic ring scavenge the radicals by donating a proton, and thus terminate the radical reaction chain. These amino acid residues constitute approximately two-thirds of the peptide chains in this study (VRP, LKY, VRY, and VVHPKESF), and might contribute to their ability to quench reactive oxygen species (ROS). Quite a few peptides have been reported to exert antioxidant effect by activating antioxidant enzymes (Du et al., 2016; Tsai et al., 2020; Yi et al., 2020). Among the four peptides, only V-F upregulated the expression of SOD2 and GPx4, two important endogenous antioxidant enzymes that transform ROS into stable and less reactive

molecules and thereby protect cells against oxidative damages (Figure 6.2C-D). Unlike the antioxidant effect exhibited by the four peptides, only V-F attenuated inflammation (iNOS, COX2, and VCAM-1) in Ang II/TNFα-stimulated VSMCs/ECs (Figure 6.3 and Figure 6.7). ROS are implicated in the development of various inflammatory responses, thus a lack of association between antioxidant and anti-inflammatory activities of these peptides indicated different mechanisms involved (Majumder et al., 2013a; Ruiz-Ortega et al., 2000). Many antioxidant and anti-inflammatory peptides have been reported in the literature (Chakrabarti, Jahandideh, & Wu, 2014; Du et al., 2016; Gu et al., 2019; Gu & Wu, 2016; Yi et al., 2020). Two egg white-derived antihypertensive peptides, IRW and IQW, reduced oxidative stress and inflammation in TNFα-stimulated ECs; the former also demonstrated these activities in Ang II-stimulated VSMCs and skeletal muscle cells as well as in lipopolysaccharide-induced RAW 264.7 Macrophages (Liao et al., 2016; Shang & Wu, 2020; Son, Chan, & Wu, 2018). Two milk-derived peptides, VPP and IPP, and a soybean-derived peptide, LSW, reduced Ang II-stimulated inflammation and oxidative stress in VSMCs; VPP inhibited both inflammation and oxidative stress while IPP and LSW reduced only oxidative stress or inflammation, respectively (Chakrabarti et al., 2017; Lin et al., 2017). To understand possible mechanisms of action underlying the protective roles of spent hen peptides in vascular cells, we further investigated their regulatory effects on intracellular signaling pathways.

In VSMCs, Ang II binds with AT_1R, resulting in many pathological responses such as inflammation, oxidative stress, migration, and apoptosis, among others (Clempus & Griendling, 2006; Griendling et al., 1994; Touyz & Schiffrin, 2000). As a protective arm of the RAS, ACE2 degrades Ang II to Ang (1-7) that binds with MasR and counteracts the harmful effects caused by the Ang II-AT_1R axis (Patel et al., 2014). Hence, in VSMCs, antioxidant and anti-inflammatory effects of bioactive peptides could be mediated by preventing Ang II from binding with AT_1R, or

by promoting binding of Ang (1-7) with MasR through elevated Ang (1-7) or upregulated MasR level (Liao et al., 2018; Wang et al., 2020). For example, IRW attenuated vascular oxidative stress, inflammation, and migration through upregulating ACE2 and MasR expressions in Ang II-stimulated VSMCs; it also reduced BP in SHR through activating the ACE2-Ang (1-7)-MasR axis (Liao et al., 2016; Liao et al., 2019; Liao et al., 2018). LRW modulated Ang II-induced dysfunction in VSMCs likely through upregulating the ACE2-Ang-(1−7)-MasR axis (Alghamri et al., 2013; Wang et al., 2020). Other peptides such as IPP, LY, RALP, GHS, IQP, and VEP have demonstrated a correlation between their antihypertensive effects and upregulated plasma or tissue ACE2 level in SHR (Ehlers et al., 2011; He et al., 2019; Pan et al., 2015; Zheng et al., 2017). In our study, V-F attenuated Ang II-induced inflammation in VSMCs (Figure 6.3). By elucidating the contribution of AT_1R or MasR through adding AT_1R or MasR antagonist, respectively, we found that the anti-inflammatory action of V-F was dependent on MasR, not AT_1R (Figure 6.6). This might be due to its ability in upregulating both ACE2 and MasR expressions and therefore inhibited Ang II-induced upregulation of iNOS and COX2. Of particular note is that LKY and VRY were also ACE2u peptides but were not able to inhibit inflammation in VSMCs upon Ang II stimulation. One possible explanation for this variation was the upregulated MasR expression by V-F in the meantime, but this speculation remains to be validated until more ACE2u peptides are investigated and characterized in the near future.

The NF-κB pathway is highly correlated with the generation of proinflammatory cytokines (such as iNOS, COX2, VCAM-1, and ICAM-1) and serves as a pivotal mediator of inflammatory responses (Tak & Firestein, 2001). Prior to stimulation, NF-κB is normally restricted in the cytoplasm and inactive; once being stimulated by certain cytokines, NF-κB members such as p65 are subsequently translocated into the nucleus and activate transcription of proinflammatory genes

(Liu et al., 2017). The MAPKs, including p38 MAPK, ERK1/2, and JNK, are also involved in Ang II- or TNFα-mediated intracellular signaling events (MacEwan, 2002; Touyz et al., 2000). We found that phosphorylation of NF-κB p65 and p38 MAPK, not of ERK1/2 and JNK, was significantly inhibited by V-F, which implied that its modulatory effect might be mediated by NF-κB and p38 MAPK pathways (Figure 6.4 and 6.8). These results were aligned with the findings that only V-F ameliorated inflammation in VSMCs and ECs (Figures 6.3 and 6.7). Many peptides have been reported to modulate MAPK and NF-κB pathways underlying their cellular anti-inflammatory actions. For instance, LSW, reduced inflammation in Ang II-induced VSMCs, through blunting phosphorylation of ERK1/2 and NF-κB p50 (Lin et al., 2017). IRW inhibited lipopolysaccharide-induced inflammatory responses in RAW 264.7 macrophages via inhibiting ERK1/2 and NF-κB p65 pathways (Shang et al., 2020). Besides, IRW protected VSMCs against Ang II stress by inhibiting the phosphorylation of p38 MAPK and NF-κB p65. Furthermore, IRW reduced TNFα-stimulated inflammation via both NF-κB p65 and p50 in ECs (Liao et al., 2016; Liao et al., 2018; Majumder et al., 2013a), but IQW, which differs in only one amino acid residue from IRW, inhibited NF-κB p50 but not p65 (Majumder et al., 2013a). These findings indicated the regulatory roles of bioactive peptides against inflammation, but these regulatory effects were likely through multiple mechanisms. Nevertheless, our results demonstrated that V-F reduced vascular inflammation, being likely mediated by inhibiting NF-κB p65 or p38 MAPK pathways, but which one plays the major role awaits further clarification.

In conclusion, this study demonstrated that the identified spent hen peptides could reduce vascular inflammation (V-F) and oxidative stress (VRP, LKY, VRY, and V-F) at the cellular level, in addition to their ACEi (VRP, LKY, and VRY) or ACE2u (LKY, VRY, and V-F) activities that were initially characterized in Chapter 5. All the four peptides showed antioxidant activity, with

VRP, LKY, and VRY acting as direct ROS scavengers whereas V-F also activating endogenous antioxidant enzymes. Only V-F showed an anti-inflammatory effect, being partially dependent on MasR and possibly involving modulation of NF-κB p65 and p38 MAPK pathways. Among a handful of ACE2u peptides reported in the literature, only two (IRW and LRW) appeared to reduce Ang II-induced inflammation in VSMCs through activating the ACE2-Ang (1-7)-MasR axis; however, two ACE2u peptides in our study, LKY and VRY, did not show any effect, indicating a gap between ACE2 upregulation and activation of ACE2-Ang (1-7)-MasR axis and that modulation of MasR might also contribute. The protective roles of VRP, LKY, VRY, and V-F in ameliorating cytokine-stimulated vascular inflammation or oxidative stress in vascular cells may contribute to BP regulation in SHR.

6.5 References

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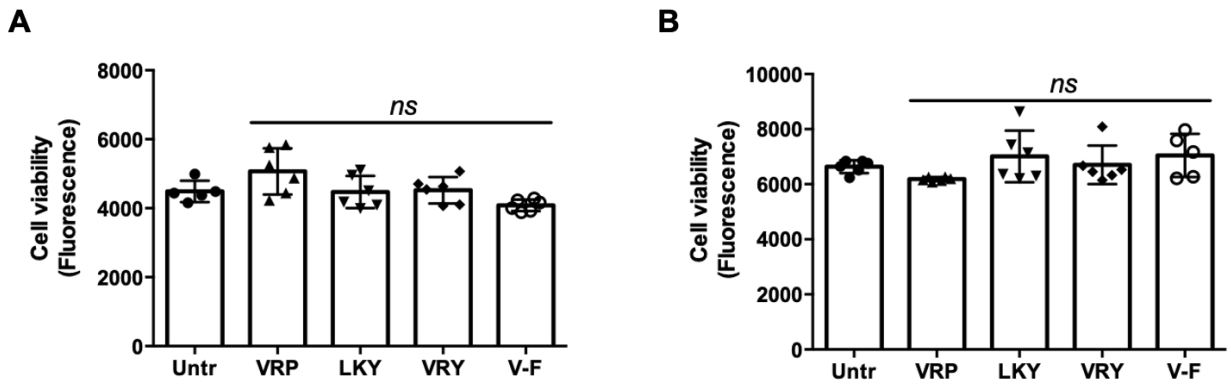


Figure 6.1 Cytotoxicity of VRP, LKY, VRY, and VVHPKESF (V-F) in VSMCs (**A**) and ECs (**B**). Both cells were treated with 100 μM of peptides for 24 hours, followed by alamarBlue® cell viability assay. Data were expressed as means ± SEMs of 5-6 independent experiments. ns, not significant.

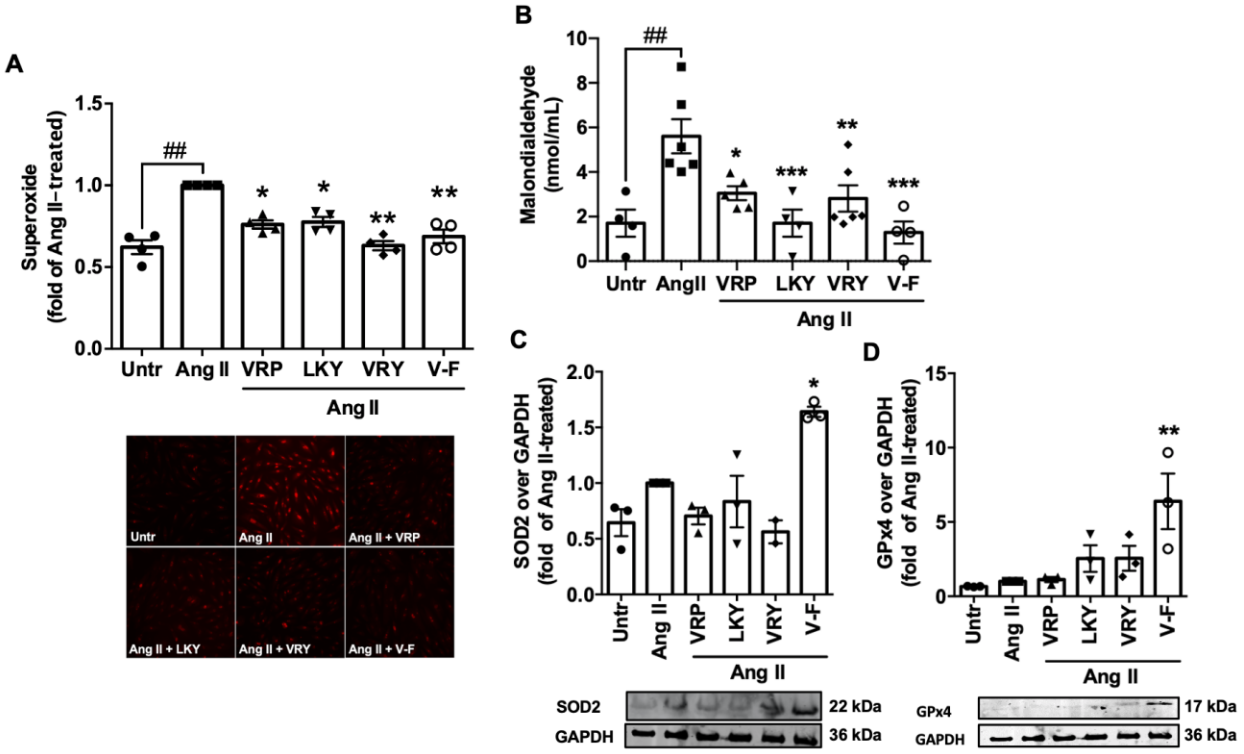


Figure 6.2 Effect of VRP, LKY, VRY, and VVHPKESF (V-F) on oxidative stress in Ang II-stimulated VSMCs. (**A**) Effect of peptides on superoxide generation; cells were treated with 50 μM of peptides for 1 h prior to treatment with 1 μM of Ang II for 30 min, followed by treatment with 20 μM of DHE for 30 min. (**B-D**) Effect of peptides on formation of malondialdehyde and expressions of two antioxidant enzymes: SOD2 and GPx4; cells were treated with 50 μM of peptides for 24 h prior to treatment with 1 μM of Ang II for 30 min. Data were expressed as mean ± SEM and normalized to the Ang II-treated group. ##, $p < 0.01$, compared to the untreated group (Untr); *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$, compared to Ang II-treated group.

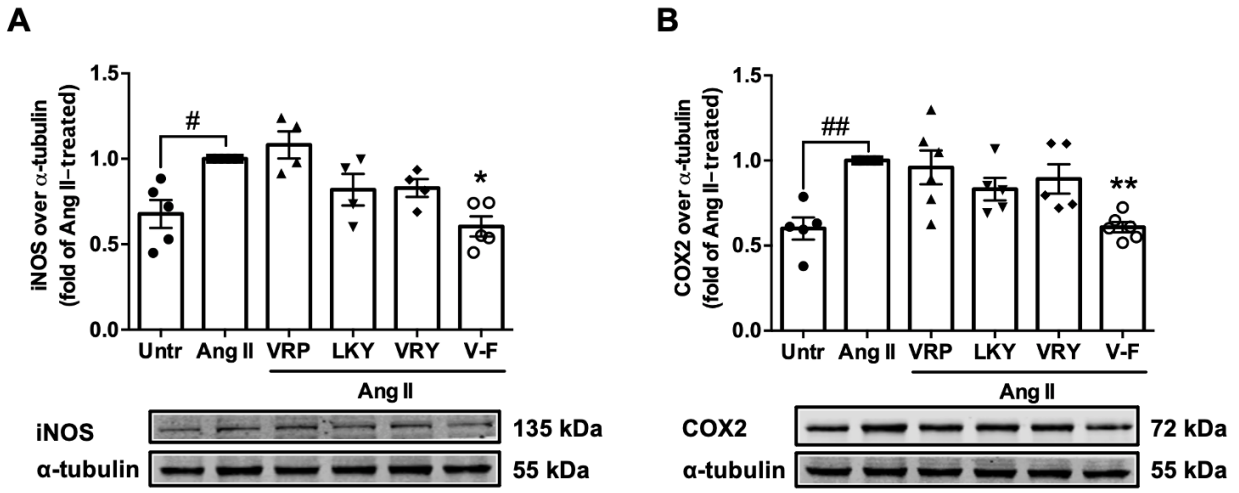


Figure 6.3 Effect of VRP, LKY, VRY, and VVHPKESF (V-F) on inflammation in Ang II-stimulated VSMCs. Cells were pre-treated with 50 μM of peptides for 1 h prior to addition of 1 μM of Ang II for 23 h. Protein bands were quantified by densitometry and normalized to α-tubulin. Data were expressed as means ± SEMs of 4-5 independent experiments, normalized to Ang II-treated group. #, $p < 0.05$, ##, $p < 0.01$, compared to the untreated group (Untr); *, $p < 0.05$, **, $p < 0.01$, compared to Ang II-treated group.

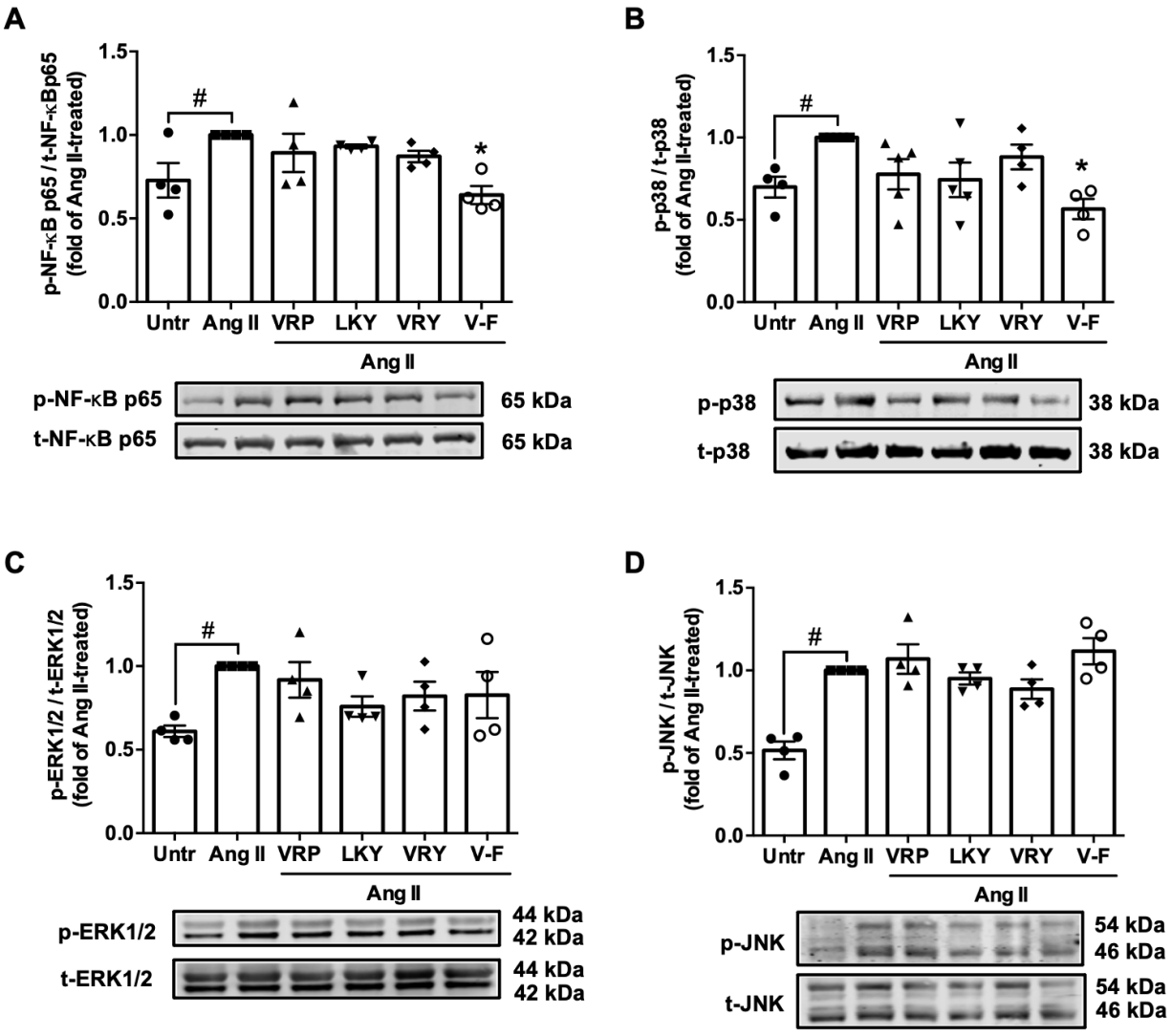


Figure 6.4 Effect of VRP, LKY, VRY, and VVHPKESF (V-F) on (**A**) NF-κB p65, (**B**) p38 MAPK, (**C**) ERK1/2, and (**D**) JNK in Ang II-stimulated VSMCs. Cells were treated with 50 μM of peptides for 1 h prior to addition of 1 μM of Ang II for 15 min. Protein bands were quantified by densitometry and normalized to their respective "total" forms. Data were expressed as means ± SEMs of 4 independent experiments and normalized to the Ang II-treated group. #, $p < 0.05$, ##, $p < 0.01$, compared to the untreated group (Untr); *, $p < 0.05$, compared to Ang II-treated group.

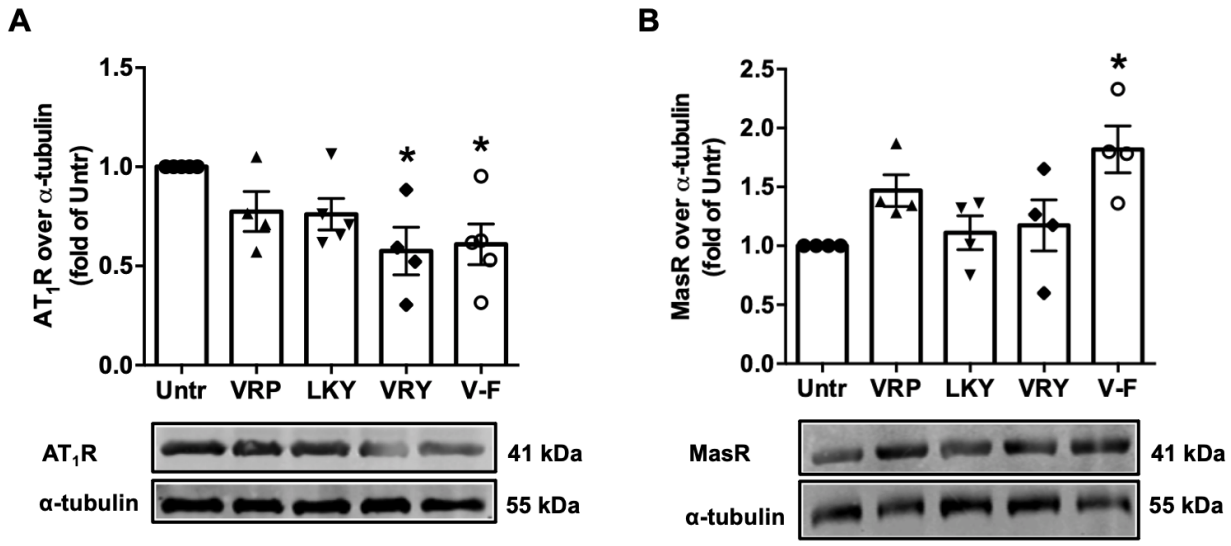


Figure 6.5 Effect of VRP, LKY, VRY, and VVHPKESF (V-F) on expressions of (**A**) AT_1R and (**B**) MasR in VSMCs. Cells were treated with 50 μM of peptides for 24 h. Protein bands were quantified by densitometry and normalized to α-tubulin. Data were expressed as means ± SEMs of 4-5 independent experiments, normalized to the untreated group (Untr). *, $p < 0.05$, compared to the untreated group.

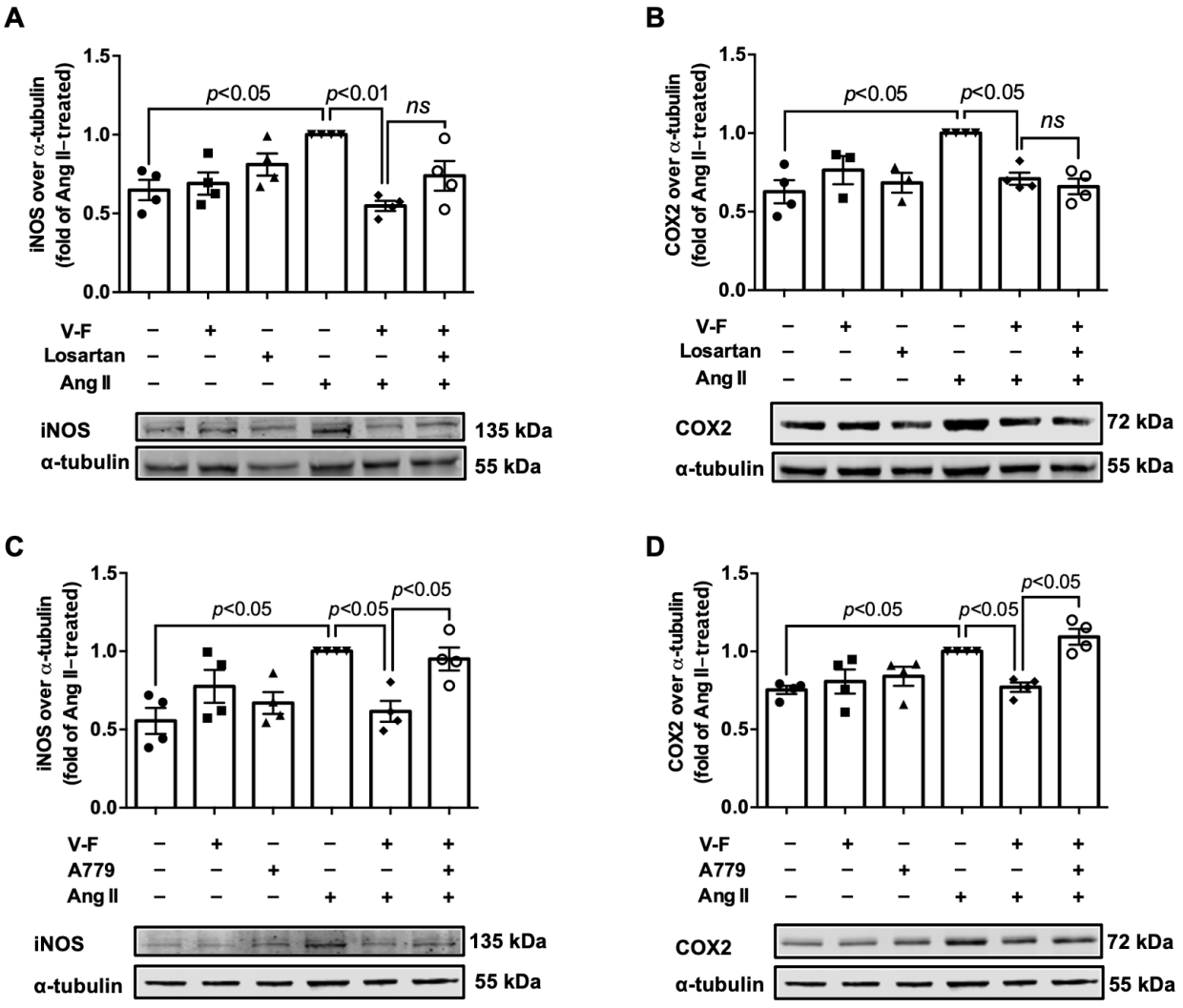


Figure 6.6 Involvement of (**A-B**) AT_1R and (**C-D**) MasR in anti-inflammatory effect of VVHPKESF (V-F) in Ang II-stimulated VSMCs. Cells were treated with 50 μM of peptides with or without 50 μM AT_1R antagonist (losartan potassium) or 1 μM MasR antagonist (A779) for 1 h prior to treatment with 1 μM of Ang II for 23 h. Protein bands were quantified by densitometry and normalized to α-tubulin. Data were expressed as means ± SEMs of 4 independent experiments, normalized to the Ang II-treated group. ns, not significant.

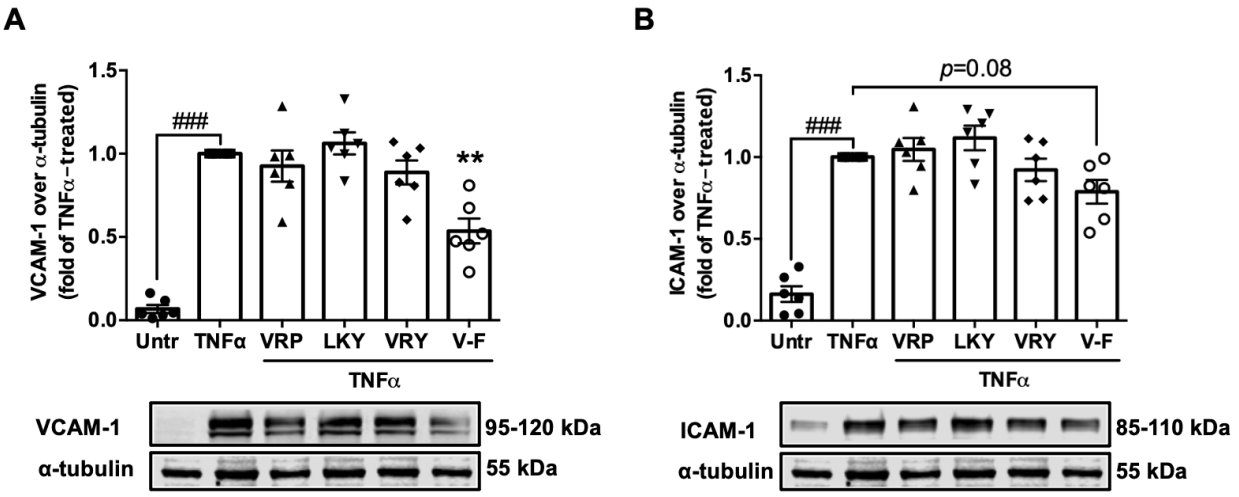


Figure 6.7 Effect of VRP, LKY, VRY, and VVHPKESF (V-F) on inflammation in TNFα-stimulated ECs. Cells were treated with 50 μM of peptides for 18 h prior to treatment with 10 ng/mL of TNFα for 6 h. Protein bands were quantified by densitometry and normalized to α-tubulin. Data were expressed as means ± SEMs of 6 independent experiments, normalized to the TNFα-treated group. ###, $p < 0.001$, compared to the untreated group (Untr); **, $p < 0.01$, compared to the TNFα-treated group.

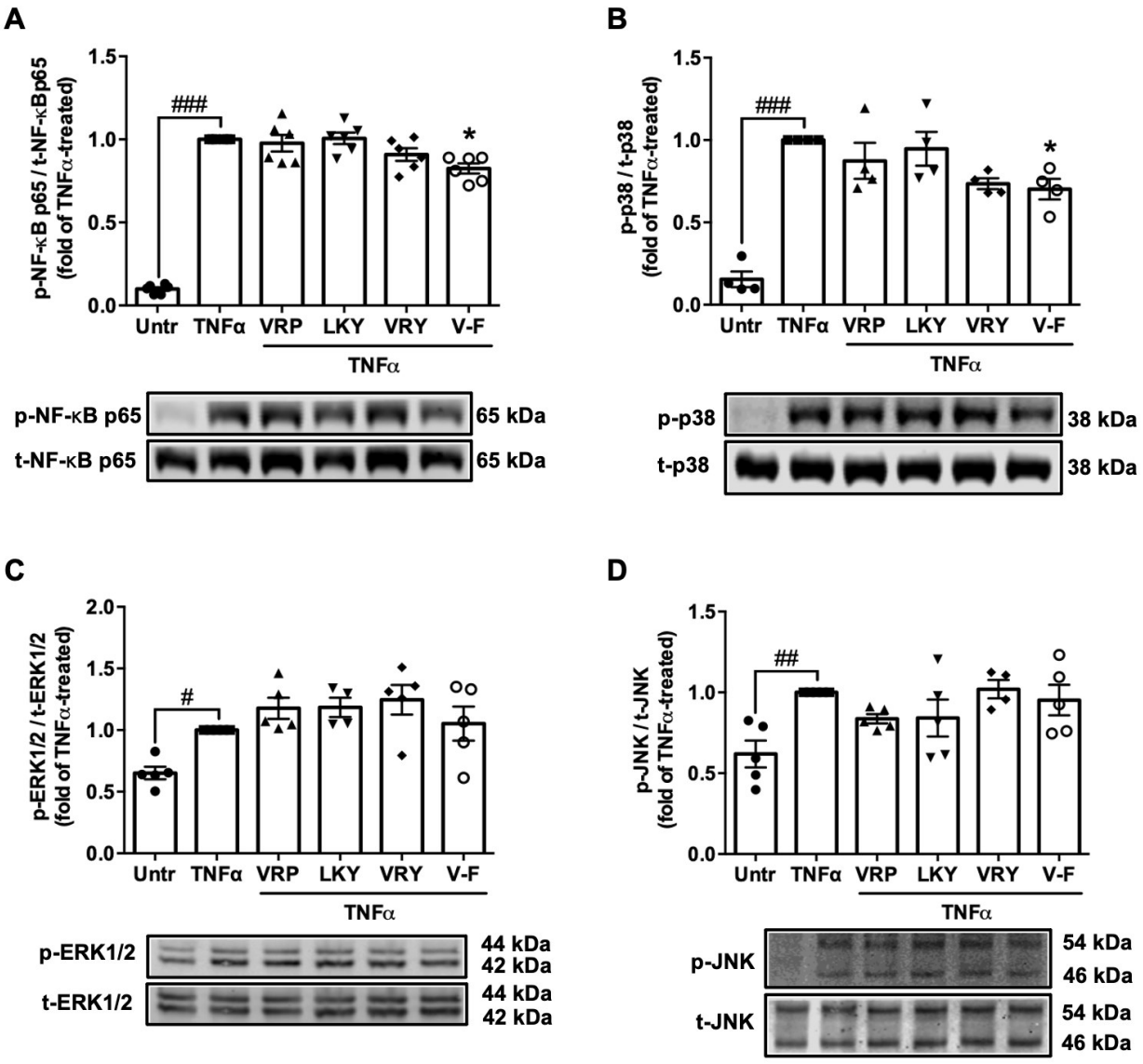
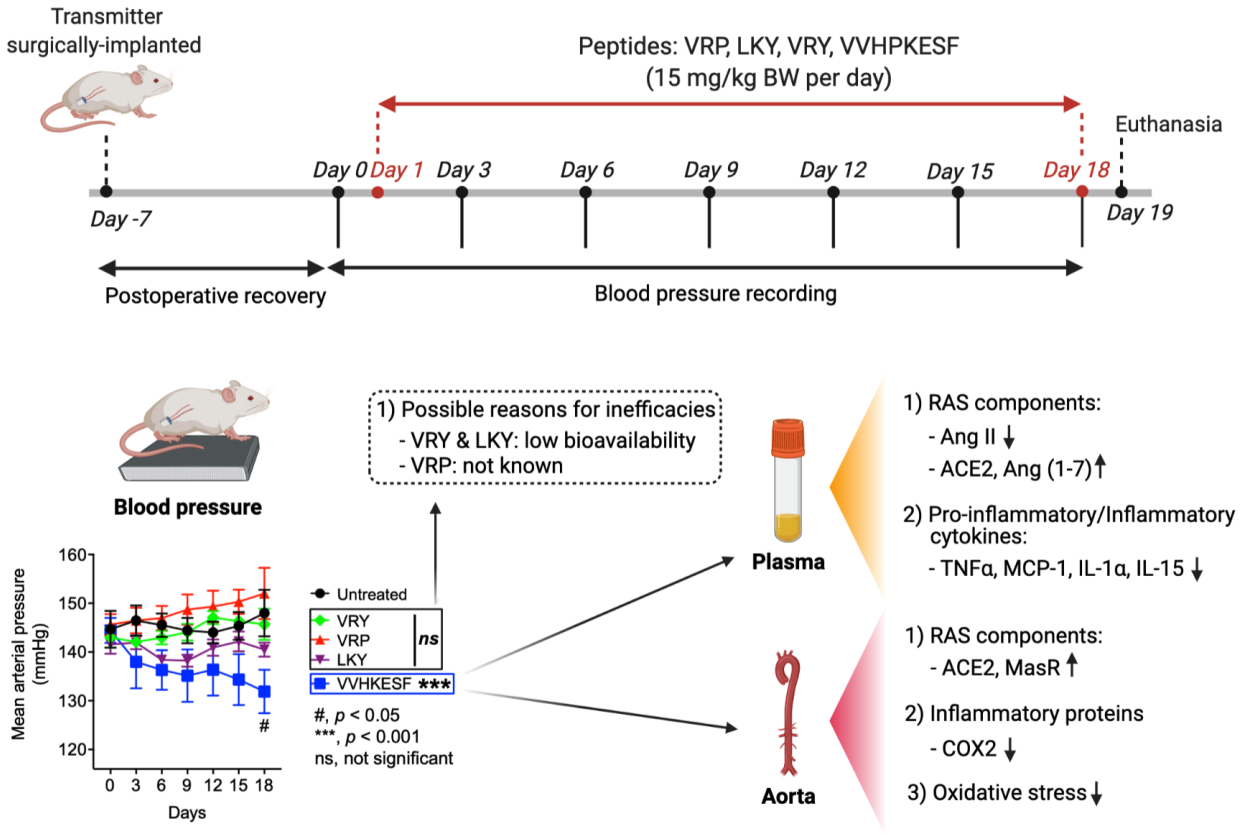


Figure 6.8 Effect of VRP, LKY, VRY, and VVHPKESF (V-F) on (**A**) NF-κB p65, (**B**) ERK1/2, (**C**) p38, and (**D**) JNK in TNFα-stimulated ECs. Cells were treated with 50 μM of peptides for 18 h prior to treatment with 10 ng/mL of TNFα for 15 min. Protein bands were quantified by densitometry and normalized to their respective "total" forms. Data were expressed as means ± SEMs of 4-6 independent experiments, normalized to the TNFα-treated group. ###, $p < 0.001$, compared to the untreated group (Untr); *, $p < 0.05$, compared to the TNFα-treated group.

CHAPTER 7 – Antihypertensive Effect of Spent Hen Muscle Protein-Derived ACE Inhibitory Peptides and ACE2 Upregulating Peptides in Spontaneously Hypertensive Rats [6]

Graphical abstract



[6] Part of this chapter will be submitted to *Molecular Nutrition & Food Research* for consideration of publication.

7.1 Introduction

Downregulation of the hyperactive renin-angiotensin system (RAS) is central to the development of antihypertensive peptides (Aluko, 2015). The primary effector hormone in the RAS, angiotensin (Ang) II, produced by angiotensin-converting enzyme (ACE), is not only a potent vasoconstrictor but also stimulates inflammatory responses and oxidative stress via Ang II type 1 receptor (AT_1R) (Unger, 2002). The current antihypertensive peptides are developed mostly based on their abilities to downregulate the ACE-Ang II-AT_1R axis mainly by inhibiting ACE activity (Wu, Liao, & Udenigwe, 2017). However, ACE2 can degrade Ang II to Ang (1-7) that binds with Mas receptor (MasR), which counteracts the harmful effects of the ACE-Ang II-AT_1R axis (Patel et al., 2014). Upregulation of ACE2 is becoming a new target for developing antihypertensive peptides. Physiologically, increased ACE2 level protects the animals against hypertension and attenuates hypertension-linked pathological responses (Díez-Freire et al., 2006; Patel et al., 2014; Zhong et al., 2011). A few bioactive peptides have been reported to reduce BP in spontaneously hypertensive rat (SHR), being accompanied with ACE2 upregulation (Ehlers et al., 2011; He et al., 2019b; Pan et al., 2015; Zheng et al., 2017). Most recently, IRW, derived from egg white, was proved to reduce BP primarily in SHR via upregulating the ACE2-Ang (1-7)-MasR axis, which also involved improvement of endothelial-dependent vasodilation and attenuated vascular inflammation (Liao et al., 2019; Majumder et al., 2015b).

In Chapter 5, four ACE inhibitory (ACEi) and/or ACE2 upregulating (ACE2u) peptides were characterized, including VRP (with ACEi activity), VVHPKESF (V-F) (with ACE2u activity), LKY and VRY (with both ACEi and ACE2u activities). In Chapter 6, we evaluated their abilities in mitigating oxidative stress and inflammatory responses in two vascular cells. As reported, many peptides identified using *in vitro* approaches are reported to lack *in vivo* antihypertensive effect,

given many complicated bioconversion processes in the body as well as an incomplete understanding of *in vivo* mechanisms of actions of peptides, which many substantially endanger the fates of peptides during oral administration (Fujita, Yokoyama, & Yoshikawa, 2000; Jensen et al., 2014; Miguel et al., 2007; Miguel et al., 2007). Therefore, the current chapter aims to study the antihypertensive effect of these peptides in SHR.

7.2 Materials and Methods

7.2.1 Materials

Dithiothreitol (DTT), Triton-X-100, pepsin (from the porcine gastric mucosa), and pancreatin (from porcine pancreas) were obtained from Sigma Aldrich (St Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), 0.25% (*w/v*) trypsin-0.53 mM EDTA, fetal bovine serum (FBS), Hanks balanced salt solution (HBSS with Ca and Mg), nonessential amino acids (NEAA), 4-(2−68 hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and penicillin−streptomycin were obtained from Gibco Invitrogen (Burlington, ON, Canada). Rat vascular smooth muscle A7r5 cells (VSMCs, CRL-1444) and human colon carcinoma cells (Caco-2, HTB-37) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Peptides (VRP, LKY, VRY, and V-F with purity > 98%) were synthesized by Genscript Corp (Piscataway, NJ, USA).

7.2.2 Ethics statement, telemetry recording, and experimental design

Rat experimental procedures were approved by the University of Alberta Animal Welfare Committee (#AUP 00001571) according with the guidelines issued by the Canadian Council on Animal Care and were adhered to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health. Twelve- to fourteen-week-old SHRs (290 ± 10 g) were obtained from the Charles River (Senneville, QC, Canada). Upon arrival, animals were

acclimatized for one week in the University of Alberta animal core facility, exposed to a 12:12 h of light:dark cycle with controlled temperature and humidity. Rats were given standard rat chow and water *ad libitum*. Then, they were surgically implanted with telemetry transmitters (HD-S10, Data Sciences International, St. Paul, MN, USA) and were allowed for a 7-day postoperative recovery as described in Chapters 3 and 4.

Animals were randomly assigned into 5 groups (n=6): the untreated group and four peptides (VRP, LKY, VRY, and V-F) (15 mg/kg/day body weight (BW)); the dose were selected based on previous studies (Liao et al., 2019; Majumder et al., 2013b; Majumder et al., 2015a). Peptides were orally administrated to animals from day 1 for 18 days dissolved in 20 mL of 10% (in ddH_2O, v/v) Ensure (Abbott Nutrition, QC, Canada); the untreated group was given 10% Ensure only (Fan et al., 2019b; Liao et al., 2019). BP was recorded for a continuous 24 h (10 seconds of every 1 min) every 3 days until day 18 (BP on day 0 was recorded as the baseline). Systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP), and heart rate (HR, bpm) were recorded. At the end of the experiment, animals were sacrificed by cardiac blood collection under anesthesia. Blood and tissues were collected and stored at –80 °C for further analysis.

7.2.3 Plasma biomarker analysis

Plasma samples were collected after centrifuging the blood samples at 1,000 × g for 20 min at 4 °C in EDTA-coated tubes (BD Vacutainer, NJ, USA) immediately after animal euthanasia. ACE activity was determined using an ACE assay kit obtained from Abcam (Toronto, ON, Canada). Concentrations of Ang II (CSB-E04494r), Ang (1-7) (CSB-E14241r), and ACE2 (CSB-E14308r) were evaluated by ELISA kits obtained from Cusabio Technology (Houston, TX, USA). Circulating cytokines were quantified using rat cytokine strips from Signosis (Santa Clara, CA, USA). All determinations were performed as per the manufacturers' manuals.

7.2.4 Immunostaining

The immunostaining protocol of aortic rings referred to steps described in Chapter 4.

7.2.5 Western blotting of animal tissues

Aortas were homogenized in using RIPA buffer containing a 1% (v/v) protease inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA, USA) for protein extraction. The homogenates were centrifuged at 15,000 × g for 15 min at 4 °C, and supernatants were collected with protein concentrations determined by bicinchoninic acid (BCA) assay (Thermo Fisher Scientific). Samples were stored at -80 °C until western blotting within 2 weeks. Total proteins were calibrated before being loaded on a 9% separating gel and transferred to a nitrocellulose membrane (diameter 0.45 μm, Bio-Rad, Montreal, QC, Canada) for incubation with antibodies. Bands of ACE (Abcam, Toronto, ON, Canada), ACE2 (Abcam), AT_1R (Invitrogen), angiotensin II type 2 receptor (AT_2R, Abcam), and MasR (Novus biologicals, Toronto, ON, CA), inducible nitric oxide synthase (iNOS; BD Biosciences, San Jose, CA, USA), cyclooxygenase 2 (COX2; Abcam), intracellular adhesion molecule-1 (ICAM-1; Novus biologicals), and vascular cell adhesion molecule-1 (VCAM-1; Abcam) were normalized to α-tubulin (Abcam) or glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Abcam). Donkey-anti-mouse 800 CW or Goat-anti-rabbit IRDye 680 RD secondary antibodies (Licor Biosciences, Lincoln, NE, USA) were used to visualize the bands in a Licor Odyssey BioImager which were quantified using Image Studio Lite 5.2 (Licor Biosciences).

7.2.6 Simulated gastrointestinal digestion of spent hen-derived peptides

Simulated gastrointestinal digestion of peptides was conducted according to Fan et al. (2019b). Peptides (100 μg/mL, w/v ddH_2O) were digested by pepsin (1% enzyme/substrate, w/w protein) for 1.5 h (37 °C, pH 2.0) (using 3 M HCl). Then, the digest was adjusted to pH 5.3 using 0.9 M

$NaHCO_3$ followed by pH 7.5 using 1 M NaOH, with one half of the digest collected as peptic digest and the other half further digested by pancreatin (1% enzyme/substrate, w/w protein) for another 1.5 h (37 °C). The reaction was terminated by heating the digest to 95 °C and maintained for 10 min. Peptide degradation profiles were evaluated using ultra-performance liquid chromatography (UPLC). Each digest was prepared individually in quadruplicate.

7.2.7 Transport of spent hen-derived peptides across Caco-2 cell monolayer

The Caco-2 culturing protocol was referring to Fan et al. (2018). All stability tests incorporated 0.5 mL apical HBSS (pH 6.0) and 1.5 mL basolateral HBSS (pH 7.4) in each well, respectively. Only wells with transepithelial electrical resistance (TEER) > 400 Ω/cm^2 before and after the study were counted. Caco-2 cells were first washed 3 times with pre-warmed (37 °C) HBSS and left at cell incubator (37 °C) for 30 min. Peptide samples (5 mM) were then added to the apical chambers. After 2 h of incubation, both apical and basolateral samples were collected and analyzed for peptide degradation using UPLC.

7.2.8 ACE2 expression in vascular smooth muscle A7r5 cells (VSMCs)

The culturing protocol of VSMCs was described in Chapter 3. Cells (passages 4-11) were treated with peptides (50 μM) for 24 h. Cells were lysed in a boiling Laemmle's buffer containing 50 mM Dithiothreitol (DTT) and 0.2% Triton-X-100. The lysates were run on a 9% separating gel and transferred to a 0.45-μm nitrocellulose membrane for antibody incubation. ACE2 expression was normalized to that of α-tubulin. Incubation of secondary antibodies and quantification of protein expressions were described in 7.2.5.

7.2.9 Statistical analysis

Data were expressed as mean values with standard errors of means (SEM). BP and HR were analyzed by two-way ANOVA by Tukey's test; all other results including plasma biomarkers and western blotting were analyzed by unpaired *t* test or one-way ANOVA using GraphPad Prism Version 6 (San Diego, CA, USA). A value of $p < 0.05$ was considered significantly different.

7.3 Results

7.3.1 Only VVHPKESF (V-F) reduced blood pressure

None of the peptide treatments affected body and organ weights over the period of 18 days (Figure 7.1). The SBP of the untreated SHRs was 173.7 mmHg on day 0 and increased up to 179.5 mmHg on day 18 ($p > 0.05$); a similar trend was also observed for MAP and DBP (Figure 7.2). V-F was the only peptide that reduced BP over the period of 18-day oral administration ($p < 0.001$), with SBP lowering to 159.9 mmHg on day 18; VRP, LKY, and VRY did not reduce BP over the treatment period ($p > 0.05$). Thus, later plasma and tissue analyses were performed for the V-F group.

7.3.2 V-F modulated the RAS components

V-F treatment reduced the circulating level of Ang II while increased those of Ang (1-7) and ACE2 significantly ($p < 0.05$); no effect on circulating ACE activity was detected (Figure 7.3). In the aorta, V-F upregulated expressions of ACE2 and MasR, without affecting those of ACE, AT_1R, and AT_2R over the treatment period (Figure 7.4).

7.3.3 V-F attenuated vascular inflammation and oxidative stress

Treatment with V-F reduced circulating inflammatory cytokines or chemokines including tumor necrosis factor alpha (TNFα), MCP-1 (monocyte chemoattractant protein-1), interleukin-1α (IL-1α), and interleukin-15 (IL-15) ($p < 0.05$) (Figure 7.5A-D). In aorta, the treatment inhibited expression of COX2 ($p < 0.01$) but not iNOS, ICAM-1 and VCAM-1 ($p > 0.05$) (Figure 7.5E-F). V-F treatment also reduced nitrotyrosine/nitrosative stress in aorta (($p < 0.01$) (Figure 7.6).

7.3.4 Possible explanations for lack of blood pressure reduction in animals treated with VRP, LKY, and VRY

VRP, LKY, and VRY showed high *in vitro* ACEi activity (Chapter 5) and also antioxidant activity in VSMCs (Chapter 6), however, no BP reduction was observed in these groups of animals ($p > 0.05$) (Figure 7.2). The circulating ACE activity in SHR treated with VRP was reduced, while those of LKY and VRY were not affected (p=0.48 or 0.31) (Figure 7.7). The stability tests against simulated gastrointestinal digestion and Caco-2 cell monolayers showed the poor stability of LKY and VRY, whereas VRP was relatively stable (Table 7.1).

7.3.5 The gastrointestinal digest of V-F upregulated ACE2 expression

V-F was found to be susceptible to trypsin digestion, during which two fragmentary peptides, VVHPK and ESF, were formed (Table 7.1); VVHPK, not ESF, maintained the ACE2u activity of V-F (Figure 7.8).

7.4 Discussion

SHR is the most widely used rodent model for studying essential hypertension due to similar pathogenesis (Liu, 2009; Trippodo & Frohlich, 1981; Wu & Juurlink, 2002). SHR develops

persistent high BP at early adulthood (~9-15 weeks) till up to >180 mmHg (16-28 weeks), as opposed to normotensive Wistar Kyoto rat which develops and maintains BP at ~120 mmHg after 9-10 weeks (Luo et al., 2008). Asides from the elevated RAS activity, SHR also develops increased oxidative stress and inflammation in the vasculature (Unger, 2002; Wu & Juurlink, 2002). Many bioactive peptides with *in vitro* RAS-regulating, antioxidant, or anti-inflammatory activity were assessed for their *in viv*o activities in SHR (Chen et al., 2020; He et al., 2019a; Jahandideh et al., 2016; Majumder et al., 2013b; Majumder et al., 2015a; Tsai et al., 2020). Four ACEi and/or ACE2u peptides, VRP, LKY, VRY, and V-F, possessing antioxidant effect in VSMCs, were characterized in previous chapters; V-F also protected vascular endothelial cells (ECs) and VSMCs against inflammation. These peptides were administrated to SHRs; through an 18-day oral administration at a daily dose at 15 mg/kg BW, only V-F reduced BP without affecting HR of SHRs (Figure 7.2). It should be noted that the BP reduction by V-F was achieved at a lower molar dose (due to a longer peptide length) compared with the other three peptides, since they were administrated at the same mass dose.

Bioactive peptides have shown great potential in controlling BP (Aluko, 2015). In this study, treatment with V-F significantly increased the circulating ACE2 and Ang (1-7) but reduced Ang II levels ($p < 0.05$) (Figure 7.3A-C), representing the evidence of ACE2 upregulation by V-F. Likewise, V-F upregulated the expression of ACE2 and MasR in the aorta ($p < 0.05$) (Figure 7.4A-B). Both amplification of plasma and vascular ACE2 contribute to BP reduction (Rentzsch et al., 2008; Wysocki et al., 2010). The treatment did not change the circulating ACE activity as well as aortic expressions of ACE, AT_1R, and AT_2R ($p > 0.05$) (Figure 7.3D and Figure 7.4C-E). It was well documented the beneficial effects of upregulating the ACE2-Ang (1-7)-MasR axis in managing hypertension and many cardiovascular events (Benter et al., 1995; Patel et al., 2014). A

decade ago, a small molecule drug, xanthenone has been identified as a potent ACE2 activator through structure-based *in silico* screening; its ability in reducing MAP by ~30-70 mmHg was successfully achieved at a single dose of 5-10 mg/kg BW in SHR (Prada et al., 2008). Recently, a few bioactive peptides have been reported to reduce BP associated with upregulated ACE2, Ang (1-7), or MasR at various tissue levels including the vascular wall, heart, and kidney (Ehlers et al., 2011; He et al., 2019b; Liao et al., 2018; Zheng et al., 2017). IRW was the first peptide that reduces BP in SHR predominantly via the ACE2-Ang (1-7)-MasR axis, despite being initially characterized as an ACEi peptide (Liao et al., 2019). V-F was identified as an ACE2u peptide while was also found to upregulate MasR expression and ameliorate inflammation dependent on MasR in VSMCs upon Ang II stress (Chapter 5-6). The ability of V-F in reducing BP in SHR was possibly mediated by upregulating the ACE2-Ang (1-7)-MasR axis.

Vascular inflammation is recognized as an important contributor to vascular remodeling and hypertension (Savoia et al., 2011; Virdis & Schiffrin, 2003). V-F reduced the circulating level of inflammatory cytokines or chemokines including TNFα, MCP-1, IL-1α, and IL-15, as well as vascular expression of COX2 (Figure 7.5); expressions of iNOS and VCAM-1 were not affected, differing from the results reported from the previous cell study (Chapter 6). V-F also reduced nitrotyrosine level in the aorta, an indicator of oxidative injury formed by a highly reactive oxygen species (ROS), peroxynitrite (Figure 7.6) (Escobales & Crespo, 2005). Oxidative stress and associated oxidative damage are mediators of vascular injury in many cardiovascular diseases including hypertension (Cuzzocrea et al., 2004; Schulz, Gori, & Münzel, 2011; Touyz & Schiffrin, 2004). Antioxidant agents could regress vascular remodeling and reduce BP in animals (Touyz & Schiffrin, 2004). V-F attenuated nitrotyrosine level in the aorta, indicating an antioxidant effect occurred in the vasculature. Bioactive peptides from various food sources with antioxidant and

anti-inflammatory effects have been reported. Egg-derived hydrolysates and peptides, IQW and IRW, reduced BP and attenuated vascular inflammation and oxidative stress in ECs or SHRs (Jahandideh et al., 2014; Majumder et al., 2013b; Majumder et al., 2015a). Three rapeseed protein-derived peptides, LY, RALP, and GHS, exerted antioxidant and anti-inflammatory effects in RAW 264.7 cells and SHRs (He et al., 2019a). A potato-derived peptide, IF, improved antioxidant defense against renal damage, contributing to BP reduction in SHRs (Tsai et al., 2020). V-F reduced inflammation and oxidative stress in vascular cells in Chapter 6, while this study further demonstrated these beneficial effects *in vivo*. V-F treatment might achieve these effects through reducing the circulating Ang II level and thus Ang II signaling that stimulates inflammation and oxidative stress in the vasculature. Besides, increased circulating levels of ACE2 and Ang (1-7) and aortic expressions of ACE2 and MasR might also contribute to its antioxidant and anti-inflammatory effects, since upregulation of the ACE2-Ang (1-7)-MasR axis implicates these effects (Prestes et al., 2017; Simões e Silva et al., 2013; Zhang et al., 2015). Furthermore, peptides might exert antioxidant effects through acting as direct ROS scavengers (Liu et al., 2016); their anti-inflammatory effects may be achieved through interacting with nucleus or certain unknown receptors (Fan, Liao, & Wu, 2019a; Majumder et al., 2013a; Wu, 2020). Therefore, to what degrees were the antioxidant and anti-inflammatory effects of V-F dependent on the ACE2-Ang (1-7)-MasR axis is yet to be investigated.

To our surprise, VRP, LKY, and VRY did not reduce BP (Figure 7.2), as opposed to their potent *in vitro* ACEi activities (LKY and VRY possessed also ACE2u activity) and antioxidant activities (Chapter 5-6). Furthermore, only the circulating ACE activity in SHRs treated with VRP, not LKY or VRY, was reduced (Figure 7.7). It was therefore hypothesized that LKY and VRY might not reach the circulation and then the action sites at effective doses. We found that LKY and

VRY were susceptible to digestion by trypsin and α-chymotrypsin; besides, both of them could be cleaved by unidentified peptidases, possibly a carboxypeptidase, secreted by the polarized Caco-2 cell monolayers, an *in vitro* intestinal transepithelial transport model for pharmaceuticals or food nutrients (Table 7.1) (Hidalgo, Raub, & Borchardt, 1989; Hooton et al., 2015; Howell, Kenny, & Turner, 1992). Gastrointestinal stability presents as an important factor for the bioavailability and *in vivo* bioactivity of peptides (Vermeirssen, Van Camp, & Verstraete, 2004; Xu et al., 2019). The poor gastrointestinal stability of LKY and VRY might explain a lack of BP reduction in SHRs. However, VRP showed high stability against simulated gastrointestinal digestion and the polarized Caco-2 cell monolayers; a reduced circulating ACE activity in VRP-treated SHRs indicated its capability in reaching the circulation. However, VRP failed to exert an antihypertensive effect; the underlying mechanisms were not known at the current stage, which relies more on future studies such as its stability against serum peptidases and *in vivo* metabolism (Jenssen & Aspmo, 2008). Besides, V-F can be digested by trypsin into two fragments, ESF and VVHPK, and the latter was also an ACE2u peptide; this indicated that V-F is indeed a "pro-drug" type ACE2u peptide (Figure 7.8) (Fujita, Yokoyama, & Yoshikawa, 2000). The antihypertensive activity of V-F may be partially due to that of VVHPK, but this speculation requires further clarification.

In summary, only V-F reduced BP in SHRs among the four spent hen peptides and its activity might involve upregulation of the ACE2-Ang (1-7)-MasR axis as well as amelioration of vascular inflammation and oxidative stress. V-F is a "pro-drug" ACE2u peptide and its gastrointestinal-degraded fragment, VVHPK, might partially contribute to its bioactive functions. Therefore, the contribution of the ACE2-Ang (1-7)-MasR axis to the antihypertensive activity of V-F, as well as whether or not the beneficial effects of V-F were mediated by VVHPK, are yet to be answered in future studies. Lack of BP reduction in SHR treated with LKY and VRY was possibly due to their

poor gastrointestinal stability. Besides, VRP was resistant to gastrointestinal digestion, reducing the circulating ACE activity in SHR but without causing any BP reduction; the underlying reasons await further clarification. Our findings further emphasized the importance of the gastrointestinal stability of peptides in dictating their *in vivo* efficacies. Furthermore, this study reported for the first time the antihypertensive activity of an ACE2u peptide identified *in vitro*. The presence of antihypertensive peptides in spent hens further supports their potential uses as antihypertensive functional food ingredients and nutraceuticals.

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Table 7.1 Stability of spent hen peptides against simulated gastrointestinal digestion and Caco-2 cell monolayers

	VRP	LKY	VRY	V-F
ACEi peptide	✓	✓	✓	
ACE2u peptide		✓	✓	✓
Simulated gastrointestinal digestion	VRP (N.D.)	L|K||Y (59±4.0%)	V|R||Y (46±9.1%)	VVHPK||ESF (92±0.40%)
Caco-2 incubation	VRP (N.D.)	LK|||Y (32±13%)	VR|||Y (17±2.0%)	VVHPKESF (N.D.)

Simulated gastrointestinal digestion: peptides (100 μg/mL) were digested by pepsin (enzyme/substrate, E/S, 1%, w/w) for 1.5 h followed by pancreatic (α-chymotrypsin and trypsin) digestion (E/S, 1%, w/w) for another 1.5 h; Caco-2 incubation: peptides (5 mM) were incubated with Caco-2 cell monolayer (day 21) for 2 h and both apical and basolateral samples were analyzed (n=4). Values in the parentheses indicated the % of peptides degraded at the respective stage of digestion (|, || and |||, indicated cleavage sites by α-chymotrypsin, trypsin and an unidentified peptidase, respectively). N.D., not degraded, indicated no degradation or degradation < 5%.

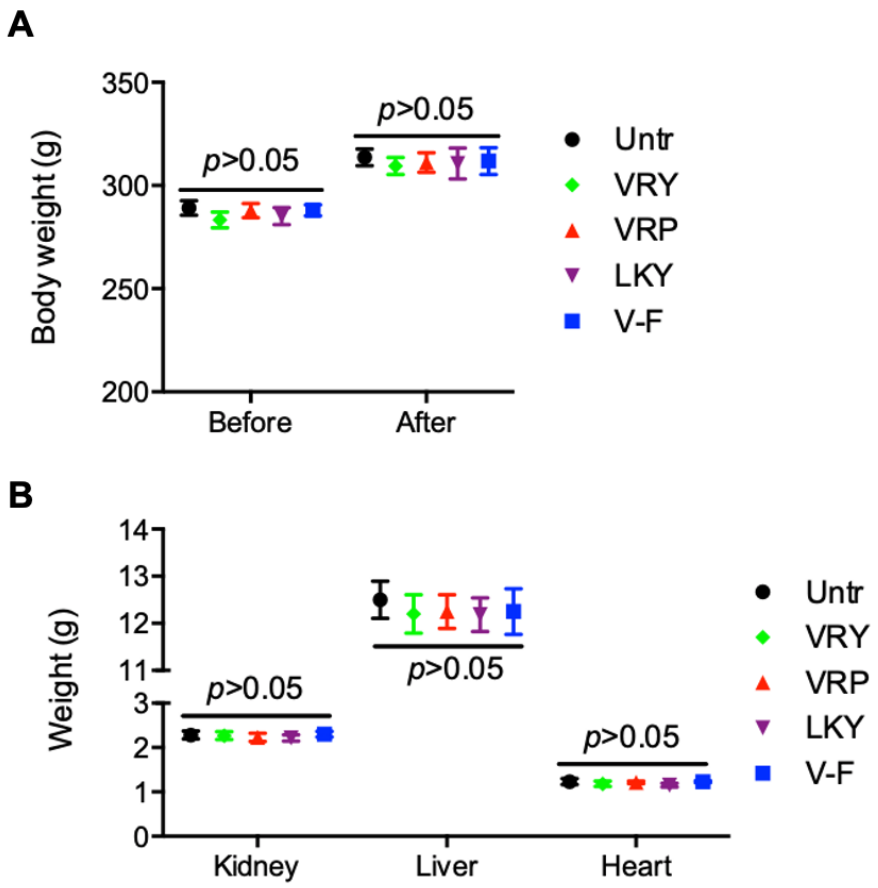

Figure 7.1 Effect of oral administration of peptides on body and organ weights in SHRs. **(A)** Body weight was obtained before and after (at the end point of the experiment) 18 days of oral administration of peptides to SHRs. **(B)** Organ weight was obtained after euthanizing the animals. Data were expressed as means ± SEMs from n=6 animals per group. Peptide (15 mg/kg) were orally administrated once a day over 18 days. Untr, untreated group.

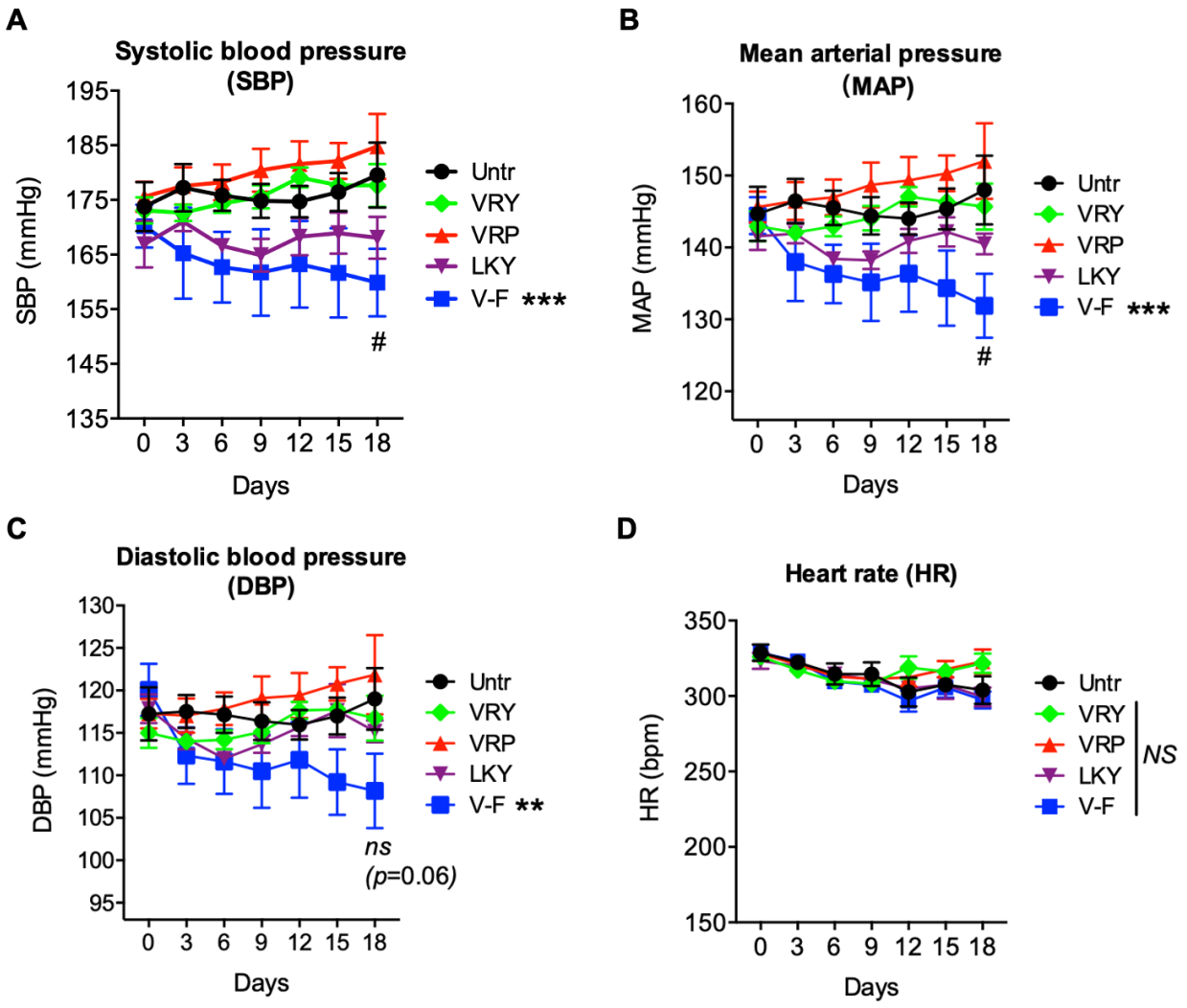

Figure 7.2 Only VVHPKESF (V-F) reduced BP in SHRs. Systolic blood pressure (SBP), mean arterial pressure (MAP), diastolic blood pressure (DBP) and heart rate (HR) on each day represented the mean values recorded over a 24-h period. Data were expressed as means ± SEMs from n=6 animals per group. Peptide (15 mg/kg) were orally administrated once a day over 18 days. **, ***, and NS indicated $p < 0.01$, $p < 0.001$, and not significant ($p > 0.05$), respectively, compared to the untreated group (Untr) over the entire treatment period; #, $p < 0.05$, ns, not significant ($p > 0.05$), compared to the untreated group at a specific time point.

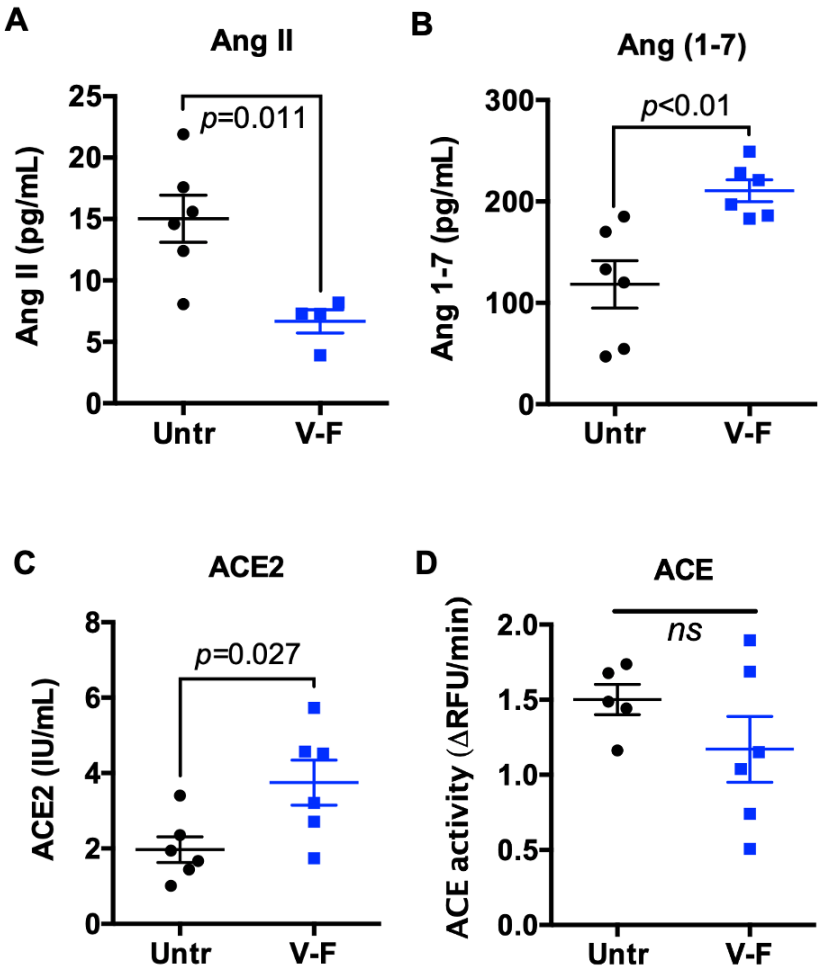

Figure 7.3 VVHPKESF (V-F) treatment modulated the circulating RAS components. Plasmas were collected at the end point for evaluating the circulating (**A**) Ang II concentration, (B) Ang (1-7) concentration, (**C**) ACE2 concentration, and (**D**) ACE activity. Data were represented as mean ± SEM from 5-6 animals per group. ns, not significant.

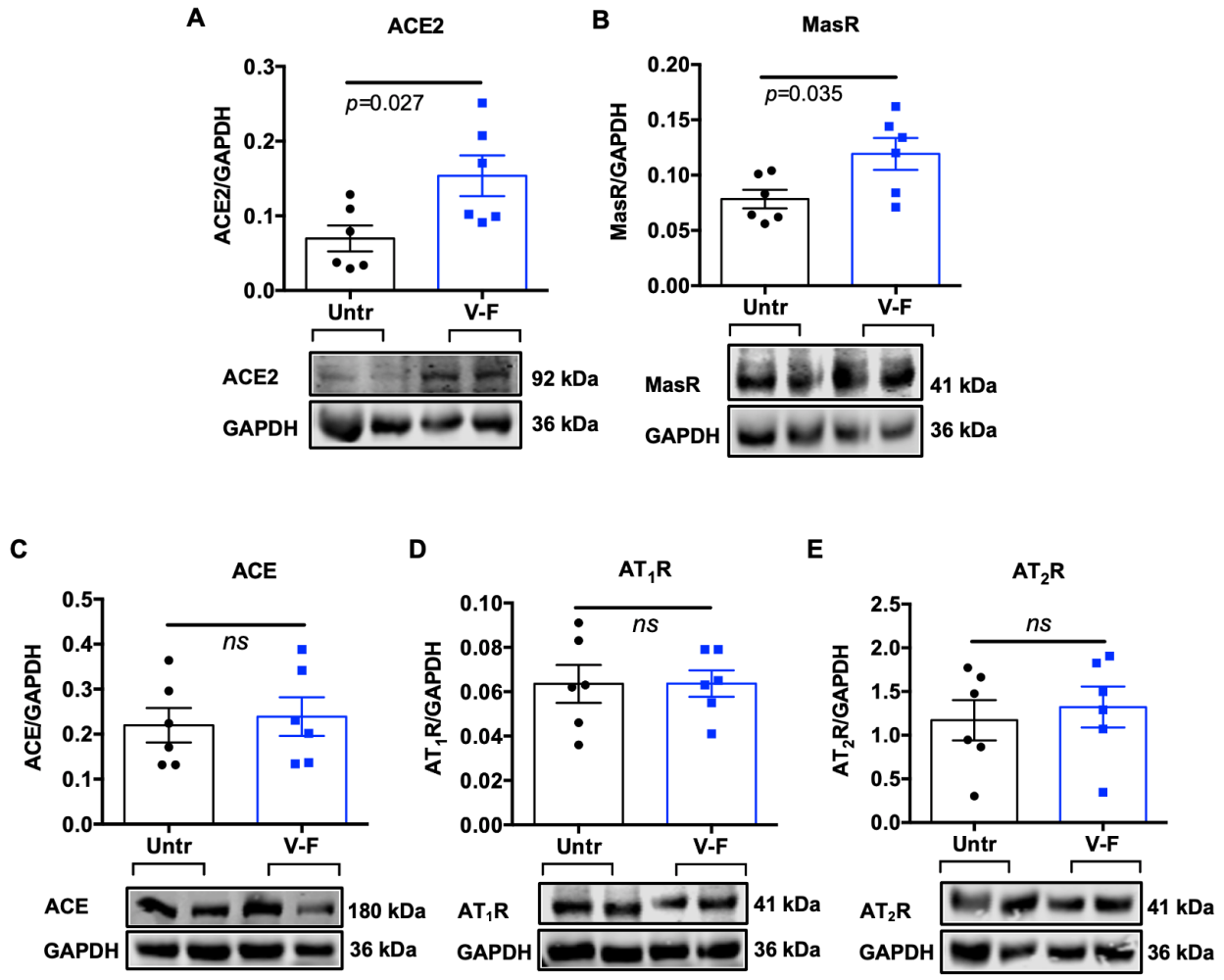

Figure 7.4 VVHPKESF (V-F) treatment modulated the RAS components in aorta. Expressions of (**A**) ACE2, (**B**) MasR, (**C**) ACE, (**D**) AT_1R and (**E**) AT_2R were normalized to GAPDH. Data were expressed as means ± SEMs from 6 animals per group. Untr, untreated group. ns, not significant.

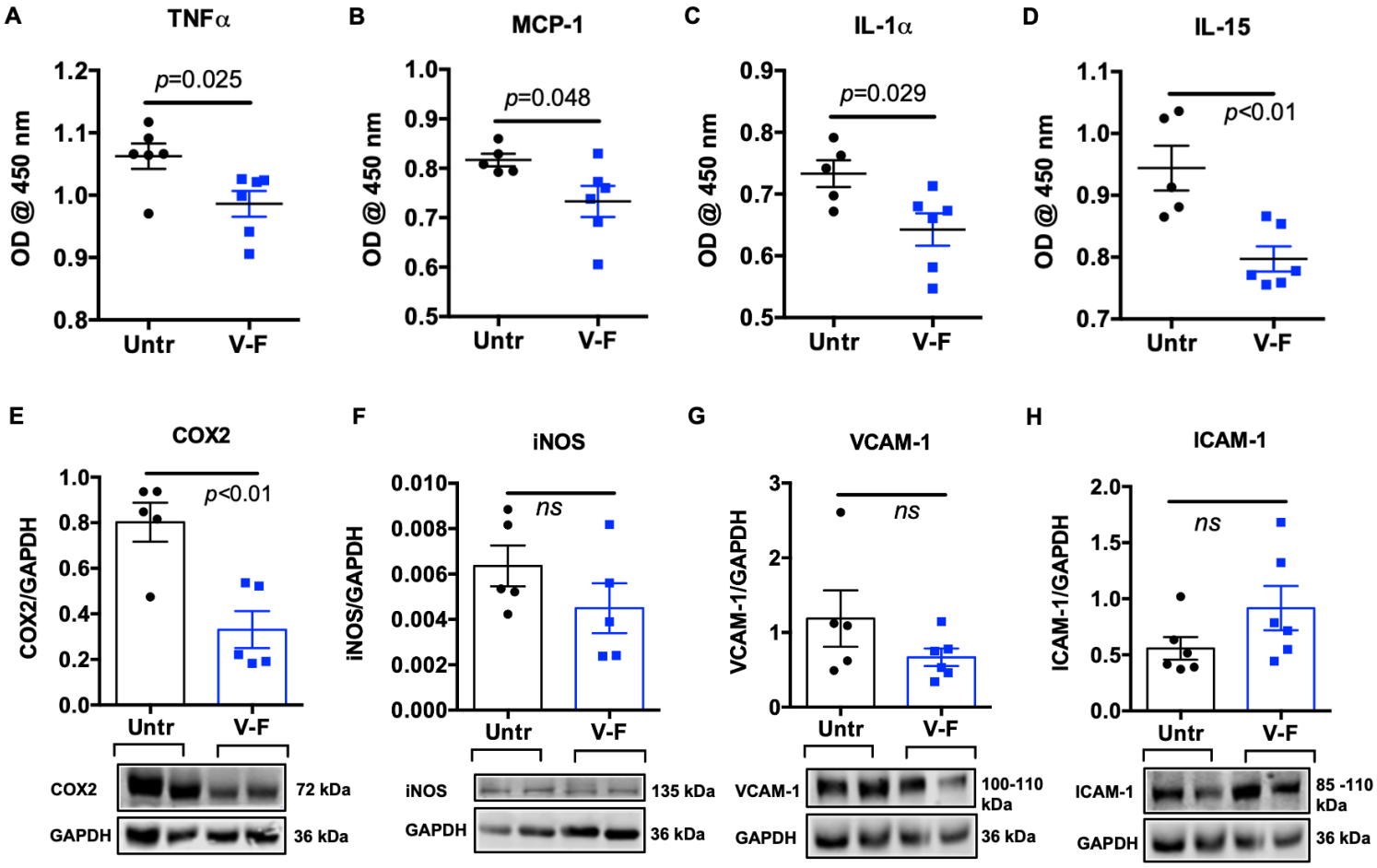

Figure 7.5 VVHPKESF (V-F) treatment attenuated circulating inflammatory cytokines and vascular inflammation. Plasmas were collected at the end point for evaluating the circulating levels of (**A**) TNFα, (**B**) MCP-1, (**C**) IL-1α, and (**D**) IL-15. Expressions of (**E**) COX2, (**F**) iNOS, (**G**) VCAM-1 and (**H**) ICAM-1 in aortas were normalized to GAPDH. Data were expressed as means ± SEMs from 5-6 animals per group, Untr, untreated group. ns, not significant.

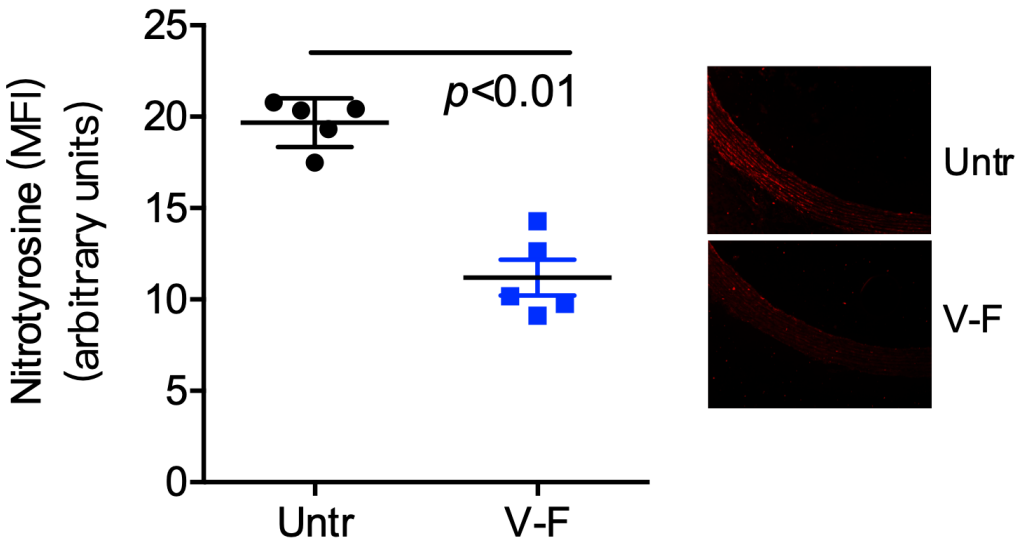

Figure 7.6 VVHPKESF (V-F) treatment ameliorated nitrosative stress in aorta. Nitrotyrosine in aortic sections of the untreated (Untr) or V-F group were immunostained. Data were expressed as means ± SEMs from 5 animals per group.

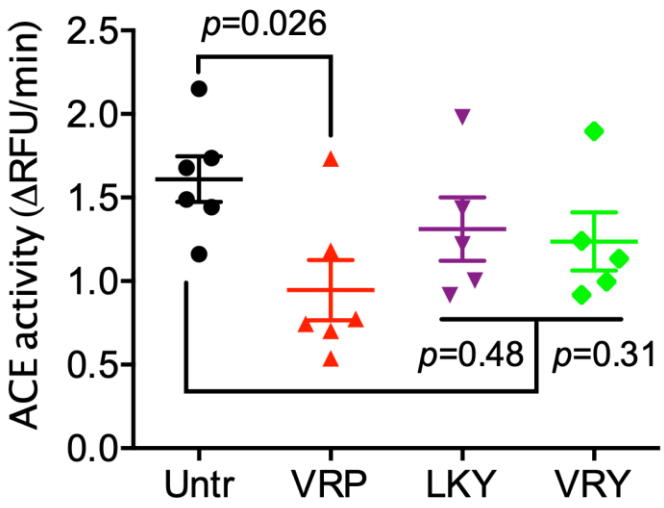

Figure 7.7 Effect of VRP, LKY and VRY on the circulating ACE activity of SHR**.** Data were represented as mean ± SEM from 5-6 animals per group.

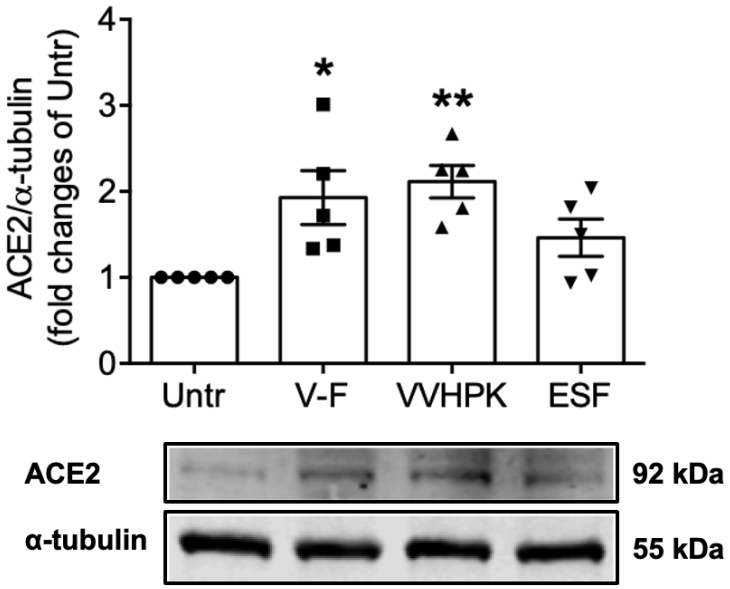

Figure 7.8 The gastrointestinal digest of VVHPKESF (V-F) upregulated ACE2 expression. Rat vascular smooth muscle A7r5 cells (VSMCs) were treated with 50 μM of peptides for 24 h. Protein bands were quantified by densitometry and normalized to α-tubulin. Data were expressed as means ± SEMs of 5 independent experiments, normalized to untreated group (Untr). *, $p < 0.05$; **, $p < 0.01$, compared to the untreated group (Untr).

CHAPTER 8 – Conclusions and Recommendations

8.1 Key findings of the present research

1) A spent hen muscle protein hydrolysate (SPH) prepared by thermose PC10F (SPH-T) with antihypertensive activity was prepared (Chapters 3 and 4)

The aim of Chapter 3 was to prepare an SPH with antihypertensive effect, SPH-T, while that of Chapter 4 was to further investigate its antihypertensive activity and the underlying mechanisms. To achieve these goals, 18 SPHs, prepared by 9 enzymes in isolation or in combination, were assessed initially for their *in vitro* angiotensin-converting enzyme (ACE) inhibitory (ACEi) activities, and three SPHs, SPH-T, SPH-P, and SPH-26L, showed the highest activity. To screen the most promising SPH with antihypertensive effect, a multiple evaluation approach, which considered not only ACEi activity but also ACE2 upregulating (ACE2u), antioxidant, and anti-inflammatory activities, coupled with their fates during simulated gastrointestinal digestion and transepithelial transport, was applied. As a result, SPH-T was found to have the highest potential. Note that ACEi activity is still the most widely used *in vitro* index for screening peptides for *in vivo* study, SPH-T, SPH-P, and SPH-26L were all administrated to spontaneously hypertensive rat (SHR) (n=3). However, only SPH-T reduced blood pressure (BP) at the dose of 1,000 mg/kg body weight (BW) over a period of 20 days. These results indicated the importance of gastrointestinal stability in dictating the *in vivo* efficacies of antihypertensive peptides (Fujita, Yokoyama, & Yoshikawa, 2000; Jensen et al., 2014).

In Chapter 4, more animals were included (n=6-7) to study the antihypertensive effect and mechanisms of action of SPH-T; two doses were used: high dose (1,000 mg/kg BW) and low dose (250 mg/kg BW). Results showed that both treatments significantly reduced BP ($p < 0.05$),

especially the high-dose SPH-T ($p < 0.01$). Associated with BP reduction was increased circulating ACE2 and Ang (1-7) but reduced Ang II levels, upregulated aortic ACE2 and downregulated AT_1R expressions, as well as ameliorated vascular inflammation, oxidative stress, and fibrosis.

2) ACE inhibitory peptides and ACE2 upregulating *peptides were purified from spent hen muscle protein hydrolysate prepared by thermoase (Chapter 5)*

ACEi peptides and ACE2u peptides in SPH-T were purified after a line of ultrafiltration and chromatographic technologies, five potent ACEi peptides, VRP, LKY, VRY, KYKA, and LKYKA (IC_{50} values of 0.034–5.77 μg/mL), and four ACE2u peptides, VKW, VHPKESF, VVHPKESF (V-F), and VAQWRTKYETDAIQRTEELEEAKKK (increased ACE2 expression by 0.52–0.84 folds in vascular smooth muscle A7r5 cells [VSMCs]) were identified. ACE2u peptides are a new type of antihypertensive peptides, and therefore this study supported the presence of ACE2u peptides in food proteins and the feasibility for isolation. It also demonstrated the presence of bioactive peptides with multiple bioactivities in spent hen muscle proteins. Among the purified ACEi peptides, LKY and VRY were also ACE2u peptides, upregulating ACE2 expression by ~0.80 folds in VSMCs. This implied that certain common structural features might be shared by ACE2u peptides and ACEi peptides, suggesting a possibility of identifying ACE2u peptides from the current ACEi peptides. Previous ACE2u peptides were all initially characterized as ACEi peptides, including IQP, VEP, IRW, LY, GHS, and a few others (He et al., 2019; Majumder et al., 2015; Zheng et al., 2017). Besides, several ACEi drugs were also found to upregulate the ACE2 level (Ferrario et al., 2005; Yang et al., 2013). To date, it is impossible to propose any structure-function relationship for ACE2u peptides, due to a very limited number of candidates reported in the literature. Thus, continuous discovery of ACE2u peptides will remain to be crucial, through either isolation from food proteins or screening from the current ACEi peptides. Among the

identified peptides, only those with the highest ACEi or ACE2u activity and from the major muscle proteins were considered for animal work; they were characterized into 3 categories: VRP (with ACEi activity), V-F (with ACE2u activity), and LKY and VRY (with both ACEi and ACE2u activities).

3) ACE inhibitory peptides and/or ACE2 upregulating *peptides showed antioxidant and anti-inflammatory effects in vascular cells (Chapter 6)*

The attenuation of vascular oxidative stress and inflammation by bioactive peptides contributes to BP regulation (Wu, Liao, & Udenigwe, 2017). Thus, we further assessed the antioxidant or anti-inflammatory activities of VRP, LKY, VRY, and V-F in VSMCs or endothelial EA.hy926 cells (ECs), upon stimulation by Ang II or TNFα, respectively, before being administrated to SHR (Fan, Liao, & Wu, 2019; Majumder et al., 2013). The antioxidant activity was assessed in VSMCs whereas the anti-inflammatory activity was evaluated in both VSMCs and ECs. Results showed that VRP, LKY, VRY, and V-F reduced oxidative stress mainly by acting as free radical scavengers, but V-F also involved activation of endogenous antioxidant enzymes (glutathione peroxidase 4, GPx4 and superoxide dismutase 2, SOD2). However, only V-F attenuated inflammation in both cells, manifested by the downregulated inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX2) in VSMCs and vascular cell adhesion molecule 1 (VCAM-1) in ECs; its anti-inflammatory action was likely mediated by modulation of NF-κB p65 and p38 MAPK pathways. Except for upregulating ACE2, V-F also upregulated Mas receptor (MasR) in VSMCs and its anti-inflammatory effect was partially dependent on the ACE2-MasR axis. Notably, LKY and VRY, which are also ACE2u peptides, did not show any effect. These variations hinted that ACE2 upregulation does not necessarily mean the upregulation of the ACE2-MasR axis, neither does the anti-inflammatory activity against Ang II stress. Nevertheless, the antioxidant or

anti-inflammatory effects of the four peptides demonstrated their multifunctional properties, which could contribute to their antihypertensive effects in SHR. Table 8.1 specifies the bioactivities of the identified ACEi and ACE2u peptides.

4) The ACE2 upregulating *peptide VVHPKESF (V-F) reduced blood pressure in spontaneously hypertensive rats (Chapter 7)*

VRP, LKY, VRY, and V-F were orally administrated to SHR. Only V-F reduced BP significantly at the dose of 15 mg/kg BW over the treatment period of 18 days. BP reduction was likely through the increased circulating levels of ACE2 and Ang (1-7), upregulated vascular ACE2 and MasR expressions, as well as the ameliorated vascular inflammation and oxidative stress. We also analyzed the possible reasons for the lack of BP reduction by VRP, LKY, and VRY. LKY and VRY had poor gastrointestinal stability and were unlikely to reach the circulation and the action sites at their respective effective doses. VRP was resistant to gastrointestinal digestion, appearing to inhibit the circulating ACE activity ($p < 0.05$); the reasons behind its lack of BP reduction were not known which require further clarification such as the susceptibility to serum peptidases (Jenssen & Aspmo, 2008). Besides, although several previously-reported ACE2u peptides were purified from pea proteins, their *in vivo* antihypertensive effects were not studied (Liao et al., 2019). Therefore, this study reported for the first time that an ACE2u peptide identified using the conventional activity-guided fractionation reduced BP in SHR**.** Interestingly, V-F was not stable to gastrointestinal digestion and can be degraded into VVHPK, which is also an ACE2u peptide. Therefore, whether the antihypertensive effect of V-F was mediated by that of VVHPK remains to be answered in future studies. The beneficial effects of V-F in two vascular cells and SHRs are concluded in Figure 8.1.

8.2 Significance of the present research

1) Significance to the egg industry

Over the last decade, the production of spent hens continued increasing in Canada and exceeded 35 million in 2019 (AAFC, 2020). Spent hens are treated as a byproduct in the egg industry and are of little market value. The current disposal methods by burial, composting, and incineration are often infeasible and raise environmental and animal welfare concerns; meanwhile, the producers are liable for paying the associated cost of transportation and disposal (Newberry et al., 1999). Recently, various value-added uses have been developed for spent hens including being utilized as starting materials for biomaterial and functional food applications (Esparza et al., 2018; Fan et al., 2020; Hong et al., 2018; Safder, Temelli, & Ullah, 2019; Yu, Field, & Wu, 2018). In this study, a spent hen muscle protein hydrolysate using thermoase, SPH-T, and its derived peptide (V-F) were developed, both reducing BP significantly in SHR ($p < 0.05$). This provided the scientific foundation that spent hens can be processed into antihypertensive nutraceuticals or functional food ingredients. This value-added application with enhanced nutritional value and health benefits will contribute to alleviating the burden of waste disposal and generating extra revenue for the Canadian egg industry. Besides, the present work can shed light on other protein-rich agricultural byproducts for possible value-added uses.

2) Significance to research of antihypertensive peptides

Hypertension afflicts more than 20% of adults worldwide (DeGuire et al., 2019). In Canada, the cost attributed to hypertension was approximately $13.9 billion in 2010 and was estimated to be $20.5 billion in 2020, accounting for more than 10% of the health expenditure (DeGuire et al., 2019; Weaver et al., 2015). Given the cost and side effects of pharmacological treatment over the

long-term use, developing natural bioactive compounds have gained increasing interest in the past decades. This study aims to develop antihypertensive peptides using a cheap and underutilized source of proteins, spent hens, as less expensive and safer alternatives for hypertensive treatment. Previous research on discovering antihypertensive peptides was mainly focusing on identifying ACEi peptides, whereas this study also targeted ACE2u peptides, and more importantly, reported for the first time that an ACE2u peptide identified using *in vitro* method exerted *in vivo* BP-lowering effect. Targeting ACE2 upregulation is an emerging mechanism of antihypertensive peptides, thus the discovery and characterization of ACE2u peptides in this study demonstrated the presence of this new category of antihypertensive peptides in food proteins, the feasibility of isolation, and their potential in regulating hypertension.

3) Significance to the regulation of coronavirus diseases (COVID) infection by bioactive peptides

The COVID 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has posed a threat to global public health (Shah et al., 2020). Since ACE2 is a crucial functional receptor for SARS-CoV-2 entry (Hoffmann et al., 2020), there is an ongoing debate on the pros/cons of continuous use of antihypertensive medications, such as ACE inhibitors and AT_1R blockers which generally upregulate ACE2 expression, on COVID implications (Sanchis-Gomar et al., 2020). Upregulated ACE2 level provides more viral entry (Hoffmann et al., 2020), but on the other hand, it elevates Ang (1-7) level which mediates vasodilatory, anti-inflammatory, and antioxidant effects, and thus may protect from the virus infection (Tikellis & Thomas, 2012). The virus-ACE2 binding is known to attenuate ACE2 level or activity, which accumulates Ang II, shifting the RAS towards a predominant ACE-Ang II-AT_1R axis, exacerbating pulmonary inflammatory and oxidative organ damage (Sanchis-Gomar et al., 2020). Some studies demonstrated the protective roles of ACE2 upregulation in fighting against COVID.

For example, the Ang (1-7)/Ang I ratio was significantly higher in survivors than in non-survivors, suggesting a higher ACE2 activity in survivors (Reddy et al., 2019). Administrating recombinant ACE2 in mice protected the animals from acute respiratory distress syndrome, possibly by competitively binding with viral particles that would otherwise bind with membrane-bound ACE2 (Gu et al., 2016). Nevertheless, it is recommended to continue the current antihypertensive medication regardless of the COVID status at present (Sanchis-Gomar et al., 2020).

The present work reported SPH-T and V-F which increased both circulating level and vascular expression of ACE2 in SHR, providing preliminary data on bioactive peptides regulating ACE2 level *in vivo*. It awaits further investigation on how ACE2 is regulated in other organs such as the small intestine and kidney where ACE2 is highly expressed (Hikmet et al., 2020). Currently, there is very rare information on the effect of antihypertensive peptides on COVID, therefore more fundamental research is warranted before being able to elucidate their roles especially RAS-regulating peptides (*e.g.,* ACE2u peptides) in the COVID scenario.

8.3 Recommendations for future research

It is imperative to point out some limitations of this thesis. For example, we did not use the whole spent hen carcass but spent hen meat for preparing muscle proteins, which requires additional labor and cost for separating meat from the carcass. Besides, the animal used in this study is young male SHR, therefore it is not known whether or not the antihypertensive effect of spent hen-derived peptides may vary among different age groups. In addition, it is unknown whether the antihypertensive effect of V-F was via upregulating the ACE2-Ang (1-7)-MasR axis. As noted, V-F was degraded into VVHPK during the gastrointestinal digestion, therefore whether the in *vivo* activity of V-F was due to that of VVHPK remains unclarified. Besides these, we have not identified all peptides derived from SPH-T in SHR, which might lead to missing other

promising antihypertensive peptides. In consideration of the findings and limitations of this thesis, future studies are recommended:

1) To further minimize the cost of scale-up production, using the whole spent hen carcass instead of only meat for muscle protein extraction is anticipated (Lin et al., 2011; Mccurdy et al., 1986). However, the composition of the extracted proteins as well as the profile of the generated peptides and their antihypertensive effects need to be compared and validated.
2) SPH-T triggered more BP reduction (Chapter 4) than that of V-F (Chapter 7) in SHR; there is a possibility that other active antihypertensive peptides may also contribute to the BP-lowering activity. Therefore, it is meaningful to continue studying the antihypertensive effects of the other peptides with potent ACEi or ACE2u activity (in Chapter 5), other than the four (VRP, LKY, VRY, and V-F).
3) V-F was susceptible to gastrointestinal digestion, and a fragment, VVHPK, which is also an ACE2u peptide, can be released; therefore VVHPK is suggested to be administrated to SHR and to study to what extent is the antihypertensive effect of V-F mediated by VVHPK. It is also crucial to monitor the absorption and metabolism of V-F in SHR, especially whether V-F or VVHPK can reach the circulation (Tanaka et al., 2015). Besides, the peptide dosage through oral administration needs to be further optimized. Future research is also warranted to study the absorption, distribution, metabolism, and excretion of spent hen peptides using *ex vivo*, *in situ*, and *in vivo* animal models; some techniques such as LC-MS/MS and isotope labeling may help detect possible modification and metabolism of peptides after oral administration in animals (Tanaka et al., 2015).
4) Two cell lines, Ea.hy926 (passages 3-10) and A7r5 (passages 4-11), were used to study the protective effect of peptides against vascular inflammation or oxidative stress upon

stimulation by TNFα or Ang II, respectively. Primary cells generate more relevant data representing living systems but have a very limited life span and low passages (up to 4-5). Use of cell lines, *i.e.* Ea.hy926 and A7r5, is theoretically able to mimic part of the nature of vascular cells at low passages (recommended to be passage ~10 by the manufacturer), which were also validated in our previous studies using both cells until passage 11-12 (Gu et al., 2019; Liao et al., 2018). For example, Ea.hy926 cells, produced by fusing human umbilical vein endothelial cells with the permanent human cell line A549, was reported to possess a similar nature of adhesion molecule expressions such as ICAM-1 and VCAM-1 (Thornhill et al, 1993); similar findings were also reported for A7r5 cells (Kimes & Brandt (1976). Nevertheless, it is also meaningful to study the protective efficacies of spent hen peptides against vascular inflammation and oxidative stress in primary cells, since they represents a closer physiological state of cells *in vivo*.

5) Though the addition of MasR antagonist (A779) provides evidence that the anti-inflammatory and antioxidant effects of V-F were partially dependent on the ACE2-Ang (1-7)-MasR axis in VSMCs, it is not known whether or not these effects were due to increased Ang (1-7) level. Future studies need to ascertain whether or not the Ang (1-7) level and cellular ACE2 activity were increased by V-F treatment.
6) The identified peptides targeting ACE2 were characterized based on their capabilities in upregulating ACE2 expression not ACE2 activity which also regulates the conversion of Ang II to Ang (1-7) and thus the bioactivities of spent hen peptides in cells and animals. Future studies are warranted to determine the effect of spent hen peptides on ACE2 activity; knowing this may contribute to the characterization of new ACE2-regulating peptides and

provide interpretation of the varying bioactivities of the characterized ACE2u peptides in this study (LKY, VRY, and V-F) in VSMCs and SHRs.

7) The animal model used in this study is young male SHR. To broaden the horizon on the antihypertensive peptide research, it is meaningful to study the antihypertensive effects of SPH-T and V-F in other animal models, *e.g.* Ang II-induced rats, female or aged SHR, etc. (Girgih et al., 2014; Sriramula et al., 2011; Zimmerman, Harris, & Sullivan, 2014); female SHRs are reported to be more dependent on Ang (1-7), while aged SHRs are associated a progressive decline in nitric oxide (NO) generation and bioavailability (especially for SPH-T which shifted vasodilation toward NO-dependent manner as depicted in Chapter 4) (Cau, Carneiro, & Tostes, 2012; Smith et al., 2006; Zimmerman, Harris, & Sullivan, 2014). In addition, both SPH-T and V-F upregulated plasma and vascular ACE2 levels in SHR, it is thus warranted to know whether or not their BP-lowering effects were mainly via upregulating the ACE2-Ang (1-7)-MasR axis, for which infusion of MasR antagonist in SHR or use of ACE2 knockout animal models is recommended (Alghamri et al., 2013; Liao et al., 2019).

8) There are very limited studies exploring the effect of bioactive peptides on SARS-CoV-2 infection. Considering the crucial role of ACE2 in coronavirus infection, potentially harmful consequences of ACE2 upregulation have recently been the subject of much debate (discussed in 8.2) (Reddy et al., 2019). However, current evidence does not indicate a negative effect of administration of RAS regulators which increase ACE2 levels on the susceptibility and prognosis of SARS-CoV-2 infection (Ni et al., 2020; Sanchis-Gomar et al., 2020). The increased circulating ACE2 level by spent hen peptides may be due to a higher gene expression or an enhanced ACE2 shedding process at the tissue level, which

requires further investigation. Besides, ACE2 expression varies significantly among different organs, therefore it is also important to study the effect of SPH-T and V-F on ACE2 expression in other tissues (Hikmet et al., 2020). The impact of ACE2 upregulation by SPH-T and V-F in the COVID-19 scenario represents an interesting topic, which is warranted to be studied using *in vitro* and *in vivo* models in future studies.

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Table 8.1 Bioactivities of SPH-T-derived ACE inhibitory and/or ACE2 upregulating peptides

Peptides	VRP	LKY	VRY	VVHPKESF (V-F)
ACE inhibitory activity	✓ - IC_{50} value: 0.64 μg/mL	✓ - IC_{50} value: 0.81 μg/mL	✓ - IC_{50} value: 5.77 μg/mL	
ACE2 upregulatng activity		✓ - upregulate by 0.79 folds	✓ - upregulate by 0.78 folds	✓ - upregulate by 0.84 folds
Antioxidative stress	✓ - scavenge ROS directly	✓ - scavenge ROS directly	✓ - scavenge ROS directly	✓ - scavenge ROS directly - activate expressions of GPx4 and SOD2
Anti-inflammatory activity				✓ - VCAM-1 downregulation (ECs) - iNOS & COX2 downregulation V(SMCs) - via downregulating NF-κB p65 and p38 MAPK pathways (ECs and VSMCs) - iNOS & COX2 downregulation was via ACE2-Ang (1-7)-MasR pathway (VSMCs)
Antihypertensive activity				✓ - SBP reduction by 19.6 mmHg

ACE inhibitory activity was determined using a biochemical assay while ACE2 upregulating activity was determined as fold change of ACE2 expression compared with untreated control after treatment with 50 μM of peptides for 24 h (Chapter 5). Antioxidative stress and anti-inflammatory activity were determined in vascular smooth muscle A7r5 cells (VSMCs; pre-treatment with 50 μM of peptides for 1 h before stimulation by Ang II for 23 h) and endothelial EA.hy926 cells (ECs; pre-treatment with 50 μM of peptides for 18 h before stimulation by TNF-α for 6 h) (Chapter 6). Antihypertensive activity was determined in spontaneously hypertensive rats (dose of 15 mg/kg per day for 18 days) (Chapter 7). COX2, cyclooxygenase 2; GPx4, glutathione peroxidase 4; iNOS, inducible nitric oxide synthase; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor kappa B; ROS, reactive oxygen species; SBP, systolic blood pressure; SOD2, superoxide dismutase 2; SPH-T, spent hen muscle protein hydrolysate prepared by thermoase PC10F; VCAM-1, vacular cell adhension mocelule-1.

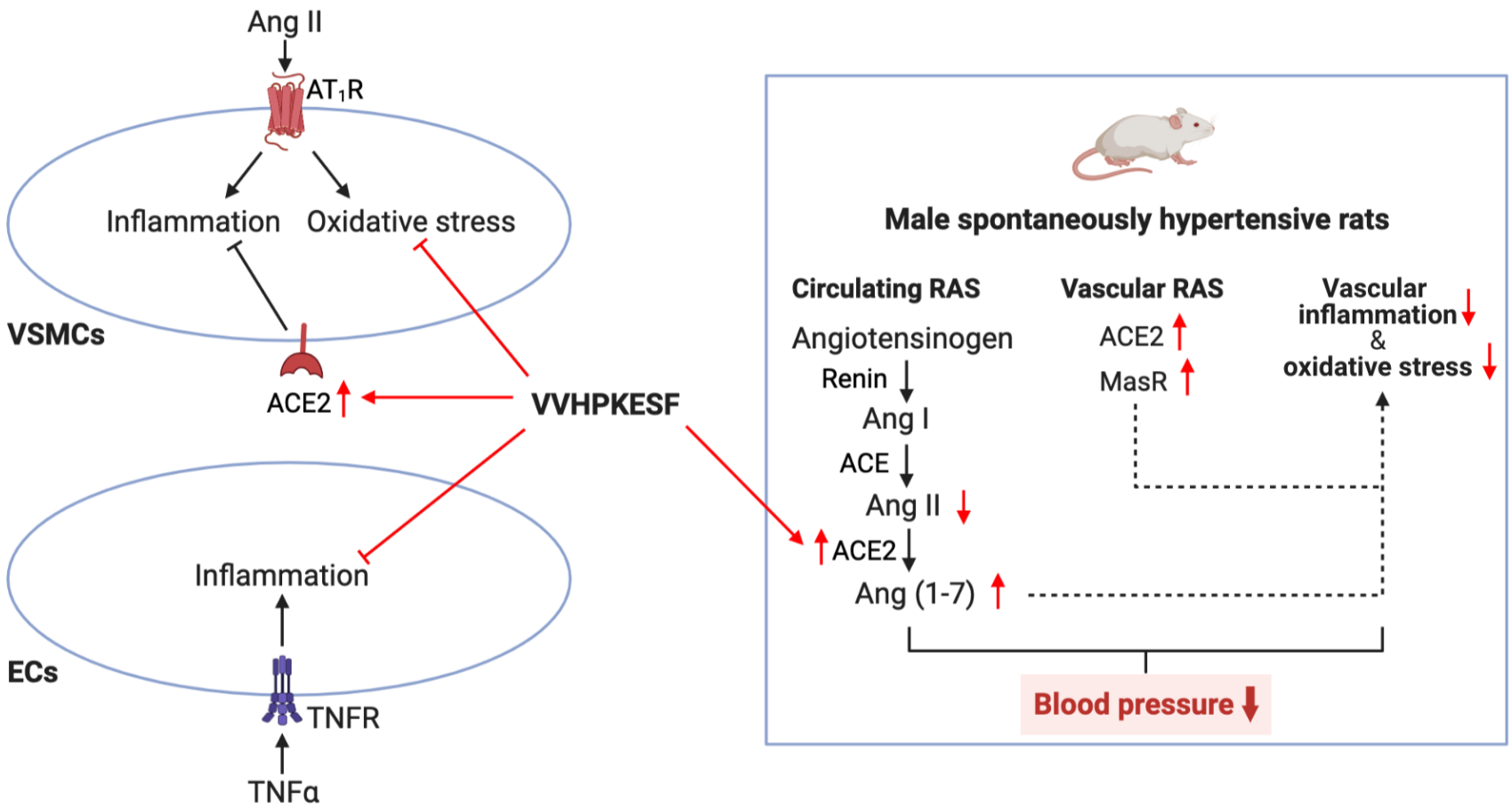

Figure 8.1 Beneficial effects of VVHPKESF in vascular cells and spontaneously hypertensive rats. ACE2, angiotensin-converting enzyme 2; Ang I/ II/(1-7), angiotensin I/II/(1-7); AT_1R, Ang II type 1 receptor; MasR, mas receptor; RAS, renin-angiotensin system; TNFα, tumor necrosis factor alpha; TNFR, TNFα receptor; ECs, endothelial EA.hy926 cells; VSMCs, vascular smooth muscle A7r5 cells. The dash line means possible pathways required to be verified.

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APPENDIX

Supplementary Table 3.1 Working parameters of one-enzyme hydrolysis

One-enzyme	Parameters		
	pH	Temperature (°C)	E/S (%, *w/w*)
Alcalase	8	50	4%
Protex 6L	8	37	4%
Protease S	8	37	4%
Thermoase	8	60	4%
Trypsin	8	60	4%
Protease M	8	60	4%
Pepsin	2	60	4%
Protex 50FP	3	60	4%
Protex 26L	3	60	4%

3 h hydrolysis. E/S (%, w/w) was based on protein mass.

Supplementary Table 3.2 Working parameters of two-enzyme hydrolysis

Two-enzymes	Enzyme 1	Parameters			Enzyme 2	Parameters		
		pH	Temperature (°C)	E/S (%, *w/w*)		pH	Temperature (°C)	E/S (%, *w/w*)
A + 6L	Alcalase (A)	8	60	2%	Protex 6L (6L)	8	60	2%
A + S	Alcalase (A)	8	60	2%	Protease S (S)	8	60	2%
A + T	Alcalase (A)	8	60	2%	Thermoase (T)	8	60	2%
6L + S	Protex 6L (6L)	8	60	2%	Protease S (S)	8	60	2%
6L + T	Protex 6L (6L)	8	60	2%	Thermoase (T)	8	60	2%
S + T	Protease S (S)	8	60	2%	Thermoase (T)	8	60	2%
50FP + 26L	Protex 50FP (50FP)	3	50	2%	Protex 26L (26L)	3	50	2%
50FP + P	Protex 50FP (50FP)	3	50	2%	Pepsin (P)	2	37	2%
26L + P	Protex 26L(26L)	3	50	2%	Pepsin (P)	2	37	2%

3 h hydrolysis: two enzymes together for 3 h, except for (50FP + pepsin) and (26L + pepsin), which were hydrolyzed by the first enzyme for 1.5 h, and then the second enzyme for another 1.5 h without inactivating the first enzyme. E/S (%, w/w) was based on protein mass.