IDENTIFICATION AND CHARACTERIZATION OF BIOACTIVE PEPTIDES DERIVED FROM PEA PROTEINS

by

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A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfilment of the requirements of the degree of

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Department of Human Nutritional Sciences

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ABSTRACT

Functional foods and nutraceuticals developed with food protein hydrolysates/peptides have received considerable attention for their effectiveness in the prevention and treatment of chronic diseases. The aim of this work was to identify and characterize bioactive peptides derived from pea proteins. Utilization of pea proteins can greatly contribute to the economic development of the prairie provinces.

First, sensory properties of an enzymatic pea protein hydrolysate (PPH, <3 kDa) was evaluated using a human test panel. It was found that bitterness of PPH decreased with decreased concentration until it reached minimum response threshold (2.5 mg/mL). Results of amino acid composition showed that high percentage of hydrophobic amino acids may contribute to the observed bitterness property.

Second, bioavailability and short-term influence of PPH (<3 kDa) on levels of angiotensin converting enzyme (ACE) and angiotensin II in normotensive rats were examined. Results showed that in PPH fed rats compared with control, ACE activity in lung was dose-dependently inhibited, while there were decreased angiotensin II levels in lung and plasma.

Third, the long-term effectiveness of PPH (<3 kDa) on blood pressure and renin-angiotensin system (RAS) was investigated. Development of hypertension was attenuated in both groups treated with PPH compared

with that of casein-treated group, and plasma angiotensin II level was also decreased in the PPH-treated groups.

Fourth, pea protein was hydrolyzed with alcalase and peptides present in the <1 kDa PPH were separated and purified by solid phase extraction (SPE) followed by two consecutive rounds of reverse-phase high performance liquid chromatography (RP-HPLC). Amino acid sequences of purified peptides were identified, and inhibitions of calmodulin-dependent phosphodiesterase 1 (CaMPDE1), ACE and renin by synthesized equivalent peptides were determined. Three multifunctional dipeptides, IR, KF, and EF were identified, and shown to exhibit inhibitory activities against ACE, renin, and CaMPDE1.

In conclusion, this work presented a detailed investigation of the effectiveness and influence of PPH on hypertension and RAS in addition to identification of multifunctional bioactive peptides present in the active PPH fractions. For the first time in the literature, bioavailability of PPH was studied in a normotensive Long-Evans rat model, influence of PPH on hypertension was investigated in a chronic renal disease rat model, and multifunctional bioactive peptide sequences were identified from pea proteins.

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DEDICATION

I wish to dedicate this thesis to

My wonderful parents, Qing Li and Guohua Zhang
For your unconditional love and support
Every step of the way
Through good times and bad

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Abbreviations

ACE	angiotensin-converting enzyme
ACEI	ACE inhibitor
AngII	angiotensin II
AUC	area under the curve
ARB	angiotensin receptor blocker
BP	blood pressure
bw	body weight
CaM	calmodulin
CaMPDE1	calmodulin-dependent phosphodiesterase 1
CKD	chronic kidney disease
DBP	diastolic blood pressure
FI	fluorescence intensity
GIT	gastrointestinal tract
HHL	Hippuryl-His-Leu
HPLC	high performance liquid chromatography
iv	intravenous
NK	natural killer
PBS	phosphate buffered saline
PKD	polycystic kidney disease
RAS	renin-angiotensin system

SBP	systolic blood pressure
SHR	spontaneously hypertensive rat
TCA	trichloroacetic acid
TFA	trifluoroacetic acid

Amino acid abbreviations

Amino acid	3-Letter abbreviation	1-Letter abbreviation
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Iso	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V
Unspecified/unknown	Xaa	X

CHAPTER 1

INTRODUCTION

1.1 GENERAL INTRODUCTION

From nutritional point of view, proteins provide to human body energy and amino acids which are essential and crucial for growth and maintenance. In the last two decades, functional properties of proteins have attracted great attention of scientists and been widely discussed. Among them is the role of proteins as physiologically active components benefiting human health. Many proteins exert their physiological actions either directly as an intact form or upon enzymatic hydrolysis *in vivo* or *in vitro*. It has been commonly recognized that food proteins are rich sources of biologically active peptides. These peptides are inactive within the raw parent proteins, but can be liberated through gastrointestinal digestion or during food processing and become physiologically active. In recent years, various functions of these peptides have been recognized and understood, such as anti-hypertensive, anti-oxidative, anti-microbial, anti-appetizing, hypocholesterolemic, immunomodulatoy, opiate, etc (Qian *et al.*, 2008; Cerovsky *et al.*, 2008; Fang *et al.*, 2007; Kim *et al.*, 2007; Seppo *et al.*, 2003; Shin *et al.*, 2001).

Chronic diseases such as hypertension, chronic kidney disease, and cancer are still the top health problems in Canada. Hypertension is directly

connected to stroke, heart attack, and kidney failure. Cancer death rates for both men and women have decreased from 1988 to 2005, but cancer incidence rate has increased for women, due largely to lung cancer and breast cancer. Expenditure for treatment is huge, which makes prevention crucial. As a result, consumer demand for healthy nutritious food with additional health-promoting functions, such as functional foods and nutraceuticals, increases. Bioactive peptides have been shown to be able to provide specific beneficial effects going beyond basic nutrient supply; therefore, incorporation of these peptides as ingredients into functional foods and nutraceutical products is of great potential with increased consumer awareness and promotion of healthy eating and lifestyle.

1.2 OBJECTIVES

The objectives of this research are:

1) Evaluation of bitterness properties of thermolysin-digested PPH (<3 kDa);
2) Determination of bioavailability of thermolysin-digested PPH (< 3 kDa), and its short-term effects on ACE activity and angiotensin II levels in the blood and organs of normotensive rats;
3) Determination of long-term antihypertensive effects of thermolysin-digested PPH (< 3 kDa) in a chronic kidney disease rat model;
4) Separation and purification of low molecular weight (< 1 kDa) peptides from an alcalase-digested PPH;
5) Determination of enzyme (ACE, renin, CaMPDE1) inhibitory activities of the peptide fractions;
6) Identification of amino acid sequences of the purified peptides;
7) Determination of enzyme inhibitory activities and IC_{50} values of synthetic forms of the sequenced peptides.

1.3 HYPOTHESES

1) Thermolysin digest of pea proteins contains high levels of hydrophobic amino acids which contribute to the bitterness of PPH.

2) Bioactive peptides in the PPH (<3 kDa) are bioavailable and can influence blood pressure, ACE activity, and angiotensin II levels on both short-term and long-term feeding basis.

3) PPI can be hydrolyzed by alcalase to produce small molecular weight (<1 kDa) peptides with inhibitory activities against ACE, renin, and CaMPDE1.

CHAPTER 2

LITERATURE REVIEW

2.1 PEA PROTEINS

Pea is defined as the small spherical seed of the legume plant *Pisum sativum*. It is a natural edible plant traceable to thousands of years ago. Pea seeds have relatively high protein content (Fan *et al.*, 1994); however, they contain at the same time a variety of anti-nutritional factors that may negatively influence protein quality (Fredrikson *et al.*, 2001). Therefore, obtaining high quality pea protein isolate with improved nutritional property is of great importance. Total protein content of pea is between 18-30 wt%, and the technology for producing pea protein isolate has been well studied and documented (Adsule *et al.*, 1989; Gueguen & Barbot, 1988; Schroeder, 1982). Pea protein is composed mainly of albumin (18-25%) and globulin (55-65%) as the major storage proteins (Gueguen & Cerletti, 1994). Tryptophan and sulphur-containing amino acids, such as methionine and cysteine are deficient in pea proteins, while the levels of lysine and arginine are quite high. The level of acidic acid (aspartic acid, asparagines, glutamic acid, glutamine) is also high and is similar to that of other seed storage proteins. Although nutritional values of pea proteins are less studied compared to other food proteins, it has been demonstrated that albumin and globulin fractions of pea protein are of good nutritional values

for human, such as constituting highly digestible proteins and providing essential amino acids. Some of them have been previously reviewed (Mariotti *et al.*, 2001; Savage & Deo, 1989).

Peas represent the fifth largest field crop of Canada after wheat, barley, canola, and oat. Peas are mainly grown in Manitoba, Saskatchewan, and Alberta. Majority of peas produced in Canada are yellow and green peas, other types include marrowfat and maple peas. There's no difference of nutrient content between yellow and green peas, however, there may be some small difference between some varieties due to the size of pea and thickness of the hulls (Pulse Canada). For example, maples peas have smaller seed size than yellow, green, and marrowfat peas, and seeds of marrowfat peas contain higher level of tannin and therefore usually used for bird feed. Peas grown in Canada have an average of 23% crude protein and 1.67% high level of essential amino acid lysine (Pulse Canada).

2.2 BIOACTIVE PEPTIDES

2.2.1 Definition

As increased interest has been paid to identify biologically active components that physiologically benefit human body, the term "bioactive" needs to be clarified. According to Schrezenmeir *et al.* (2000), bioactive components are *food components that can affect biological processes or*

substrates and hence have an impact on body function or condition and ultimately health. Besides, 1) *for a dietary component to be considered 'bioactive' it should impart a measurable biological effect at a physiologically realistic level*, and 2) *the 'bioactivity' measured has the potential (at least) to affect health in a beneficial way, thus excluding from this definition potentially damaging effects.*

2.2.2 Food Sources of Bioactive Peptides

—*"Bioactive substances in milk and colostrums is mother language on a substrate basis"* (Schrezenmeir *et al.*, 2000). By far, bovine milk seems to be the richest food source of bioactive peptides. A great number of peptides with various physiological functions have been identified and purified from bovine milk. However, apart from milk, bioactive peptides have been discovered from other food sources such as egg, animal meat, gelatine, all kinds of fish (tuna, salmon, sardine, herring, sole, mudfish, shark, wakame, jelly fish, etc), wheat, soybean, rice, rye, oat, pumpkin, pea, sesame, spinach, etc. Table 1 lists a number of identified peptides and their food sources.

2.2.3 Bioavailability of Bioactive Peptides

It is difficult to establish a direct relationship between *in vivo* and *in vitro* activities of bioactive peptides because *in vitro* activities do not always reflect their real activities *in vivo*. This is due to the bioavailability of these

peptides after oral consumption. In order to exert *in vivo* effects, bioactive peptides need to be absorbed from the gastrointestinal tract (GIT) and reach target organs in minimum effective concentrations. It has long been known that intact di- and tri-peptides can be easily absorbed through the intestine. An early study provided evidence for the intact absorption of dipeptides in the gut lumen (Adibi, 1971). Intact absorption of dipeptides VY was also investigated by Matsui and his team. After oral administration, plasma VY level increased with dose administered, indicating that exogenous VY could be absorbed intact into human blood (Matsui *et al.*, 2002). Intact tripeptide VPP was observed to be transported across the Caco-2 cell monolayer in a significant amount, and this transport was hardly inhibited by any competitive substrate (Satake *et al.*, 2002).

Actually, absorption of small peptides, mainly di- and tripeptides, across brush border membrane are even faster than that of free amino acids (Webb, 1990). Peptide transporters PepT1 and PepT2 were identified to use a transmembrane electrochemical proton gradient as the driving force and had broad substrate specificity (Matsui *et al.*, 2002; Yang *et al.*, 1999). Other transport systems for intact small peptides such as paracellular and transcellular routes have also been observed but are under debate for their significance. Knowledge about the absorption of bioactive peptides with large molecular weight is not very much known.

Table 1. Food sources of bioactive peptides.

Food sources	Bioactive peptides example	Reference
Milk	VPP	Nakamura *et al.*, 1995
	IPP	
Bovine placenta	YXFLGLPGXT	Fang *et al.*, 2007
Egg	FFGRCVSP	Fujita *et al.*, 2000
Pork	VKKVLGNP	Katayama *et al.*, 2007
Chicken	LKP	Fujita *et al.*, 2000
Royal jelly	IY, VY, IVY	Tokunaga *et al.*, 2004
Squid	YALPHA	Wako *et al.*, 1996
Sardine	KVLAGM	Ariyoshi *et al.*, 1993
Mudfish	RQRVEELSKFSKKGAAARRRK	Park *et al.*, 1997
Oyster	LKQELEDLLEKQE	Qian *et al.*, 2008
Bonito	LRP	Matsumura *et al.*, 1993
Wakame	YH, KY, FY, IY	Suetsuna *et al.*, 2004
Broccoli	YPK	Lee *et al.*, 2006
Soy	LLPHH	Chen *et al.*, 1998
Wheat	YPISL	Fukudome *et al.*, 1993

Rice	TQVY	Li *et al.*, 2007
Sesame	LVY, LQP, LKY	Nakano *et al.*, 2006
Spinach	MRWRD, MRW, LRIPVA, IAYKPAG	Yang *et al.*, 2003
Rapeseed	IY, RIY, VW, VWIS	Marcazk *et al.*, 2003
Pea	LKP	Vermeirssn, 2003
Maize	IRA	Miyoshi, 1991
Garlic	FY	Suetsuna, 1998
Fig	LYPVK	Ariyoshi *et al.*, 1993
Sake lee	YW	Saito *et al.*, 1994

However, *in vivo* potency of peptides decreases as the chain length increases due to potential breakdown by gastrointestinal proteases. It is not completely known if there is any chance that some of these peptides can be absorbed integrally or in fact whether they are not absorbed in the intestinal tract but act either directly in the intestinal tract or through certain receptors and cell signalling in the intestine. Further studies are needed to add to the current state of knowledge on the relationship between peptide size and bioactive potential following oral administration.

Once inside the enterocyte, peptides are hydrolyzed to free amino acids by various intracellular peptidases. However, a significant amount of small peptides can escape the digestion and enter the blood circulatory system in intact form via two major transport routes: paracellular and transcellular routes (Grimble, 2000; Gardner, 1984; Gardner, 1983). Large water-soluble peptides are able to pass the tight junctions between cells paracellularly, and highly lipid-soluble peptides can diffuse via the transcellular route. Blood contains substantial activities of peptidase; therefore half-life of some peptides can be very short (Gardner, 1998).

Masuda *et al.* (1996) showed that six hours after oral administration of Calpis™ sour milk (contained VPP and IPP) to spontaneously hypertensive rats (SHR), ACE activity in abdominal aorta was significantly lower than that of the rats given saline; VPP and IPP peptides were detected by HPLC in aortal tissues, which confirmed absorption. In

another study, tissue distribution of the anti-hypertensive dipeptide VY was investigated in SHR after single oral administration of 10 mg/kg body weight (bw). For the first time, it was reported that VY was highly and widely accumulated in the tissues, which included kidney, lung, liver, heart, abdominal aorta, and mesenteric artery, rather than just in the circulatory system (Matsui *et al.*, 2004). And the major accumulation of VY occurred in the kidneys and lungs. Besides, the tyrosine residue of VY peptide was not sulphated when incubated with a liver homogenate taken from the SHR, implying that the dipeptide was not sulfated in the liver but was secreted into the blood circulatory system in an intact form.

Various activities of bioactive peptides are influenced by their bioavailability due to susceptibility to digestion, absorption, and metabolism. Currently, few studies have been undertaken to investigate the absorption and the metabolism of bioactive peptides. More research is needed to elucidate mechanism of peptide metabolism with emphasis on *in vivo* bioavailability so as to make the peptides relevant for the formulation of functional foods and nutraceuticals.

2.2.4 Classification of Bioactive Peptides

Bioactive peptides can usually be classified into 3 groups based on their fate after oral consumption (Fujita *et al.*, 2000).

1) True inhibitor type: these peptides are resistant to cleavage by

target enzymes and gastrointestinal proteases, and inhibitory concentration that reduced enzyme activity by 50% (IC_{50}) values is not affected after pre-incubation with these enzymes. Such peptides include IY, IKP, LKP, and IWH from bonito, VPP and IPP from milk, and IKW from chicken.

2) Substrate type: these peptides are susceptible to degradation by either gastrointestinal proteases or target enzymes, and release peptides with lower or even no activity and higher IC_{50} values. IC_{50} value of peptide FKGRYYP from chicken increased from 0.55 μM to 34 μM after incubation with ACE, and the released peptide fragments showed no hypotensive effect after oral administration in SHR.

3) Pro-drug type: these peptides, usually long-chain precursor peptides, are hydrolysed by either gastrointestinal proteases or target enzymes to produce peptides with true activities and lower IC_{50} values. Characteristics include bioactivity *in vivo* without sufficient activity *in vitro* and long-lasting effect after oral administration. The most well known is peptide LKPMN (IC_{50} = 2.4 μM) which produced LKP (IC_{50} = 0.32 μM) and had prominent antihypertensive activity in SHR. Peptide IVGRPRHQG did not have antihypertensive effect until it was digested in the GIT by chymotrypsin to produce HQG and IVGRPR.

2.2.5 Various Health Benefits of Bioactive Peptides

A wide range of health benefits of bioactive peptides has been discovered, investigated and described in some details (Table 2), including anti-hypertensive, anti-oxidative, anti-lipemic, anti-microbial, anti-thrombotic, opiate, hypocholesterolemic, immunomodulating, mineral-binding, growth promoting, etc.

2.2.5.1 Anti-hypertensive bioactive peptides

Hypertension is one of the most common chronic diseases worldwide, and the number one killer in North America. Over five million Canadians have hypertension, and about 20% of the world's adult population suffer from hypertension. ACE is an enzyme within the RAS that plays an important role in regulating human blood pressure (BP). Within RAS, angiotensinogen is hydrolyzed by renin to yield angiotensin I, which is then hydrolyzed by ACE to give angiotensin II, a very potent vasoconstrictor. Excessive activities of ACE lead to undesirably high levels of angiotensin II and development of hypertension (Segall *et al.*, 2007; Yang *et al.*, 1970). Therefore, ACE inhibitors are effective in lowering BP and prevent cardiovascular diseases. The anti-hypertensive activity of ACE–inhibitory peptides has been confirmed both *in vitro* and *in vivo* through various reports.

Isolation of this type of peptides was first reported by Ferreira (1970) though at that time the ability of these peptides to act as ACE inhibitors

was not realized. ACE inhibitors were not reported until 1971 when Ondetti and his colleagues first isolated ACE-inhibitory peptides from venom of the snake *Bothrops jararaca* and designed a series of ACE inhibitors based on known chemical and kinetic properties of the enzyme (Ondetti *et al.*, 1977; Ondetti *et al.*, 1971). Since then a great number of ACE-inhibitory bioactive peptides have been isolated and purified from various sources, especially food proteins. Table 3 lists some of the food-derived ACE inhibitory peptides and their *in vitro* potency while Table 4 shows hypotensive effects of ACE-inhibitory peptides after oral administration to animals and human beings. As shown in Table 4, several BP-lowering ACE-inhibitory peptides are small in size (usually less than 8 amino acid residues) with low molecular weights; most of them being di- and tri-peptides. This is consistent with previous studies that showed di- and tri-peptides could be absorbed intact and quickly through the small intestine without being decomposed by digestive enzymes and were then transported to various organs (Daniel, 2004; Matsui *et al.*, 2004; Matsui *et al.*, 2002; Webb, 1990). The data in Table 4 also agree with the suggestion that the active site of ACE can not accommodate large peptide molecules (Natesh *et al.*, 2003).

Table 2. Health benefits of food protein-derived bioactive peptides.

Cardiovascular system	Gastrointestinal system	Immune System	Nervous system
Anti-hypertensive	Anti-microbial	Anti-microbial	Opioid agonist
Anti-oxidative	Anti-appetizing	Immunomodulatoy	Opioid-antagonist
Anti-thrombotic	Mineral binding	Cytomodulatory	
Anti-lipidemic	Opioid agonist	Opioid agonist	
Hypocholesterolemic	Opioid antagonist	Opioid antagonist	

Table 3. Food protein-derived ACE-inhibitory peptides with *in vitro* IC_{50} values less than 50 μM.

Peptide sequence	Source	IC_{50} (μM)	Reference
Bovine milk			
VAP	αs_1-casein (f25-27)	2.0	Maruyama *et al.*, 1987
ALKAWSVAR	Serum albumin (f208-216)	3.0	Chiba *et al.*, 1991
YELQNKIHPFAQT QSLVYPFPGPIPNS	β-casein	4.0	Yamamoto *et al.*, 1994
FALPQY	αs_2-casein (f174-179)	4.3	Tauzin *et al.*, 2002
FALPQYLK	αs_2-casein (f174-181)	4.3	Tauzin *et al.*, 2002
IPP	β-casein (f74-76)	5.0	Nakamura *et al.*, 1995
KVLPVP	β-casein (f169-174)	5.0	Maeno *et al.*, 1996
FFVAP	αs_1-casein (f23-27)	6.0	Maruyama *et al.*, 1985
GPAGAHyP	gelatine	8.3	Oshima *et al.*, 1979
GPPGAHyP	gelatine	8.3	Oshima *et al.*, 1979
VPP	β-casein (f84-86) κ-casein (f108-110)	9.0	Nakamura et al, 1995
FVAP	αs_1-casein (f24-27)	10.0	Maruyama *et al.*, 1987
GPHyPGTDGAHyP	gelatine	10.5	Oshima *et al.*, 1979
GPHyPGAPHyP	gelatine	11.3	Oshima *et al.*, 1979
FPQYLQY	αs_2-casein (f92-98)	14.0	Tauzin *et al.*, 2002
TVY	αs_2-casein (f182-184)	15.0	Tauzin *et al.*, 2002
AVPYPQP	β-casein (f177-183)	15.0	Maruyama *et al.*, 1987
TTMPLW	αs_1-casein (f194-199)	16.0	Maruyama *et*

			al., 1987
IHPFAQTQSLVYP	β-casein (f49-61)	19.0	Kohmura *et al.*, 1990
LLYQQPVLGPVRG PFPIIV	β-casein	21.0	Yamamoto *et al.*, 1994
YKVPQL	αs_1-casein (f104-109)	22.0	Maeno *et al.*, 1996
FAQTQSLVYP	β-casein (f52-61)	25.0	Kohmura *et al.*, 1990
PPQSVLSLSQSKV LPVPE	β-casein	25.0	Yamamoto *et al.*, 1994
HPFAQTQSLVYP	β-casein (f50-61)	26.0	Kohmura *et al.*, 1990
GPIGSVGAHyP	gelatine	31.8	Oshima *et al.*, 1979
PLW	αs_1-casein (f197-199)	36.0	Maruyama *et al.*, 1987
GPAGAPGAA	gelatine	37.0	Oshima *et al.*, 1979
KIHPFAQTQSLVYP	β-casein (f48-61)	39.0	Kohmura *et al.*, 1990
SKVLPVPE	β-casein	39.0	Yamamoto *et al.*, 1994
SLVYP	β-casein (f57-61)	40.0	Kohmura *et al.*, 1990
QSLVYP	β-casein (f56-61)	41.0	Kohmura *et al.*, 1990
ALPMHIR	β-lactoglobulin (f142-148)	42.6	Mullally *et al.*, 1997
VYP	β-casein (f59-61)	44.0	Kohmura *et al.*, 1990
LW	αs_1-casein (f198-199)	50	Maruyama *et al.*, 1987
Human milk			
SFQPQPLIYP	human β-casein (f43-52)	1.4	Kohmura *et al.*, 1990
VRP	human κ–casein (f63-65)	2.2	Kohmura *et al.*, 1990
TAP	human κ–casein (f18-20)	3.5	Kohmura *et al.*, 1990
IPP	human κ–casein (f96-98)	5.0	Kohmura *et al.*, 1990
YANPNVVRP	human κ–casein (f57-65)	7.8	Kohmura *et al.*, 1990

KIYPSFQPQPLIYP	human β–casein (f39-52)	8.6	Kohmura *et al.*, 1990
MYY	human κ–casein (f24-26)	9.6	Kohmura *et al.*, 1990
PAVVRP	human κ–casein (f60-65)	18.0	Kohmura *et al.*, 1990
NPAVVRP	human κ–casein (f59-65)	19.0	Kohmura *et al.*, 1990
YYPQIMQY	human αs1-casein (f136-143)	24.8	Kohmura *et al.*, 1990
ANPNVVRP	human κ–casein (f58-65)	25.0	Kohmura *et al.*, 1990
QKTAP	human κ–casein (f16-20)	30.0	Kohmura *et al.*, 1990
PTPAP	human κ–casein (f118-122)	33.0	Kohmura *et al.*, 1990
KTAP	human κ–casein (f17-20)	37.0	Kohmura *et al.*, 1990
NNVMLQW	human αs1-casein (f164-170)	41.0	Kim *et al.*, 1999
Animal			
LKP	chicken muscle	0.3	Fujita *et al.*, 2000
LKP	Bonito	0.32	Yoshikawa *et al.*, 2000
FKGRYYP	chicken muscle	0.6	Fujita *et al.*, 2000
LRP	Bonito	1.0	Matsumura *et al.*, 1993
IKP	Bonito	1.6	Fujita *et al.*, 2000
IRP	Bonito	1.8	Matsumura *et al.*, 1993
GKKVLQ	porcine hemoglobin	1.9	Mito *et al.*, 1996
IY	Bonito	2.1	Fujita *et al.*, 2000
FQKVVAK	porcine hemoglobin	2.1	Mito *et al.*, 1996
VRP	Bonito	2.2	Matsumura *et al.*, 1993
LKPMV	Bonito	2.4	Yoshikawa *et al.*, 2000
IVGRPRHQG	chicken muscle	2.4	Fujita *et al.*,

			2000
GPL	pollack skin gelatin	2.65	Byun *et al.*, 2002
LAP	chicken muscle	3.5	Fujita *et al.*, 2000
IWH	Bonito	3.5	Fujita *et al.*, 2000
IWHHT	Bonito	5.8	Fujita *et al.*, 2000
FQKVVA	porcine hemoglobin	5.8	Mito *et al.*, 1996
FQKVVAG	porcine hemoglobin	7.4	Mito *et al.*, 1996
LKA	chicken muscle	8.5	Fujita *et al.*, 2000
YALPHA	squid liver	9.8	Wako *et al.*, 1996
LKVGVKQY	Sardine	11	Ariyoshi *et al.*, 1993
FQKPKR	chicken muscle	14	Fujita *et al.*, 2000
PANIKWGD	Porcine	21	Ariyoshi *et al.*, 1993
WPEAAELMMEVDP	tuna dark muscle	21.6	Qian *et al.*, 2007
CWLPVY	dried tuna	22.2	Astawan *et al.*, 1995
GYALPHA	squid liver	27.3	Wako *et al.*, 1996
KVLAGM	Sardine	30	Ariyoshi *et al.*, 1993
VAWKL	Tuna	31.8	Astawan *et al.*, 1995
Egg			
FFGRCVSP	Ovalbumin	0.4	Fujita *et al.*, 2000
ERKIKVYL	Ovalbumin	1.2	Fujita *et al.*, 2000
FGRCVSP	Ovalbumin	6.2	Fujita *et al.*, 2000
LW	Ovalbumin	6.8	Fujita *et al.*, 2000
FCF	Ovalbumin	11.0	Fujita *et al.*, 2000

NIFYCP	Ovalbumin	15.0	Fujita *et al.*, 2000
Plant			
LKP	pea vicilin	0.3	Vermeirssn, 2003
LRP	maize α-zein	0.3	Miyoshi, 1991
LRIPVA	Spinach	0.38	Yang *et al.*, 2003
IVY	wheat germ	0.48	Saito *et al.*, 1994
MRW	Spinach	0.6	Yang *et al.*, 2003
LGP	pea albumin	0.7	Vermeirssn, 2003
GGY	Sake	1.3	Saito *et al.*, 1994
VW	sake lees	1.4	Saito *et al.*, 1994
AKYSY	red algae	1.5	Suetsuna, 1998
IW	Wakame	1.5	Sato *et al.*, 2002
LSP	maize α-zein	1.7	Miyoshi, 1991
LQP	maize α-zein	1.9	Miyoshi, 1991
IY	pea vicilin	2.1	Vermeirssn, 2003
MRWRD	Spinach	2.1	Yang *et al.*, 2003
IY	sake lees	2.4	Saito *et al.*, 1994
PRY	sake lees	2.5	Saito *et al.*, 1994
IY	Wakame	2.7	Suetsuna & Nakano, 2000
IY	red algae	2.7	Suetsuna, 1998
IAP	wheat gliadin	2.7	Motoi *et al.*, 2003
VW	Wakame	3.3	Sato *et al.*, 2002
FY	Garlic	3.7	Suetsuna, 1998
FY	Wakame	3.7	Suetsuna & Nakano, 2000
LAY	maize α-zein	3.9	Miyoshi, 1991
PR	sake lees	4.1	Saito *et al.*, 1994
IAYKPAG	Spinach	6.2	Yang *et al.*, 2003

LYPVK	fig tree latex	4.5	Ariyoshi *et al.*, 1993
DLP	Soy	4.8	Wu & Ding, 2002
HHL	Soy	5.0	Shin *et al.*, 2001
LRY	red algae	5.1	Suetsuna, 1998
VY	wheat germ	5.2	Saito *et al.*, 1994
IY	Wakame	6.1	Sato *et al.*, 2002
IRA	maize α-zein	6.4	Miyoshi, 1991
VY	Sake	7.1	Saito *et al.*, 1994
MKY	red algae	7.3	Suetsuna, 1998
VWY	sake lees	9.4	Saito *et al.*, 1994
IYPR	sake lees	10.0	Saito *et al.*, 1994
YW	pea albumin	10.0	Vermeirssn, 2003
VY	pea albumin	10.0	Vermeirssn, 2003
VSP	maize α-zein	10.0	Miyoshi, 1991
RY	sake lees	10.5	Saito *et al.*, 1994
YW	sake lees	10.5	Saito *et al.*, 1994
DG	pea albumin	12.0	Vermeirssn, 2003
DG	Soy	12.3	Wu & Ding, 2002
VK	pea vicilin	13.0	Vermeirssn, 2003
LAA	maize α-zein	13.0	Miyoshi, 1991
VAA	maize α-zein	13.0	Miyoshi, 1991
AVNPIR	fig tree latex	13.0	Ariyoshi *et al.*, 1993
KY	wakame	13.0	Suetsuna & Nakano, 2000
LVR	fig tree latex	14.0	Ariyoshi, 1993
AF	pea vicilin	15.2	Vermeirssn, 2003
VAY	maize α-zein	16.0	Miyoshi, 1991
YPR	sake lees	16.5	Saito *et al.*,

YPRY	sake lees	17.4	1994 Saito *et al.*,
LY	pea albumin	18.0	1994 Vermeirssn,
TQVY	rice protein	18.2	2003 Li *et al.*, 2007
FWN	sake lees	18.3	Saito *et al.*,
AW	Wakame	18.8	1994 Sato *et al.*, 2002
YNKL	Wakame	21.0	Suetsuna &
FY	maize α-zein	25.0	Nakano, 2000 Miyoshi, 1991
HY	sake lees	26.1	Saito *et al.*,
KF	Wakame	28.3	1994 Suetsuna &
NY	Garlic	32.6	Nakano, 2000 Suetsuna, 1998
LNP	maize α-zein	43.0	Miyoshi, 1991
YKY	Wakame	43.5	Suetsuna &
MF	pea albumin	45.0	Nakano, 2000 Vermeirssn,
KFY	Wakame	45.0	2003 Suetsuna &
NF	Garlic	46.3	Nakano, 2000 Suetsuna, 1998

*: IC_{50} refers to peptide concentration that inhibits 50% of ACE activity.

Table 4. Hypotensive effects of ACE-inhibitory protein hydrolysates and peptides in animal and human beings.

Source	Peptide	Subjects	Dose (oral)	BP change (mmHg)	References
Human studies					
Whey protein hydrolysate	___	30 subjects with borderline hypertension	20 g/d for 6 wks	SBP↓8 DBP↓5.5	Pins & Keenan, 2006
Sour milk powder	VPP IPP	40 subjects with high normal BP (HN), 40 subjects with mild hypertension (MH)	12 g/d for 4 wks	HN: DBP↓5 MH: SBP↓11.2, DBP↓6.5	Aihara *et al.*, 2005
Sour milk	VPP IPP	96 subjects with hypertension	250 mL twice/d for 10 wks	SBP↓5.1 DBP↓1.1	Jauhiainen *et al.*, 2005
Casein hydrolysate	VPP IPP	144 subjects with hypertension	200 mL test drink/d (1.47 mg VPP+1.6 mg IPP)	SBP↓5.9 at 12 wks DBP↓3.2 at 12 wks	Sano *et al.*, 2005
Casein hydrolysate	VPP IPP	131 subjects with hypertension (4 groups)	a. 0 mg for 6 wks b. 1.8 mg for 6 wks c. 2.5 mg for 6 wks d. 3.6 mg for 6 wks	a. SBP↓1.7 b. SBP↓6.3 c. SBP↓6.7 d. SBP↓10.1	Mizuno *et al.*, 2005
Sour milk	VPP IPP	46 male with hypertension, 23-59 y	160 g/d for 4 wks (~2 mL/kg bw)	SBP↓5.2 DBP↓2.0	Mizushima *et al.*, 2004
Sour milk	VPP IPP	36 male and 24 female with hypertension	Study I: 5 dL/d for 8-10 wks Study II: 5 dL/d for	Study I: SBP↓15.8, DBP↓10.3 Study II:	Tuomilehto *et al.*, 2004

			5 wks	SBP↓10.5, DBP↓5.7	
Liquid yogurt	VPP IPP	34 male and 72 female with hypertension	150 g × 2/d for 12 wks	SBP↓6.1 DBP↓3.8	Nakamura *et al.*, 2004
Sour milk	VPP IPP	39 subjects with hypertension, 32-61 y	150 mL (5.25 mg peptide)/d for 21 wks	SBP↓6.7 DBP↓3.6	Seppo *et al.*, 2003
Sour milk	VPP IPP	17 subjects with mild hypertension	150 mL (5.25-6 mg peptide)/d for 8 wks	SBP↓10.1 DBP↓9.4	Seppo *et al.*, 2002
Sardine muscle	VY	12 male subjects with mild hypertension, 39-59 y	a. 6 mg b. 12 mg in 100mL drink	No significant or marked BP reduction observed	Matsui *et al.*, 2002
Dried bonito	LKPNM	61 subjects with borderline and mild hypertension	1.5 g (5 mg peptide)/d for 5 wks	SBP↓11.7 DBP↓6.9	Fujita *et al.*, 2001
Sardine muscle	VY	29 subjects with high normal or mild hypertension	3 mg × 2/d for 4 wks	SBP↓9.7& DBP↓5.3 at 1 wk; SBP↓9.3& DBP↓5.2 at 4 wks	Kawasaki *et al.*, 2000
Dried bonito	LKPNM LKP	30 subjects with hypertension or borderline hypertension	3 g/d for 8 wks	SBP↓12.7	Fujita & Yoshikawa, 1999
Sour milk	VPP IPP	30 subjects, 40-86 y	95 mL/d for 8 wks (~2 mL/kg bw)	SBP↓14.1 DBP↓6.9	Hata *et al.*, 1996
Casein hydrolysate	—	28 subjects with mild hypertension	10 g × 2/d for 4wks	SBP↓4.6 DBP↓6.6	Sekiya *et al.*, 1992
Animal studies					
Porcine skeletal muscle	VKKVLGNP	Male SHR, 12 wks	10 mg/kg bw	SBP↓24 at 3 h	Katayama *et al.*, 2007

Tuna dark muscle hydrolysate	WPEAAELMME VDP	Male SHR, 10 wks	10 mg/kg bw	SBP↓~18 at 3 h	Qian *et al.*, 2007
Sesame protein hydrolysate	LVY LQP LKY	Male SHR, 13 wks	a. 1 mg/kg bw b. 10 mg/kg bw	a. SBP↓~30 at 8 h b. SBP↓~25 at 8 h	Nakano *et al.*, 2006
Porcine muscle hydrolysate	—	Male SHR, 10 wks	a. 210 mg/kg bw b. 2100 mg/kg bw	a. SBP↓15.8 at 6 h b. SBP↓16.3 at 6 h, 22.9 at 8 h	Nakashima *et al.*, 2006
a. Captopril b. Sour milk	—	Male SHR	17wks	a. SBP↓111, DBP↓112 b. SBP↓37, DBP↓17	Miguel *et al.*, 2005
Casein hydrolysate	XP and XPP	SHR	a. 9.6 mg/kg bw b. 32 mg/kg bw c. 96 mg/kg bw	a. SBP↓14.4 at 5h b. SBP↓25.1 at 5h c. SBP↓31.0 at 5h	Mizuno *et al.*, 2004
Whey	WE80BG (commercial product), ®-lactosin B (ALPM)	SHR	2 mg/mL	SBP↓21.2 at 6 h SBP↓21.4 at 8 h	Murakami *et al.*, 2004
Sardine muscle	VY	Male SHR, 18 wks	10 mg/kg bw	SBP↓13.8 at 1 h, SBP↓44 at 9 h	Matsui *et al.*, 2004
Wakame	YH, KY, FY, IY	SHR	50 mg/kg bw	SBP↓50 at 3 h	Suetsuna *et al.*, 2004
Royal jelly hydrolysate	IY VY IVY	Male SHR, 10 wks	a. 10 mg/kg bw b. 50 mg/kg bw c. 100 mg/kg bw	a. SBP↓0.8 at 1 h b. SBP↓8.9 at 1 h c. SBP↓18.4 at 1 h	Tokunaga *et al.*, 2004
Spinach	a. MRW	Male SHR, 16-24 wks	a. 20 mg/kg bw	a. SBP↓20	Yang *et al.*,

	b. MRWRD		b. 30 mg/kg bw	at 2 h b. SBP↓15 at 4 h	2003
Wakame	a. VY b. IY c. FY	Male SHR, 18 wks	10 mg/kg bw	a. SBP↓18 at 9 h b. SBP↓19 at 9 h c. SBP↓ 21at 9 h	Sato *et al.*, 2002
Wakame hydrolysate	Peptide mixture	Male SHR, 7 wks	a. 0.1% hydrolysate b. 1% hydrolysate for 10 wks	a. SBP↓17 b. SBP↓17 compared to control at 10 wks	Sato *et al.*, 2002
Royal jelly hydrolysate	Peptides mixture	Male SHR, 10 wks	1 g/kg bw	SBP↓22.7 at 2 h	Matsui *et al.*, 2002
Sour milk	VPP IPP	SHR, 6 wks	(0.4 mg IPP + 0.6 mg VPP)/d for 14 wks	SBP↓21	Sipola *et al.*, 2002
a. Sour milk b. Purified peptides	VPP IPP	Female Wistar rats, 10-11/group	2.5-3.5 mg/kg/d for 12 wks	a. SBP↓17 b. SBP↓12	Sipola *et al.*, 2001
Soy protein hydrolysate	Peptide mixture	Female SHR, 7 wks	a. 100 mg/kg bw for1 mon b. 1000 mg/kg bw for 1 mon	a. SBP↓37.8 at 1 mon b. SBP↓39 at 1 mon	Wu & Ding, 2001
Dried bonito	LKPNM	SHR	a. 125 mg/kg bw b. 250 mg/kg bw c. 500 mg/kg bw	a. SBP↓7 at 4 h b. SBP↓9 at 2 h c. SBP↓15 at 4 h	Fujita *et al.*, 2001
Soybean paste	HHL	Male SHR	5 mg/kg bw (i.v.)×3 with interval of 20 min	SBP↓61 after 3rd injection	Shin *et al.*, 2001
Wheat germ	IVY	SHR, 12 wks	5 mg/kg bw (i.v.)	Mean BP↓19.2 at 8 min	Matsui *et*

hydrolysate					*al.*, 2000
Wakame	AIYK, YKYY KFYG, YNKL	Male SHR, 15 wks	50 mg/kg bw	SBP↓40-50 at 1-2 h for all peptides	Suetsuna & Nakano, 2000
Chicken muscle	a. LAP b. IKW c. LKP	SHR	10 mg/kg bw (i.v.)	a. SBP↓40 b. SBP↓50 c. SBP↓75	Fujita *et al.*, 2000
Ovalbumin hydrolysate	a. LW b. FFGRCVSP	SHR	10 mg/kg bw (i.v.)	a. SBP↓40 b. SBP↓50	Fujita *et al.*, 2000
Gouda cheese peptides	a. RPKHPIKHQ b. YPFPGPIPN	Male SHR, 12-16 wks	6.1-7.5 mg/kg bw	a. SBP↓9.3 at 6h b. SBP↓7 at 6h	Saito *et al.*, 2000
α-Lactalbumin	YGLF	Male SHR, 18-26 wks	a. 0.1mg/kg bw b. 1mg/kg bw	a. SBP↓23, DBP↓ 17 at 50-100 min b. SBP↓21, DBP↓ 15 at 50-100 min	Nurminen *et al.*, 2000
Dried bonito	LKPNM LKP	Male SHR, 16-25 wks	a. 8 mg/kg bw b. 2.25 mg/kg bw	a. LKPNM,SBP↓8 at 4h b. LKP, SBP↓7 at 2h	Fujita & Yoshikawa, 1999
Ovalbumin hydrolysate	RADHP	SHR, 15-30 wks	20 mg/kg bw	BP↓12.5 at 6 h	Matoba *et al.*, 1999
Red alga	IY, AKYSY LRY, MKY	Female SHR	200 mg/kg bw	BP↓53 at 1 h	Suetsuna, 1998
Whey protein	a. Proteinase K hydrolysate b. Actinase E hydrolysate c. Trypsin	Male SHR, 12 wks	8 mg/kg bw	a. SBP↓55 at 6 h b. SBP↓55 at 6 h c. SBP↓51 at 6 h	Abubarka *et al.*, 1998

	hydrolysate d. Papain hydrolysate e. Pepsin hydrolysate f. Thermolysin hydrolysate g. Chymotrypsin hydrolysate			d. SBP↓47 at 6 h e. SBP↓47 at 6 h f. SBP↓42 at 6 h g. SBP↓40 at 6 h	
Sour milk	VPP IPP	SHR	10 mL/kg bw	SBP↓26.4 at 6 h	Masuda *et al.*, 1996
Sour milk	—	Male SHR	—	SBP↓19.1	Nakamura *et al.*, 1996
Casein hydrolysate	a. KVLPVPQ b. LQSW c. RELEEL d. TKVIP e. AMKPW f. YKVPQL g. MKPWIQPK h. LLYQQPV i. AYFYP j. AMKPWIQPK	Male SHR, 18-25 wks	2 mg/kg bw	a. SBP↓24.1 at 6 h b. SBP↓1.6 at 6 h c. SBP↑1.3 at 6 h d. SBP↓9.2 at 6 h e. SBP↓4.6 at 6 h f. SBP↓12.5 at 6 h g. SBP↓2.9 at 6 h h. SBP↑1.2 at 6 h i. SBP↓4.3 at 6 h j. SBP↓0.6 at 6 h	Maeno *et al.*, 1996
Swine hemoglobine	a. FQKVVA b. GKKVLQ	SHR, 12-13 wks	50 mg/kg bw	a. SBP↓30 at 180 min b. SBP↓30 at 180 min	Mito *et al.*, 1996

a. Sour milk b. Purified peptides	VPP IPP	Male SHR	a. 5 mL/kg bw b. 0.6 mg/kg bw VPP, 0.3 mg/kg bw IPP	a. SBP↓17.7 at 8 h b. VPP: SBP↓26.7 at 8 h; IPP: SBP↓28.3 at 8 h	Nakamura *et al.*, 1995
Sardine muscle	VY	SHR, 12 wks	a. 20 mg/kg bw b. 50 mg/kg bw	a. SBP↓7.2, DBP↓28.8 b. SBP↓18, DBP↓35	Matsufuji *et al.*, 1995
Sour milk	IPP VPP	SHR, 20 wks	a. 5 mL/kg bw sour milk b. 0.6 mg VPP/kg bw c. 0.3 mg IPP/kg bw	a. SBP↓20 at 4 h, 21.8 at 6 h, 17.7 at 8 h; b. SBP↓24.6 at 4 h, 32.1 at 6 h, 26.7 at 8 h; c. SBP↓16.2 at 4 h, 21.7 at 6 h, 24.1 at 8 h	Nakamura *et al.*, 1995
Casein hydrolysate	25 peptides mixture	SHR, 20-23 wks	a. 75 mg/kg bw b. 15 mg/kg bw	a. SBP↓29.8 at 4 h, 24.1 at 10 h b. SBP↓14.2 at 4 h, 16.8 at 10 h	Yamamoto *et al.*, 1994
Sake and sake lees	a. IYPRY b. YGGY c. IY d. YP e. RY	SHR, 9 wks	100 mg/kg bw	a. SBP↓21 at 6 h b. SBP had no change c. SBP↓18 at 6 h d. SBP↓13 at 6 h e. SBP↓17 at 6 h	Saito *et al.*, 1994
Bonito	GVYPHK IRPVQ	SHR	100-200 mg/kg bw	BP depressor effects	Karaki et al, 1993
Maize α-zein	LRP	SHR, 11 wks	30 mg/kg bw (i.v.)	SBP↓15 at 2 min	Miyoshi, 1991

2.2.5.2 Anti-oxidative bioactive peptides

Certain bioactive peptides derived from food proteins have been confirmed to be able to prevent enzymatic and non-enzymatic peroxidation of essential fatty acids. Kim and co-workers purified from hoki frame protein a peptide, ESTVPERTHPACPDFN, which inhibited lipid peroxidation, decreased free radical-mediated cytotoxicity on MRC-5 cells and protected free radical-induced DNA damage (Kim *et al.*, 2007). Another peptide with sequence HGPLGPL was purified from trypsin digested hoki skin gelatine hydrolysate, and was proved to act as an antioxidant against linoleic acid peroxidation and increased anti-oxidative enzyme levels in cultured human hepatoma cells (Mendis *et al.*, 2005). Peptide fragments with anti-oxidative activities have been identified from soybean, milk, and other fish proteins as well (Kim *et al.*, 2001; Rival *et al.*, 2001a; Rival *et al.*, 2001b; Chen *et al.*, 1998). Table 5 lists some anti-oxidative protein hydrolysates and peptides from food sources.

Table 5. Food protein-derived bioactive peptides with anti-oxidative activities.

Peptide	Food source	IC_{50} (μM)	Reference
Protein waste hydrolysate	algae	superoxide: 7.5 hydroxyl: 8.3 DPPH: 58	Sheih *et al.*, 2009
LKQELEDLLEKQE	oyster	superoxide: 78.97 hydroxyl: 28.76	Qian *et al.*, 2008a
LEELEEELEGCE	bullfrog skin	superoxide: 34 hydroxyl: 12.8 peroxyl: 32.6 DPPH: 16.1	Qian *et al.*, 2008b
H-LNLPTAVYMVT-OH	tuna dark muscle	superoxide: 65.5 hydroxyl: 29.4 DPPH: 40.1	Je *et al.*, 2008
LEQQVDDLEGSLEQEKK	bullfrog muscle	superoxide: 176.1 hydroxyl: 162.7	Je *et al.*, 2007
ESTVPERTHPACPDFN	hoki	superoxide: 172.1 hydroxyl: 17.77 peroxyl: 18.99	Kim *et al.*, 2007
NADFGLNGLEGLA NGLEGLK	squid muscle	superoxide: 669.34, 573.83 hydroxyl: 497.32, 428.54	Rajapaske *et al.*, 2005
HGPLGPL	hoki	superoxide: 28.8 DPPH: 156.8	Mendis *et al.*, 2005a
FDSGPAGVL NGPLQAGQPGER	jumbo squid	hydroxyl: 90.9, 100.72	Mendis *et al.*, 2005b
YFYPEL	casein	superoxide: 79.2 hydroxyl: 251 DPPH: 98	Suetsuna *et al.*, 2000

2.2.5.3 Anti-lipidemic and hypocholesterolemic bioactive peptides

A hydrolysate from bovine hemoglobin was indicated to act as lipase inhibitor (Kagawa *et al.*, 1996). Peptide VVYP, which was one of the constituents, possessed most of the observed lipase-inhibitory effects. VVYP significantly decreased postprandial triglycerides in human subjects (Kagawa *et al.*, 1998). Several animal studies also demonstrated the anti-lipidemic effects of other types of food-derived bioactive peptides. After feeding a fish protein hydrolysate to Zucker (fa/fa) rats, lower total cholesterol level, higher HDL proportion, and lower acyl-CoA:cholesterol acyltransferase (ACAT) activity were observed compared to those fed a casein diet (Wergedahl *et al.*, 2004). Same effects were reported in other studies (Sirtori *et al.*, 2004; Tsutsumi *et al.*, 2000; Tani *et al.*, 1994; Borel *et al.*, 1989; Satouchi *et al.*, 1974) as shown in Table 6. Lipases and co-lipases are essential for the cleavage of triglycerides and absorption of fatty acids in the GIT; therefore, inhibition of these enzymes can impede triglyceride digestion and absorption, and lead to decreased lipid level in blood.

Bioactive peptides with hypocholesterolemic effect have been reported for milk protein-derived peptides as well as for soybean and other protein derived peptides (Nakade *et al.*, 2009; Hori G *et al.*, 2001; Nagaoka *et al.*, 2001; Nagaoka *et al.*, 1999). Although it has been suggested that possible mechanisms include suppression of cholesterol absorption by direct interaction between cholesterol-mixed micelles and bioactive

peptides, and the inhibition of absorption of bile acid in the ileum as well, further studies are needed to clarify the exact mechanisms.

2.2.5.4 Anti-microbial bioactive peptides

Anti-microbial peptides have been identified and isolated from many food proteins, especially milk. The mostly well-understood is lactoferrin, which is found in human and bovine milk. Lactoferrin is a good bioactive peptide precursor as it can be hydrolyzed by gastric acid and pepsin to lactoferricin (Chan & Li-Chan, 2005; Wakabayashi *et al.*, 2003), which is a bioactive peptide. Lactoferrin is also present in various fish, such as sardine and tuna. Initially, anti-microbial activity was associated with iron-sequestering activity, and later it was discovered that anti-bacterial peptides at the N-terminal fragment 1-47 of human lactoferrin and 17-41 of bovine lactoferrin represented domains with anti-microbial activity (Bellamy, 1992). *Misgurin* and *pardaxin* are anti-microbial bioactive peptides identified from loach and moses sole fish (Park *et al.*, 1997; Oren & Shai, 1996; Shai, 1994). Anti-microbial bioactive peptides are also found in many plants such as pumpkin, barley, rye, wheat, and oats (Segura *et al.*, 1999; Garcia-Olmedo, 1998). Some of the food-derived peptides with anti-microbial activities are listed in Table 7.

Anti-microbial activity is usually related with the disintegration of cell membrane where the bilayer of the cell membrane is the major target of the active peptide. A previous study showed that α–helix structure and

cationic character contribute to the anti-microbial activity of peptides (Epand & Vogel, 1999). Cationic property enables the peptides to bind with the anionic phospholipids-rich membrane which then triggers cell lysis.

2.2.5.5 Anti-thrombotic bioactive peptides

Bioactive peptides with anti-thrombotic activity are not properly understood and few reports exist on their properties. Several were reported to be encrypted within the glycomacropeptide that was released from κ-casein through cleavage by rennin, a milk clotting enzyme (Chabance *et al.*, 1995; Fiat *et al.*, 1993; Jollès *et al.*, 1986). They inhibited blood platelet aggregation and dose-dependently inhibited binding of fibrinogen γ-chain binding to platelet surface fibrinogen receptors. The main anti-thrombotic peptide corresponds to position 106-116 of κ-casein with the amino acid sequence as MAIPPKKNQDK, and some smaller fragment can be found at position 106-112, 112-116, and 113-116.

2.2.5.6 Immunomodulatory bioactive peptides

Many peptides, especially those derived from milk, display immunomodulatory functions, measured as lymphocyte proliferation, antibody synthesis, cytokine regulation and natural killer cell activity (Horiguchi *et al.*, 2005; Matar *et al.*, 2003; Meisel & FitzGerald, 2003; Gill *et al.*, 2000; Meisel, 1997; Fiat *et al.*, 1993). After intravenous administration, peptides derived from s_1-casein stimulated phagocytosis of sheep red blood cell and protected the mice from Klebsiella pneumoniae

infection (Migliore-Samour *et al.*, 1989). Hydrolysis of β-casein released a hexapeptide PGPIPN (residue 63-68) which promoted antibody formation and accelerated phagocytosis in vitro, and a tripeptide LLY (residue 191-193) which in addition to promoting antibody formation and accelerating phagocytosis, also enhanced antigen-dependent T-cell proliferation (Jollès *et al.*, 1988; Jollès & Migliore-Samour, 1986). Murine humoral immune response of mice was found enhanced after feeding fed hydrolysed α-lactalbumin from whey, and this effect involved modulation of both B lymphocyte and T helper cell activities (Bounous & Kongshavn, 1985). From bovine placenta, Fang *et al.* (2007) isolated a thermostable peptide which dose-dependently stimulated lymphocytes proliferation. It has been revealed that these peptides may alleviate allergic reactions in atopic humans and enhance mucosal immunity in the GIT, in which way they may regulate the development of the immune system in newborn infants (Korhonen & Pihlanto, 2003). Additionally, immunomodulatory peptides have been suggested to have anti-tumor effects. Structure-activity relationship and the mechanism by which food protein-derived peptides exert their immunoregulatory effects are not clearly known. Some of the immunoregulatory peptides are listed in Table 8.

Table 6. Anti-lipidemic and hypocholesterolemic effects of food protein-derived bioactive peptides.

Peptide	Food source	Subject	Dose (oral)	Anti-lipidemic & Hypocholesterolemic Effect	Reference
Human					
Protein hydrolysate	Soy	21 Male with serum total cholesterol > 220 mg/dL	3 g/d for 3 mon	Total cholesterol↓15%, LDL↓27.7%, (LDL/HDL)↓47.4%	Hori *et al.*, 2001
VVYP	Globin	22 Healthy male, 20-24y	1 or 4 g	Increase in chylomicron triglyceride concentration was significantly lower than control group	Kagawa *et al.*, 1998
Animal					
Protein hydrolysate	Cattle heart	Male Wistar rats, 5 wks	116.6 g/kg for 7 d	Total cholesterol, (LDL+VLDL), and liver total lipid↓ compared to casein group	Nakade *et al.*, 2009
Protein extract	White lupin	Male Sprague-Dawley rats	50 mg/d for 2 wks	(VLDL+LDL)↓30%	Sirtori *et al.*, 2004
Protein	Salmon	Male Zucker (fa/fa)	20 g per 100g	Cholesterol↓34%	Wergedahl *et al.*,

hydrolysate		rats	crude protein for 22-23d	Hepatic lipid level↓36%	2004
β–Lactoglobulin hydrolysate (IIAEK)	Milk	Male Wistar rats, 4 wks	30 % (w/w) for 3d	Compared with control, serum total cholesterol was 39 mg/dl lower, (VLDL+LDL) was 57 mg/dL lower, HDL was 18.5 mg/dL higher	Nagaoka *et al.*, 2001
Water extract of defatted rice bran	Rice bran	Male Sprague-Dawley rats, 4 wks	a. 0.5 g/mL/kg bw b. 1g/mL/kg bw c. 10 % for 4 wks	a. triglycerides↓2 mg/dL b. triglycerides↓10 mg/dL c. Visceral fat weight was reduced	Tsutsumi *et al.*, 2000
VVYP	Globin	Male ICR mice, 6 wks Male Wistar rats, 6 wks Male and female Beagle dogs, 8-10 kg	250 mg/mL mixed with olive oil (250 mg for mice, 1 g for rat, 20 g for dog)	Dose dependent hypotriglyceridemic effect	Kagawa *et al.*, 1996

a. Isolated soy protein (ISP) b. Soy protein concentrate (SPC)	Soy	29 Male golden Syrian hamster	25 g/100 g protein for 35 d	Serum total cholesterol ↓in ISP and SPC compared with casein group	Potter *et al.*, 1996
Ex Vivo					
FVVNATSN FKTNDRPSIGN SSPDIYNPQAGS DTPMIGT	Soy	—	—	Stimulated LDL-receptor transcription	Choe *et al.*, 2008
WGAPSL	Soy	—	—	Inhibited cholesterol micellar solubility	Zhong *et al.*, 2007
IAVPGEVA	Soy globulin	—	—	Inhibited cholesterol uptake	Pak *et al.*, 2005
IIAEK GLDIQK ALPMH VYVEELKPTPEG- DLEILLQK	Milk β-lactoglobuli	—	—	Inhibited cholesterol uptake	Nagaoka *et al.*, 2001
LRVPAGTTFYVV- NPDNDENLRMIA	Soy globulin	—	—	Increased LDL-receptor mediated LDL uptake	Lovati *et al.*, 2000

Table 7. Food protein-derived peptides with anti-microbial activities.

Peptide sequence	Food source	Anti-microbial activity	Reference
a: IKHQGLPQE b: VLNENLLR c: SDIPNPIGSENSEK	Casein	a: MIC* of 0.05 mM against *E. coli* b: MIC of 0.22 mM against *E. coli* c: MIC of 1.0 mM against *E. coli*	Hayes *et al.*, 2006
Cp1: LRLKKYKVPQL	Casein	Cp1: MIC of 125 µg/mL against Gram-positive, and 125 to >1000µg/mL against Gram-negative	McCann *et al.*, 2006
Cp2: VYQHQKQMKPWIQ-PKTKVIPYVRYL		Cp2: MIC of 21 µg/mL against Gram-positive, and 332 to >664 µg/mL against Gram-negative	
IVSDGDGMDAW HGLDNYR	Egg	Inhibited most Gram-negative	Mine *et al.*, 2004
Oncorhyncin III	Rainbow trout	MIC of 0.06 to 0.5µM against Gram-positive, MIC of 0.25 to >0.5µM against Gram-negative	Fernandes *et al.*, 2003

MAAP: a polypeptide with 94 amino acid residues	Loach	Inhibitory activity against *E. coli*, *B. subtilis*, and *Staphylococcus carnosus*	Dong *et al.*, 2002
K: GLWSKIKAAGKEAAKA-AAKAAHKAALNAVSEAV L: ALWKTLLKNVGKAAGK-AALNAKTDMVNQ Q1: ALWKNMLKGIGKLAG-QAALGAVKTKVGAES	Frog	K: MIC of 0.6μM against *E.coli*, and 4.7μM against *Staphylococcus carnosus* L: MIC of 2.5 μM against *E.coli*, and 1.3 μM against *Staphylococcus carnosus* Q1: MIC of 1.3 μM against *E.coli*, and 2.7 μM against *Staphylococcus carnosus*	Batista *et al.*, 1999
Snakin-1: a polypeptide with 63 amino acid residues	Potato	Antimicrobial activity against bacterial and fungal pathogens from potato at concentration <10 μM	Segura *et al.*, 1999
RQRVEELSKFSKKGAAA-RRRK	Loach	MIC of 8 μg/mL against most Gram-positive and negative, also inhibited fungi growth	Park et al., 1997
GFFALIPKIISSPLFKTLL-SAVGSALSSSGEQE	Sole fish	MIC of 13 μM against *E.coli*, 3 μM against *A.calcoaceticus*, 5 μM against *B.subtilis*, 25 μM against *P.aeruginosa*	Oren & Shai, 1996

KTKLTEEEKNRLNFLKKIS-QRYQKFALPQYLKTVYQH-QK	Casein-α_{s2}	Inhibitory activity against *E. coli* and *Staphylococcus carnosus*	Zucht *et al.*, 1995
RSGRGECRRQCLRRHEGQ-PWETQECMRRCRRRG	Maize	Dose dependent antimicrobial activity	Duvick *et al.*, 1992
Lactoferrin	Milk	Effective against most Gram-positive and negative at concentration between 0.3 to 3 µM	Bellamy *et al.*, 1992

*, MIC: minimum inhibitory concentration.

2.2.5.7 Opioid bioactive peptides

Opioid peptides, which were first discovered in the late 1970s play an active role in the nervous system. After absorption into the blood, opioid peptides can travel to the brain and other organs, bind to opiate receptors, and exert effects similar to opium or morphine. However, this effect can be reversed by opiate antagonist. Milk, wheat, and spinach have been shown to be able to release opioid peptides (Pihlanto *et al.*, 2004; Teschemacher *et al.*, 2003; Paroli, 1988; Loukas *et al.*, 1983; Branl *et al.*, 1979; Henschen *et al.*, 1979). First characterized opioid peptides from milk were β-casomorphin-7 (YPFPGPI) and β-casomorphin-5 (YPFPG) with β-casomorphin-5 being about 5 times more active than β-casomorphin-7 (Henschen *et al.*, 1979). Fragments from human β-casein showed opiate activity as well (Brantle, 1984). Wheat gluten was able to release opioid peptides with very low IC_{50} values in guinea pig ileum (GPI) and mouse vas deferens (MVD) assays and with resistance toward some proteolysis enzymes (Fukudome & Yoshikawa, 1993, 1992). Two δ-selective opioid peptides, rubiscolin-5 and rubiscolin-6, were isolated from spinach, and proved to enhance learning performance, memory consolidation, and showed anxiolytic effect in mice through mediating δ-opioid receptor (Hirata *et al.*, 2007; Yoshikawa *et al.*, 2003; Yang *et al.*, 2003, 2001). Some other peptides produced by the breakdown of κ-casein, on the other hand,

Table 8. Food protein-derived peptides with immunoregulatory activities.

Peptide sequence	Food source	Immunoregulatory activity	Reference
YXFLGLPGXT (partial sequence)	Bovine placenta	Dose-dependently stimulated lymphocyte proliferation	Fang *et al.*, 2007
Wheat gluten hydrolysate	Wheat	↑NK cell activity	Horiguchi *et al.*, 2005
EVFGK	Bovine milk	Response to *Klebsiella pneumoniae* infection	Meisel, 1997 Fiat *et al.*, 1993 Jollès *et al.*, 1992
YG YGG	Bovine milk	↑Proliferation of human peripheral blood lymphocytes	Kayser & Meisel, 1996
PNSL	β-Casein	↑Mice resistance to *Klebsiella pneumoniae* infection	Kayser & Meisel, 1996
Pancreatin and trypsin digest	Casein-α_{s1}	↓Proliferation of lymphocytes	Otani & Hata, 1995
TTMPLY	Casein-α_{s1}	↓*Klebsiella pneumoniae* infection	Schlimme & Meisel, 1995
TTMPLY	Casein-α_{s1}	↑Antibody formation and phagocytosis	Jollès *et al.*, 1992
PGPIPN	β-Casein	↑Antibody formation and phagocytosis	Jollès & Migliore-Samour, 1986
LLY	β-Casein	↑Antibody formation and phagocytosis, ↑antigen dependent T cell proliferation	Jollès & Migliore-Samour, 1986
Hydrolysed α-lactalbumin	Whey	↑Mice immune response by modulating B lymphocyte and T helper cell activities	Bounous & Kongshavn, 1985

Abbreviation: NK, natural killer.

act like opioid antagonists, and inhibit the effect of morphine like substances. Three fragments from κ-casein, YPSYGLN, YPYY, and YIPIQYVLSR showed opioid antagonist activity at 200, 100, and 5 μM, respectively (Chiba *et al.*, 1989). Peptides with opioid antagonist activity were also isolated from human lactoferrin (Tani *et al.*, 1990). Table 9 shows some food-derived peptides with opioid or opioid antagonist activities.

Although structure-activity relation has not been fully defined, it is indicated that peptides with opioid activities usually exhibit a tyrosine residue at N-terminal and the presence of another aromatic residue in the third or fourth position from N-terminal (Hartmann & Meisel, 2007). Furthermore, proline residue at second position is also important for the three-dimensional orientation of the tyrosine and phenelalanine sidechains.

Table 9. Food protein-derived peptides with opioid effect activities.

Peptide sequence	Food source	Opioid effect activity	Reference
Rubiscolin-6: YPLDLF	Spinach	Enhanced memory consolidation and showed anxiolytic effect in mice mediated by δ-opioid receptor	Hirata *et al.*, 2007 Yang *et al.*, 2003
Gluten exorphins A5: GYYPT	Wheat gluten	Improved learning performance in mice at 300 and 100 mg/kg, respectively	Yoshikawa et al., 2003
Rubiscolin-6: YPLDLF Rubiscolin-5: YPLDL	Spinach	IC_{50} of 51 and 24.4 μM in MVD assay, IC_{50}* of 2.09 and 0.93 μM in receptor binding assay	Yang *et al.*, 2001
RYLGYL YPFPGPI SRYPSY	Casein-α_{s1} β-Casein κ-Casein	Opioid activity	Pihlanto-Leppälä *et al.*, 1994
YPISL	Wheat gluten	IC_{50} of 40 and 13.5 μM in GPI and MVD assays, respectively	Fukudome & Yoshikawa, 1993
Gluten exorphins A5: GYYPT Gluten exorphins A4:GYYP Gluten exorphins B5:YGGWL Gluten exorphins B4:YGGW	Wheat gluten	Gluten exorphins B5 showed IC_{50} of 0.5 μM in GPI, and 0.017μM in MVD assay	Fukudome & Yoshikawa, 1992
YLGSGY RYYGY KYLGPQY	Human lactoferrin	Showed opioid antagonist activity with IC_{50}* of 15, 10, and 23μM, respectively	Tani et al., 1990
Casoxin A: YPSYGLN Casoxin B: YPYY Casoxin C: YIPIQYVLSR	κ-Casein	Showed opioid antagonist activity at 200, 100, and 5 μM in GPI, respectively	Chiba *et al.*, 1989
RYLGYLE RYLGYL	α-Casein	Opioid activity with resistance toward trypsin	Loukas *et al.*, 1983

YLGYLE YLGYL	α-Casein	Opioid activity with resistance toward trypsin	Loukas *et al.*, 1983
β-casomorphin-5: YPFPG β-casomorphin-7: YPFPGPI	β-Casein	Showed opioid activity with high resistance toward proteolysis, IC_{50}* was 1.1 and 14 μM, respectively	Henschen *et al.*, 1979

*, IC_{50}, peptide concentration required to inhibit ^{3}H-ligand binding by 50%.
Abbreviation: GPI, guinea pig ileum; MVD, mouse vas deferens.

2.2.5.8 Mineral-binding bioactive peptides

Peptides with mineral binding abilities are summarized in Table 10. Majority of these peptides have been reported from casein, and hence named caseinophosphopeptides (CPPs). CPPs contain clusters of phosphorylated seryl residues, which are responsible for the interaction between the casein and calcium phosphate that lead to the formation of casein micelles. CPPs retain the ability of whole casein to stabilize calcium and phosphate ions through the formation of complexes, thus enhancing their general bioavailability. With binding, CPPs solubilize many other ions besides Ca and Fe, such as Mg, Fe, Zn, Ba, Cr, Ni, Co, and Se. A previous rat study showed that CPPs enhanced calcium absorption by increasing passive calcium transport in the distal small intestine (Erba *et al.*, 2002). CPPs were also reported to enhance vitamin D-independent bone calcification in rachitic infants (Mellander, 1950). However, data on the effects of CPPs are inconsistent.

Major CPPs include fragments from α_{s1}-casein, α_{s2}-casein, and β-casein. Because of varied degree of phosphorylation of casein, CPPs mineral chelating affinity is direct related to the degree of phosphorylation, with α_{s2}-casein having the highest affinity and β-casein having the lowest (Kitts & Weiler, 2003). CPPs vary in size and phosphoseryl groups, and are reported to resist further proteolytic digestion. Characterization of peptides from whey revealed that they are less in number and less specific in their actions (Vegarud *et al.*, 2000).

Table 10. Food protein-derived peptides with mineral binding activities.

Peptide position	Food source	Mineral binding	Reference
β-Lactalbumin digest	β-Lactalbumin	Fe	Svenning & Vegarud, 1998
43-59	Casein-α_{s1}	Fe, Ca	Gaucheron *et al.*, 1996
48-58			Schlimme & Meisel, 1995
59-64			
59-79			
66-74	Casein-α_{s2}	Ca	Schlimme & Meisel, 1995
1-25	β-Casein	Ca, Fe	Reynolds *et al.*, 1994
			Bouhallab *et al.*, 1991
Lactoferrin digest	Lactoferrin	Fe	Kawakami *et al.*, 1993
α-Lactalbumin digest	α-Lactalbumin	Ca, Fe, Cu, Zn	Hirai *et al.*, 1992
1-28	β-Casein	Ca	Juilerat *et al.*, 1989
33-48			
CPPs	Casein	Ca, Fe, Mn, Cu, Se	Brulé & Fauquant, 1982

2.2.5.9 Bioactive peptides with multiple functions

Many milk protein-derived peptides have more than one function. Peptide derived from β-casein sequence 60-70 showed anti-hypertensive, opioid, and immunostimulatory activities (Migliore-Samour & Jollès, 1988). α_{s1}-Casein fraction 194-199 showed both anti-hypertensive and immunomodulatory functions (Migliore-Samour *et al.*, 1989). Furthermore, the opioid peptides α- and β-lactorphin have shown ACE-inhibitory effects. Peptides with multiple functions can be used in patients to target multiple disease conditions or by consumers who have more than one health concerns. For example, peptides with multiple functions of antihypertensive, antioxidant, anti-lipidemic and hypocholesterolemic effects can contribute to the treatment of cardiovascular disease much more than peptides with only one function. Peptides with both immunoregulatory and anti-cancer activities can work more efficiently than immunoregulatory peptides in cancer treatment. Type II diabetes patients can look for peptides with both immunoregulatory and antihypertensive effects. When lifestyle-related diseases are not simple anymore and can lead to one another, there certainly is a potential for the application of bioactive peptides with multiple functions.

2.2.6 Release and Production of Bioactive Peptides

Bioactive peptides are usually inactive when encrypted in the amino acid sequence of the parent protein. However, when the parent protein is acted upon by an appropriate enzyme or microbial enzyme, bioactive peptides can be released in an intact form and become active. Naturally,

parent protein in the diet can be degraded by digestive enzymes and digestive microbial enzymes in the GIT. Or, parent protein can be degraded *in vitro* during food processing to release active peptides. The most common way is through enzymatic hydrolysis of whole protein molecules. Many of the human digestive enzymes including pepsin, trypsin, and chymotrypsin are frequently used in the production of bioactive peptides. Microbial enzymes such as thermolysin and alcalase as well as several other proteases from bacterial and fungi sources are also widely utilized. Many industries use dairy starter cultures which contain highly proteolytic microorganisms. Bioactive peptides can be generated by the starter and non-starter bacteria used in the manufacture of fermented dairy products (Korhonen & Pihlanto, 2006). Lactic acid bacteria, *Lactococcus lactis*, *Lactobacillus helveticus*, and *Lb. delbrueckii ssp. bulgaricus* are well known for their proteolytic activities. When amino acid sequence is known, bioactive peptides can be synthesized via three approaches: chemical, enzymatic, or recombinant DNA technology. Chemical synthesis is the most widely used in the laboratory on a small scale basis, and rapid production of peptide libraries for screening puposes (Korhonen & Pihlanto, 2006). Recombinant DNA technology is the preferred choice for relatively large peptides, and peptides can be obtained in a large quantity from very inexpensive starting materials.

Production of bioactive peptides in the laboratory is usually on a small-scale, but commercialization of these peptides needs suitable large-scale technologies in order to produce in large quantities. After

hydrolysis, peptides in the hydrolysate are fractionated and enriched through various methods. Currently, selective membrane ultrafiltration and ion-exchange chromatography are the most commonly used technologies for the enrichment of peptides. Employment of membrane ultrafiltration is able to obtain peptides with specific molecular weight range. Using membranes with small molecular weight cut-offs is very useful in separating small peptides (as low as 30 Da) from peptides with large molecular mass, and can enhance isolation of small-size peptides that have higher possibility to be effectively absorbed *in vivo*. Ion-exchange chromatography is a method for the isolation of purified peptides of interest. After ultrafiltration, peptides mixture with certain molecular weight range is injected into a chromatographic column. Unbound peptides are washed out first (or collected as separate fractions), and then bound peptide fractions are eluted from the column. The advantages of this process are that peptide fractions of interest can be easily separated from the inactive peptides, production can be relatively large, and it provides possibilities for enriching the products with small molecular weight peptides.

2.2.7 Commercialization of Bioactive Peptides

There is a considerable commercial interest in developing functional foods and nutraceuticals containing bioactive peptides. Up till now, many peptides, especially those released from milk, have been added or enriched as ingredients into food materials by modification of the usual manufacturing process. They are also introduced into non-food materials to

provide certain health benefits. Table 11 summarizes some commercialized functional foods and nutraceuticals that have been formulated to contain bioactive peptides.

2.2.8 Future Applications of Bioactive Peptides

Although some food-derived bioactive peptides have already been applied in functional foods and nutraceuticals, there still is a great potential and opportunities for the incorporation of these peptides into other health products, such as skin care and medical products. Commercial peptides are currently limited to cardiovascular and digestive application but other areas such as cancer, obesity, diabetes, Alzheimer's, kidney failure, etc, need further research. Because of their natural and health promoting characters, bioactive peptide-enriched foods, nutraceuticals, and pharmaceuticals have received wide attention especially by health conscious people. However, suitable technologies for massive commercial production need further exploration. Functional properties of bioactive peptides and their mechanisms of action need more extensive studies for better understanding and corresponding quality monitoring and regulations.

Table 11. Commercialized functional foods and nutraceuticals that contain bioactive peptides.

Manufacturer	Product	Food type	Bioactive peptides	Health claim
Calpis, Japan	Calpico concentrate	Milk and lactobacilli concentrate	VPP IPP	Support healthy cardiovascular function
	Calpico	Soft drink		
	Ameal bp	Capsule, tablet		
Valio, Finland	Evolus Double Effect	Fermented milk	VPP IPP	Lowers cholesterol, helps control blood pressure
Campina, Netherlands	C12 Peption ACE	Capsule, tablet, bar, beverage,	FFVAPFPEVFGK	Helps regulating blood pressure
	C12 Peption AA	Capsule, tablet		
	C12 Peption B	Beverage		
Kanebo, Japan	Peptio	Soft drink	FFVAPFPEVFGK	—
Ingredia Nutritional, France	Prodiet F200	Beverage, chocolate, cookie, dairy product	YLGYLEQLLR	Decrease anxiety, reduce stress
Davisco Foods International, USA	Biozate	Supplement	Highly purified whey protein hydrolysate containing small peptides mixture	Lowers cholesterol and blood pressure
Natural Factors, Canada	PeptACE	Capsules	Mixture of 9 bonito-derived peptides	Lowers cholesterol, lowers blood pressure, increase blood flow
Arla Foods, Denmark	Capolac	Ingredient	CPP	Improves calcium reserves
DMV,	Glutamine	Supplement	Wheat protein	Improves

Netherlands	peptide WGE 80 GPN	hydrolysate containing peptides mixture	immune response and gut performance, fewer clinical infections

2.3 PROTEIN HYDROLYSIS

Protein hydrolysis can be accomplished by acid, alkali or enzyme. However, enzymatic hydrolysis is preferred over strict chemical methods for producing hydrolysate in nutritional applications (Lahl & Braun, 1994). Enzymatic hydrolysis is preferred because it produces hydrolysates with well-defined peptide profiles, while acid and alkali hydrolysis may lead to toxic products, destroy L-form amino acids and form D-amino acids (Finot *et al.*, 1978). Enzymatic hydrolysis can produce peptides with ACE inhibitory activities, immunomodulatory, antithrombic, ion binding and opioid properties (Kim *et al.*, 2001; Yamanoto, 1997; Meisel, 1997). Previous work from Dr. Aluko's lab demonstrated the hydrolysis of pea proteins into peptide fractions that inhibited calmodulin-dependent protein kinase II (CaMKII) activity (Li & Aluko, 2005).

Peptide composition of a protein hydrolysate is dependent on hydrolysis parameters which include substrate concentration, enzyme:substrate ratio, pH, temperature, and duration of hydrolysis. Different combinations of these parameters can be used to optimize protein hydrolysis and produce preferred peptides.

2.4 ENZYME-DEPENDENT METABOLIC DISORDERS

Enzymes are proteins that catalyze about 4000 chemical and biochemical reactions (Bairoch, 2000). They accelerate the rate by lowering the activation energy of the reactions. They are involved in almost all

processes in cells to keep the reactions at specific rates. Like all other proteins, an enzyme is composed of amino acids which are connected through peptide bonds, and folded into a unique structure. The region where the enzyme binds the substrate and catalyzes the reaction is called active site. In addition to binding the substrate, the active site also binds cofactor(s), which is (are) required for enzyme activity. Cofactors can be either inorganic, such as ions, or organic, such as heme. Take CaMKII for example, it needs both Ca^{2+} and calmodulin (CaM) for activity. Enzymes are neither consumed in the reactions, nor do they change the equilibrium of the reaction. However, they are very specific for a reaction: each reaction needs a specific enzyme, and an enzyme only catalyzes one specific reaction. This property of specificity depends on many characters of enzyme, which include structures/shapes, electric charges, hydrophobicity/hydrophilicity, etc. There are more than one model that have been suggested to explain this property but with one point in common, that is, the enzyme structure is changed in response to binding the effectors, after which the enzyme activity is influenced too. The activity of enzyme is also affected by environmental elements such as temperature and pH. Because of the critical roles enzymes play in all reactions happening in human body, any malfunction of enzyme activity may lead to a disease. However, using bioactive components of natural foods to correct the enzyme malfunction has become a mainstream thought of the food and nutrition scientists. In this way, diseases can be prevented and alleviated at the same time with nutrient ingestion. For example, CaMKII is a key enzyme in cell

proliferation and overexpression may lead to excessive proliferation of cells, and thus, the resulting disease such as cancer. Previous *in vitro* studies from Dr. Aluko's lab found out that peptides from pea protein could inhibit CaMKII activity and therefore, in theory, are potential therapeutic agents (Li & Aluko, 2005). ACE and renin are two key enzymes in the RAS, and malfunction of these two enzymes will lead to hypertension. Consumption of bioactive peptides derived from food source can lower BP in both animal and human.

2.4.1 Angiotensin I-converting enzyme (ACE)

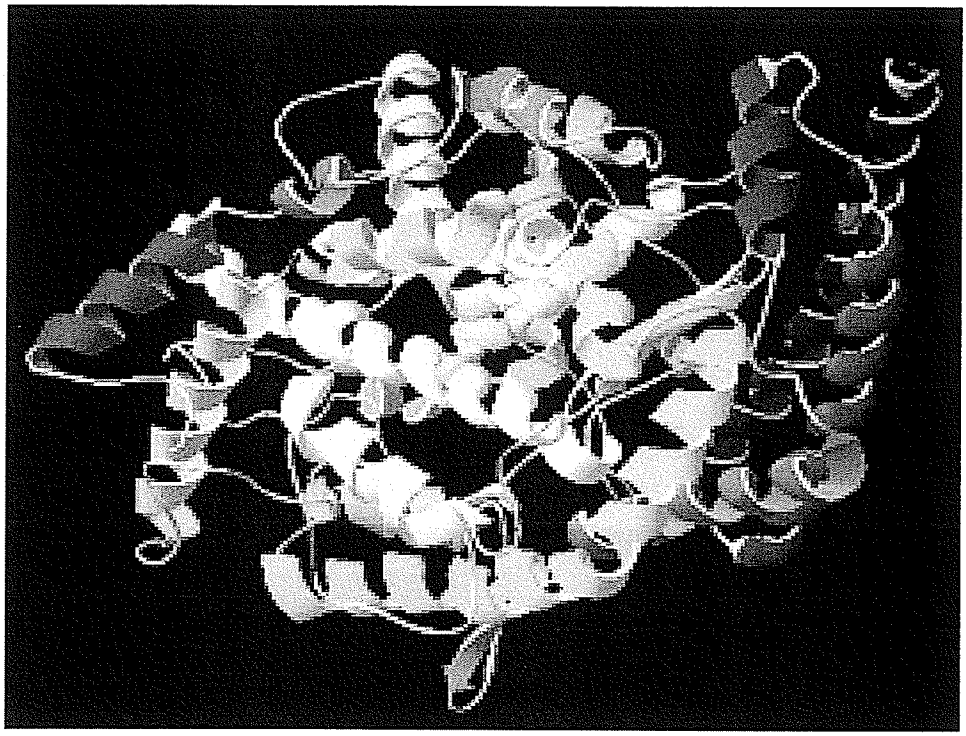
ACE is a membrane-bound dipeptidyl carboxypeptidase that catalyzes the conversion of angiotensin I to its active form, angiotensin II, which is a vasoconstrictor by removing a carboxy-terminal (C-terminal) His-Leu dipeptide. Also, ACE can inactivate the blood vessel dilating enzyme called bradykinin leading to increased vasoconstriction. Therefore, ACE plays a vital role in maintaining blood pressure control as part of the RAS, and is a potent target for prevention and treatment of hypertension, cardiovascular diseases, kidney failure and diabetics (Johnston *et al.*, 1981; Johnston *et al.*, 1979). ACE is produced by monocytes under influence from CD4 lymphocytes, and has a signal peptide that directs it to an extracellular localization which positions it optimally for interaction with its substrate angiotensin I. There are two isoforms of ACE in the human body transcribed from the same gene, somatic and testicular (Riordan, 2003). Somatic ACE is expressed in virtually all tissues, especially strong in capillaries of the lung. Testicular ACE, with only half the size of somatic ACE, is found only in

developing and mature sperms in testes.

Somatic ACE is a membrane-bound protein consisting of a 28-residue C-terminal cytosolic domain, a 22-residue hydrophobic transmembrane domain, and a 1277-residue extracellular domain that is glycosylated (Riordan, 2003). The extracellular part is further divided into the N- and C-domains. Structure studies revealed that it is composed of α–helix for most part that incorporates a zinc ion and two chloride ions. Each of the two domains contains an active site with a conserved HEMGH zinc-binding motif, where histidine is the zinc ligand (Natesh *et al.*, 2003; Soubrier *et al.*, 1988) (Figure 1). Testicular ACE, with lower MW, has 701 amino acids.

The extracellular C- and N-terminal domains have different functions, substrate specificities, inhibitors, and chloride active profiles (Wei *et al.,* 1992). Although the active sites of both domains have a similar efficiency in the hydrolysis of angiotensin I and bradykinin, studies indicate that C-domain is the dominant angiotensin I-converting domain. The C-domain is indispensable for maintaining sufficient blood pressure regulation and cardiovascular functions, whereas N-domain inhibition does not affect angiotensin I induced vasoconstriction (Van Esch *et al.,* 2005; Natesh *et al.,* 2003; Junot *et al.*, 2001; Esther *et al.,* 1997). Current ACE-inhibitory drugs act by inhibiting both domains. Testicular ACE is identical, from residue 37 to its C terminus, to the second half or C-terminal domain of the endothelial ACE sequence (Ehlers *et al.,* 1989).

Figure 1. ACE structure.



(Anthropology.net)

The exact functions of testicular ACE is not clear; however, it is believed that it can be served as a 'template' for the design of next-generation, domain-selective ACE inhibitors with the potential for greater efficacy, fewer side effects and new treatment indications for hypertension. ACE is a zinc (Zn) metallo-enzyme. Antihypertensive diuretics may decrease ACE activity by increasing Zn excretion (Lockett *et al.*, 1983). On the other hand, ACE inhibitors may cause Zn depletion (Golik *et al.*, 1998; Golik *et al.*, 1990).

Since 1980s, ACE inhibitors have achieved great success as the choice for first-line treatment of cardiovascular diseases. ACE inhibitors are "a group of pharmaceuticals that are used primarily in treatment of hypertension and congestive heart failure, in most cases as the drugs first choice" (Wikipedia). Numerous articles have been published, discussing characters, inhibition mechanisms and effects of ACE inhibitors. ACE inhibitors have both acute and long-term effects (Rasmussen *et al.*, 1985; McGrath *et al.*, 1980; Johnston *et al.*, 1979). They can lower arteriolar resistance and increase venous capacitance, increase cardiac output and cardiac index, stroke work and volume, lower renovascular resistance, and lead to increased natriuresis. The mostly used ACEI drugs include: benazepril, captopril, cilazepril, enalapril, fosinopril, lisinopril. Together with the discovery and further understanding of ACEI inhibitory drugs is the upsurge of ACE inhibitory nutraceuticals. A large number of bioactive components from natural foods, especially peptides with ACE-inhibitory activities have been reported. Compared with ACE inhibitory drugs, food

protein-derived peptides are less studied and less understood, but more natural and safer, and can be used in clinical therapy as well as preventive treatment. It has been reported that ratios of incidence of CVD in male and female with high-normal blood pressure were 1.6 and 2.5-fold higher than those with optimal blood pressure (Vasan *et al.*, 2001), and the risks of developing CVD doubles with every 20 mmHg rise in SBP and 10 in DBP (Lewington *et al.*, 2002). Hence, early management by taking nutritional supplements is very important in the prevention of hypertension and CVD.

2.4.2 Renin

Renin, which is also called angiotensinogenase, is a pepsin-like enzyme circulating in the blood and working together with ACE in the RAS that helps control blood pressure (Figure 2). The main responsibility of renin is to convert angiotensinogen to angiotensin I by cleaving angiotensinogen between its 10^{th} and 11^{th} amino acids. Renin is mainly synthesized and released by juxtaglomerular cells in kidney in response to low blood volume and low Na^+ content.

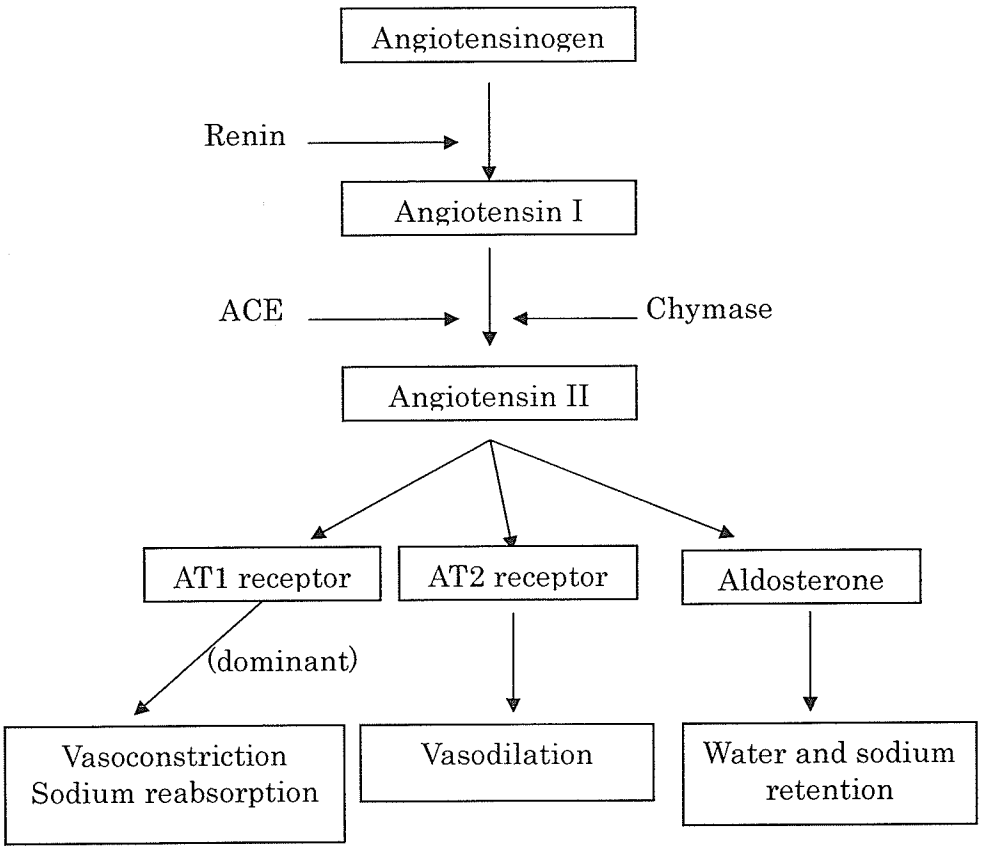
Renin is composed of 406 amino acids with MW of 45KD. It has 7 α–helices and 26 ß–sheets. Based on studies using antibodies directed to N- or C-teminus of prosegment of prorenin it was proposed that the N-terminus was hidden within the conformation of the molecule, whereas the C-terminus (activation site) was on the surface (Dzau, 1987).

The conversion of angiotensinogen to angiotensin I by renin is the rate-limiting step in angiotensin II production. An overactive renin leads to the over production of angiotensin I and angiotensin II, and subsequent

increased blood pressure. Besides, conversion of angiotensinogen to angiotensin I is the only known function of renin so far, which makes the inhibition of renin very specific and do not cause other side effects. Therefore, inhibition of renin activity can potentially be a better strategy than ACE inhibition for the treatment of hypertension and associated symptoms.

As early as 1970s, peptide-like renin inhibitors were synthesized to test their efficiency in regulating RAS. Structure study using NMR showed that formation of hydrogen bond between an inhibitory peptide and neighbouring residues in the active site of renin was an important requirement (Sarma *et al.*, 2002). Besides, extended structure is necessary for peptides to enable both cyclic and linear parts interact with residues in the active part of renin, as revealed by both NMR and x-ray diffraction studies (Sarma *et al.*, 2002; Dhanaraj *et al.*, 1992). Till now, most researches have still stayed at the level of synthesizing inhibitors for medication purposes; few of them have explored the renin inhibitors from natural food sources. Due to the fact that treatment with renin inhibitors can induce reduced blood pressure and renal vasodilation both in humans and animals (Van Paassen *et al.*, 2000; Van Paassen *et al.*, 1995; McMahon *et al.*, 1995; Azizi *et al.*, 1994; Van den Meiracker *et al.*, 1990), and that peptide-based molecules of sizable molecular mass can be absorbed intact into the system circulation (Kleinert *et al.*, 1992), it seems that renin-inhibitory peptides from natural food protein may provide a wide field for nutritional applications for antihypertensive purpose.

Figure 2. Renin-angiotensin-aldosterone system.



2.4.3 Calmodulin-dependent cyclic nucleotide phosphodiesterase 1 (CaMPDE1)

Cyclic nucleotide phosphodiesterases (PDEs) are involved in the degradation of cyclic nucleotides which regulate a variety of cellular activities. Mammalian PDEs are categorized into 11 families based on sequence homology, enzymatic properties, and sensitivity to inhibitors (Omori & Kotera, 2007), and a number of splice variants are identified in each PDE family. Their tissue expression patterns, molecular characteristics, and regulations are diverse and related to their functional roles in cells.

Calmodulin/calcium-dependent cyclic nucleotide phosphodiesterase 1 (CaMPDE1) was first discovered in rat brain and bovine simultaneously by Kakiuchi and Yamazaki and Cheung (Kakiuchi & Yamazaki, 1970; Cheung, 1970), and has been found encoded by at least 3 subfamily genes, PDE1A, PDE1B, and PDE1C. The PDE1s consist of 2 N-terminal CaM binding domain, an inhibitory domain sitting between the 2 CaM-binding domains, and a conserved C-terminal catalytic domain (Sonnenburg *et al.*, 1995). The inhibitory domain may interact with a site near the C-terminal catalytic domain so that to keep the enzyme at an inactive conformation.

Activation of CaMPDE1 requires a physiological concentration of Ca^{2+} and CaM (Wang *et al.*, 1975; Teo and Wang, 1973; Cheung, 1971; Kakiuchi & Yamazaki, 1970; Kakiuchi *et al.*, 1970; Cheung, 1970). Activation of PDE1 is initiated by binding of Ca^{2+} to CaM and converting it from inactive to an active conformation (Ca^{2+}-CaM*). CaMPDE1 then binds

to the active site of CaM to become active (Ca^{2+}-CaM*-PDE1*). CaMPDE1 isozymes differ in their CaM affinities (Sharma & Kalra, 1994; Sharma, 1991; Sharma & Wang, 1986; Hansen & Beavo, 1986), and this difference in affinity for CaM may be related to the relative concentration of CaM in the different tissues. For example, PDE1A1 has about 10-20 fold higher affinity than PDE1B1 and PDE1A2 (Sharma *et al.*, 1997; Sharma & Kalra, 1994; Hansen & Beavo, 1986; Keravis *et al.*, 1986; Mutus *et al.*, 1985). The difference in CaM affinity to isozymes extracted from different tissues indicates that the isozymes accommodate intracellular CaM concentrations of the respective tissues. As for many other CaM-dependent enzymes, activation of PDE1 needs a synergistic interaction of Ca^{2+} and CaM (Sharma & Kalra, 1994; Huang *et al.*, 1981). The Ca^{2+} concentration required for half-maximal activation is decreased when the CaM concentration is increased. When excess amount of CaM is present, EC_{50} (concentration of agonist that provokes a response halfway between the baseline and maximal response) for activation by Ca^{2+} varies from 0.27 μM (PDE1A1) to 3.02 μM (PDE1C1) (Bender, 2007). It is suggested that the differential Ca^{2+} affinity of the tissue-specific isozymes may be a mechanism by which CaM regulatory reactions are adapted in the respective tissues (Kakkar *et al.*, 1999). However, some PDE1 isozymes are not activated by general mechanisms (Wang *et al.*, 1975). These isozymes contain CaM as a subunit, and therefore, a change in CaM concentration does not influence Ca^{2+} concentration.

Expression of CaMPDE1 isozymes has been observed in different

human tissues. Expression of PDE1A1 and PDE1A4 is ubiquitous and high in kidney, liver, pancreas, and thyroid gland, PDE1A5 and PDE1A6 expression is brain specific, and PDE1A10 is only expressed in testis (Fidock *et al.*, 2002; Michibata *et al.*, 2001). PDE1B1 expression is predominant in the caudate nucleus and putamen of brain, and low in heart and skeletal muscle, whereas PDE1B2 is mostly found in spinal cord (Fidock *et al.*, 2002; Yu *et al.*, 1997). Human PDE1C1 expression is high in brain and heart, and PDE1C2 is high in olfactory epithelium (Loughney *et al.*, 1996; Yan *et al.*, 1995). PDE1C1 and PDE1C4/5 mRNA are also present in the testis (Yan *et al.*, 1996).

PDE1s regulate multiple cellular functions. PDE1A1 was reported to be up-regulated in rat aorta with the development of nitrate tolerance (Kim *et al.*, 2001). Study by Jiang *et al.* showed that PDE1B1 mRNA was expressed in RPMI-8392 cells but not in normal resting human peripheral blood lymphocytes (HPBL), and inhibition of its activity led to RPMI-8392 apoptosis (Jiang *et al.*, 1996). PDE1B mRNA is induced in phytohemagglutinin (PHA) or anti-CD3/CD28-activated human T-lymphocytes and participate in IL-13 regulation implicated in allergic diseases (Kanda & Watanabe, 2001). Reed *et al.* (2002) demonstrated that PDE1B knock-out mice exhibited exaggerated locomotor hyperactivity in response to dopamine agonist and displayed impaired spatial learning, indicating the role of PDE1B in dopaminergic signaling. As it is expressed in the central nervous system (CNS) regions, PDE1B may also contribute to other neural functions. PDE1C is absent in quiescent muscle smooth

cells (MSC), and can be induced by MSC proliferation (Rybalkin *et al.*, 2002). Inhibition of PDE1C results in suppression of MSC proliferation. Study suggested that PDE1C also played a role in insulin secretion. It was found that inhibition of PDE1C in βTC3 insulinoma cells augmented glucose-stimulated insulin secretion in a dose-dependent fashion thus demonstrated that PDE1C was the major PDE counteracting glucose-stimulated insulin secretion from βTC3 insulinoma cells (Han *et al.*, 1999).

CaMPDE1 is a good target for therapeutic purposes not only because of its tight connections with various physiological functions in the body, but also due to the fact that regulation of degradation of second messenger can make a larger and quicker change in concentration than comparable regulation of the rates of synthesis. CaMPDE1 inhibitors can either interact directly with the catalytic site of CaMPDE1 or with CaM binding site or on Ca^{2+}/CaM during activation (Ichimura *et al.*, 1996; Nokin *et al.*, 1989; Lugnier *et al.*, 1984). Early inhibitors are more likely to inhibit the activity of most, if not all, PDEs. Today, there is still no CaMPDE1 inhibitor that is both really effective and selective. For example, vinpocetine inhibits differently the various subtypes of PDE1s, and it inhibits PDE7B at the same time (Sasaki *et al.*, 2000; Yan *et al.*, 1996). Since there is a large number of CaMPDE1 isozymes, each of which with distinct structure and function, it should be possible to develop highly selective inhibitor that targets specific isozymes, and therefore functions and pathological conditions without causing non-specific side effects.

CHAPTER 3

EVALUATION OF BITTERNESS PROPERTIES OF AN ENZYMATIC PEA PROTEIN HYDROLYSATE PRODUCT

3.1 INTRODUCTION

Proteins from food origins are subjected to hydrolysis during food processes which may cause excessive bitterness and decreases the sensory quality of the products. In 1952, Murray and Baker (1952) noticed that the hydrolysate made from gelatin and casein developed a bitter and unpleasant taste, and the debittering treatment suggested that peptides rather than free amino acids were responsible for the bitter taste. After that abundant studies have demonstrated that hydrolysis significantly influences the functional and sensory properties of the resultant hydrolysates (Seo *et al.*, 2008; Humiski & Aluko, 2007; Jung *et al.*, 2005; Spellman *et al.*, 2005; Aubes-Dafau *et al.*, 1995; Kukman *et al.*, 1995; Bumberger & Belitz, 1993). Because food protein hydrolysates have a wide range of application in food production, their sensory properties are of great importance to consumer acceptability. However, many proteins such as casein, whey, soy, and pea release bitter tasting peptides during the hydrolysis process, which is a major concern and even an obstacle against the acceptance of novel products (Humiski & Aluko, 2007; Okai *et al.*, 1979; Matoba *et al.*, 1970; Arai *et al.*, 1970).

Structure-activity relationship studies have partially revealed the structural aspects related to bitter taste. It is shown that bitterness was associated with the hydrophobic amino acids of released peptides. Ney (1971) concluded that peptides <6 kDa having a high content of Leu, Phe, Pro, Tyr, Ile, and Trp residues were more likely to be bitter. Matoba and Hata (1972) reported that bitterness developed in peptides with high content of hydrophobic amino acids, which contributed independently and irrespective of their sequence and steric configuration. Bulky hydrophobic amino acids at the C-terminal and bulky basic amino acids at the N-terminal being highly related to bitterness were revealed by quantitative structure-activity relationship (QSAR) study (Kim & Li-Chan, 2006). Cho *et al.* (2004) reported that bitterness of fractionated peptides was related to molecular mass. In their study, maximum bitterness was observed at approximately 4 kDa in one soy protein hydrolysate and 2 kDa in another. Peptide fraction with molecular mass of <1 kDa showed lowest bitterness. It has been shown that the bitter taste exhibited by a peptide is also affected by the number of carbons or the hydrophobicity of the side chain (Ishibashi *et al.*, 1988). For a peptide to exhibit bitterness, the side chain of the amino acid should contain at least 3 carbon atoms. Bitterness formation during the hydrolysis seems to be related with the type of enzyme used as well (Humiski & Aluko, 2007; Kim *et al.*, 1992). In a previous study, it was

reported that alcalase led to the highest bitterness property of some food protein hydrolysates, whereas trypsin and neutrase gave relatively low bitterness scores (Humiski & Aluko, 2007; Kim *et al.*, 1992). It was reported that alcalase produced highest content of hydrophobic amino acids in the hydrolyzed peptides. Thermolysin is an enzyme with high specificity for hydrolyzing peptide bonds at the N-terminal of hydrophobic amino acids, and hence produce bitter peptides. In order to determine potential levels of incorporation of the thermolysin hydrolysate into food products, it is important to obtain information on the degree of bitterness of various aqueous concentrations.

The objective of this study was to evaluate the bitterness property of different concentrations of a thermolysin-hydrolyzed pea protein and to examine the relationship between the bitterness and the composition of amino acids.

3.2 MATERIALS AND METHODS

3.2.1 Production of Pea Protein Hydrolysate

3.2.1.1 Materials

Pea protein isolate (PPI, Propulse™) was a gift from Nutri-Pea Ltd. (Portage la Prairie, Manitoba, Canada) and contained 82% protein, < 12% carbohydrate, < 3% lipid, < 4% ash, and 6% moisture, according to the manufacturer's analysis results. Pea protein isolate was exclusively made from a mixture of seeds from the following varieties: Eclipse, Midas and Golden (Nutri-Pea Ltd, personal communication, 2009). Production of PPI involves a similar procedure to the one described by Sosulski and McCurdy (1987). In brief, the peas are dehulled and ground into flour which is passed through a screen to separate the coarse fiber particles. The flow-through flour is then extracted with a alkali solution, centrifuged and the supernatant used for isoelectric protein precipitation. Thermolysin (*Bacillus thermoproteolyticus rokko*) was purchased from Sigma (Sigma Chemicals, St. Louis, MO., USA) and has an activity of 40 units/mg enzyme powder.

3.2.1.2 Methods

PPH was prepared in a reactor with temperature and pH control devices. PPI was dispersed in distilled water to obtain a 6.0% (w/v) protein slurry. After the slurry was heated to 55°C and adjusted to pH 8.0, 0.5% thermolysin (w/w, on the basis of protein weight) was then added to initiate protein hydrolysis. The temperature and pH of the slurry was maintained at constant values for 3 h. The hydrolysis was stopped by heating the slurry at 95°C for 15 min. The hydrolysate was then centrifuged at 10,000xg for 25 min. The supernatant was further passed through a 3,000 Da molecular weight cut-off (MWCO) ultrafiltration membrane, and the resulting permeate was collected and freeze-dried for later use.

3.2.2 Determination of Bitterness Scores

Bitterness evaluation was carried out using a human taste panel with ethics approval obtained from the Joint-Faculty Research Ethics Board of the University of Manitoba.

The evaluation was done according to a previous method (Edans *et al.*, 2005) which was slightly modified as follows. A taste panel consisting of six trained volunteers recruited from among the University of Manitoba staff and students was used to determine the bitterness score of each protein hydrolysates using quinine sulfate solution as a reference. Panel

members were trained using a 30 min session to correctly score the bitterness of 15 mL quinine sulfate solution at 3 different concentrations: 1) 0.5 mg/mL; 2) 1 mg/ml; 3) 5 mg/ml. During actual test evaluation, PPH solution at six different concentrations (0.625, 1.25, 2.5, 5, 10, and 20 mg/mL) were prepared, identified with 3-digit numbers, and presented to the panel in random order using 5 mg/mL quinine sulfate solution as anchor point. Each panel member was given 15 mL PPH solution and instructed to put the solution in their mouth and swirl it around for 5 sec before expectorating into a sanitary cup. Bitterness was then scored in relation to that of the anchor solution using a 15 cm line scale. Tap water and unsalted crackers were always provided to the panel members to rinse their mouths between the samples and to be expectorated after rinse. Each sample was evaluated in duplicate on each session for two separate sessions to make a total four evaluations per sample per person.

3.2.3 Amino Acid Composition of Pea Protein Hydrolysate

The analysis was performed at the Department of Animal Science, University of Manitoba according to the Association of Official Analytical Chemists (AOAC) Official Method 994.12. An HPLC system was used to determine the amino acid compositions of PPI and PPH after the sample was hydrolyzed with 6 M HCl according to the method of Bidlingmeyer *et*

al. (1984). The cysteine and methionine contents were determined after performic acid oxidation (Gehrke *et al.*, 1985) and tryptophan content was determined after alkaline hydrolysis (Landry *et al.*, 1992).

3.2.4 Statistical analysis

Data was analyzed using general linear model of ANOVA followed by Duncan's multiple range test using SAS (SAS, USA). $P < 0.05$ was considered as statistically significant.

3.3 RESULTS

3.3.1 Bitterness Property of Pea Protein Hydrolysate

The ideal way for quantification of hydrolysate bitterness is to use sensory evaluation panels (Kim *et al.*, 2008; Edans *et al.*, 2005; Spellman *et al.*, 2005), even though this is a time and labour consuming method and requires appropriate numbers of panel members and training to detect bitterness in order to obtain statistically relevant data (FitzGerald & O'Cuinn, 2006).

Figure 3 shows that bitterness of enzymatic PPH decreased with reduced hydrolysate concentration. According to their concentrations, sample A, B, C, and D each was significantly bitter than the lower concentrated one. However, when concentration was reduced to 2.5 mg/mL (sample D), which could be the minimum response threshold (MRT) in this study, no bitterness difference among the lower concentrated samples (sample D, E, and F) could be detected. At the highest concentration of 20 mg/mL, bitterness score was 6.0 when compared to 15 for the 5 mg/mL quinine solution.

NALPE is a strong bitter peptide derived from soybean with MRT of 0.074 mM, and its analogues, NALPL, NALPW, NALPS, and NALPR were reported to have MRT values of 0.149, 0.105, 0.25, and 0.42 mM,

respectively (Kim *et al.*, 2008). Structural analysis revealed that the presence of a polar group and hydrophobic bitter amino acids, the composition of hydrophobic regions, the spatial orientation of the polar group and hydrophobic regions may be the major determinants for the taste type and bitterness intensity. Three bitter peptides, namely I^{49}-N^{68}, I^{49}-K^{97}, and G^{203}-V^{209}, were identified from β-casein hydrolysate. Threshold of the hydrolysate and the peptides was 2.67, 1.0, 1.5, and 0.175 mg/mL respectively (Bumberger & Belitz, 1993). It is interesting to notice that the more hydrophobic peptide I^{49}-N^{68} was less bitter than I^{49}-K^{97}, and this may suggest that hydrophobicity alone does not responsible for bitter potency. In another study reported by Toelstede and Hofmann (2008), a large amount of bitter peptides were formed by proteolysis of casein, among which 12 showed pronounced bitter taste with recognition threshold between 0.05 and 6.0 mM. Bovine hemoglobin hydrolysate with different enzymes exhibited a strong bitter taste concentrated in the fraction of molecular weights between 0.5-5 kDa which matched with Ney's theory that bitter peptides had a molecular weight below 6 kDa (Aubes-Dufau & Combes, 1997; Aubes-Dufau *et al.*, 1995). One bitter peptide corresponding to fragment 32-40 of the β-chain of bovine hemoglobin was identified as VVYPWTQRF, and exhibited bitterness at 0.25 mM equivalent to 0.073 mM quinine sulphate or 21 mM caffeine (Aubes-Dufau *et al.*, 1995). In a

previous study conducted in Dr. Aluko's lab, bitterness scores of PPH (3% w/v) catalyzed by α-chymotrypsin, trypsin, papain, flavourzyme, and alcalase were reported to be about 4.5, 7, 3, 8, and 11, respectively (Humiski & Aluko, 2007). In a similar study comparing the effects of different enzymes on bitterness, neutrase and papain did not produce bitter hydrolysates from bovine hemoglobin, whereas alcalase produced hydrolysate with most bitter taste followed by pepsin and proctase (Aubes-Dufau & Combes, 1997). Alcalase hydrolysate of soy protein was detected to have highest bitterness compared to soy protein hydrolysate produced by neutrase, bromelain, protamex, papain, whereas flavourzyme hardly produced any bitterness (Seo *et al.*, 2008).

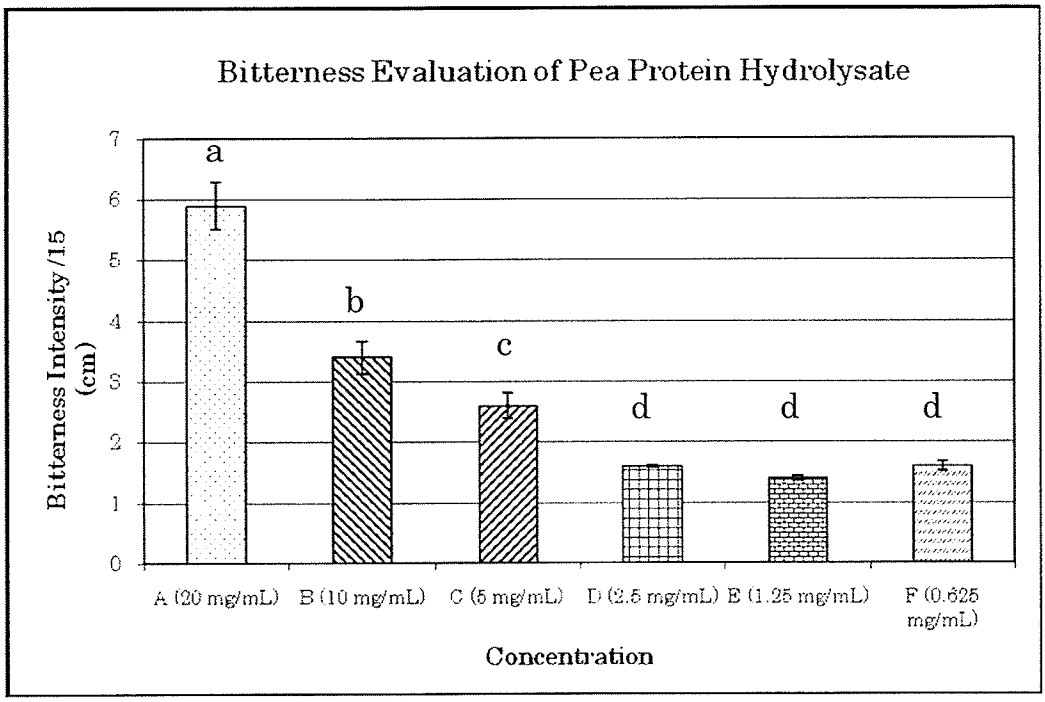
PPH produced from current study exhibited moderate bitterness compared with hydrolysates/peptides from other food proteins reported in previous studies. When compared with PPH produced from other enzymes, thermolysin resulted in less bitter hydrolysate than alcalase, similar ones with trypsin and flavorzyme, and more bitter ones than α-chymotrypsin and papain. Thermolysin specifically catalyzes hydrolysis of peptide bonds containing hydrophobic amino acids, which may be responsible for the formation of hydrolysate containing peptides with hydrophobic amino acid residues, and hence moderate bitterness of the whole hydrolysate.

3.3.2 Amino Acid Composition Analysis

Amino acid compositions of PPI and PPH are determined and shown in Table 12. Like in other seed proteins, aspartic acid and glutamic acid constitute a large portion of pea protein. Lys and Arg present in high concentration in pea, as previously stated in Chapter 2. Percentage of all amino acid residues was found similar in PPH and PPI which indicated that enzymatic hydrolysis did not significantly alter amino acid composition of pea protein. This is in agreement with previous studies that different enzymatic hydrolysis resulted in significantly altered peptide fragments but only slightly changed amino acid composition (Cheng *et al.*, 2009; Humiski & Aluko, 2007; Byun & Kim, 2001; Kim *et al.*, 2001).

Previous research showed that hydrophobic and aromatic amino acids contribute to the bitterness of enzymatic hydrolysate. Such amino acids are known to include Leu, Ile, Phe, Pro, Val, Tyr, and Trp. Gordon and Speck (1965) isolated bitter peptide fractions from whey protein, and components were identified to include hydrophobic amino acids Leu, Ile, Phe, Pro, and Val. The bitter peptide isolated by Carr *et al.* (1956) from tryptic hydrolysates of casein contained Val, Pro, Tyr, Leu, Ile, and Ala. Ichikawa *et al.* (1959) isolated a bitter peptide after treatment of casein with a proteinase from *B. subtilis*, and this peptide mainly contained Leu, Met, Pro, Phe, Tyr, and Thr. Fourteen low molecular mass hydrophobic

Figure 3. Bitterness evaluation of thermolysin catalyzed pea protein hydrolysate (<3 kDa) at various aqueous concentrations.



bitter peptides containing 3-6 amino acid residues were isolated from soybean protein, and sequence analysis identified that they were predominantly composed of hydrophobic amino acids such as Leu, Val, and Tyr (Kukman *et al.*, 1995). One bitter peptide isolated from bovine hemoglobin had a high number of hydrophobic amino acid residues and a Phe locating at the C-terminal, which met the requirements for tasting bitter (Aubes-Dufau *et al.*, 1995).

The results of the present study are comparable to those of previous works. It is obvious that except Trp, which is less than 1% in both PPI and PPH, other hydrophobic amino acids (Leu, Ile, Val, Phe, Pro, Tyr) are all present at a relatively high concentration, especially Leu, which is 9.91% in PPH. Therefore, it confirms that hydrophobicity is a major factor contributing to the bitterness of peptides.

Table 12. Amino acid composition of PPI and PPH.

Amino Acid	PPI (%)	PPH (%)
Aspartic acid	11.81	13.79
Glutamic acid	16.54	13.92
Serine	5.72	6.2
Glycine	4.09	3.76
Histidine	1.74	1.61
Arginine	8.6	6.83
Threonine	3.48	3.6
Alanine	**4.34**	**5.01**
Proline	5.49	5.15
Tyrosine	3.78	3.87
Valine	**5.19**	**5.63**
Methionine	1.12	0.91
Cysteine	0.87	0.24
Isoleucine	**4.73**	**5.43**
Leucine	**8.79**	**9.91**
Phenylalanine	**5.49**	**7.41**
Lysine	7.35	6.1
Tryptophan	0.83	0.68

3.4 CONCLUSION

Bitterness of PPH decreased with lowered concentration until 2.5 mg/mL, under which no more bitterness change was detected. PPH had a relatively high content of hydrophobic and aromatic amino acids, such as Leu, Ile, Phe, Pro, Tyr, which contribute to its bitterness. It is concluded that thermolysin hydrolyzed PPH has a moderate bitterness compared to the hydrolysates/peptides derived from other food proteins, or PPH catalyzed by other enzymes, and this bitter taste arises from the hydrophobic character of the peptides within PPH.

CHAPTER 4

BIOAVAILABILITY AND SHORT-TERM INFLUENCE OF PEA PROTEIN HYDROLYSATE ON ACE AND ANGIOTENSIN II IN NORMATENSIVE RATS

4.1 INTRODUCTION

In vitro activity of bioactive peptides does not necessarily equal to *in vivo* activity. Bioavailability, from certain aspects, determines the efficacy of bioactive peptides *in vivo*. There is plenty of scientific evidence that small-size bioactive peptides are capable of resisting digestive enzymes during passage through the GIT, and being absorbed and delivered intact to target organs to exert their efficacy. However, bioavailability should always be confirmed before conclusions about potential *in vivo* efficacy can be drawn.

Many studies have been conducted to investigate the short-term antihypertensive effects of bioactive peptides derived from food proteins (Katayama *et al.*, 2007; Li *et al.*, 2007; Nakano *et al.*, 2006). Because of the rapid digestion, absorption, and metabolism system of the rats, decreased BP can be monitored just hours after oral administration and effects can last up to 24 h. Inhibition of key disease biomarkers, enzymes, and presence of peptides in tissues can be detected as well.

Long-Evans rat model was developed by crossing Wistar female with wild gray male rats. It is a general multipurpose model which is frequently

used in behavior and metabolism studies. This study was the first one of its kind to use this rat model to exam the bioavailability of bioactive peptides from pea protein and their short-term effects on ACE activity and angiotensin II level *in vivo*.

The objective was to determine the bioavailability of PPH peptides by measuring the ACE activity and levels of angiotensin II in the plasma and major organs. If peptides present in the pea protein hydrolysate were bioavailable, it would be reflected as changes in the levels of measured parameters of treated rats when compared to the control rats that did not receive the PPH treatment. This experiment was necessary before we could embark on a long-term study to determine antihypertensive effects of the PPH in an animal model.

4.2 MATERIALS AND METHODS

4.2.1 Pea Protein Hydrolysate Preparation

PPH was prepared as described in section 3.2.1.

4.2.2 Animal Treatment

All animal procedures were approved by the University of Manitoba Committee on Animal Care and were in accordance with the guidelines of the Canadian Council on Animal Care.

Eighteen adult Long-Evans rats were maintained on standard chow diet for one week with free access to food and water before the experiment. Prior to oral administration of the PPH, rats were fasted for 6 h to minimize interference by peptides that might be arise from digestion of protein in the animal feed. However, during the fasting period, rats had free access to water. Treated rats were divided into two groups. One group was given 1 mL of 500 mg/mL PPH, which was dissolved in saline solution, by oral gavage whereas the other group received 1 mL of 1000 mg/mL hydrolysate. Rats in the control group were given only 1 mL of the saline solution. Rats were euthanized 2 h after oral administration. Brains, hearts, lungs and kidneys were collected, weighed, and frozen at -80°C until use. Blood was collected into heparinized tubes through heart puncture immediately prior to death, centrifuged at 1,500xg for 15 min, and plasma was collected and stored at -80°C until use.

4.2.3 Preparation of Tissue Extract

4.2.3.1 Materials

Rat organs were obtained as indicated in section 4.2.2. Tris-HCl (50 mM, pH 7.9) containing 0.3 M NaCl, and Tris-HCl (50 mM, pH 7.9) containing 0.5% Triton X-100 were prepared and stored appropriately before use.

4.2.3.2 Methods

Tissue extract was prepared according to Takai *et al.* (2001) with the following modifications. The frozen rat organs were weighed, chopped into small pieces and then homogenized using an ultra disperser in 50 mM cold Tris-HCl (pH 7.9) containing 0.3 M NaCl for 30 sec (4 mL/g organ), put on ice to chill, and homogenized for 30 sec again. The two steps (homogenization and chilling on ice) were alternated until the tissue became liquefied. Tissues were then rested on ice for 1 min to chill. Suspension was filtered through cheesecloth, and the filtrate was collected in a container buried in ice. The filtrate was centrifuged at 44,000xg for 90 min at 4°C, and the supernatant was discarded. The pellets were then suspended in the same cold Tris-HCl buffer (4 mL/g of original organ), centrifuged at 44,000xg again for 90 min at 4°C and supernatant discarded. The pellets were suspended in the 50 mM cold Tris-HCl buffer (pH 7.9) containing 0.5% Triton X-100 (4 mL/g of original organ) and chilled on ice. After 1 h the suspension was centrifuged at 1,000xg

for 10 min at 4°C and the supernatant, which was called the rat tissue extract, was ready to be used for further assays.

4.2.4 Determination of Protein Content in Animal Tissues

4.2.4.1 Materials

Rat tissue extract was obtained from step 4.2.3. Reagent A (2% Na_2CO_3, 0.4% NaOH, 0.16% sodium tartrate, 1% sodium dodecyl sulfate) and reagent B (4% $CuSO_4.5H_2O$) were prepared and stored at room temperature. Reagent C (100 parts reagent A mixed with 1 part reagent B) was prepared on the day of analysis. Bovine serum albumin (BSA) and Folin-Ciocalteu phenol reagent were purchased from Sigma (Sigma Chemicals, St. Louise, MO., U.S.A.).

4.2.4.2 Methods

Standard solutions containing 10-100 μg/mL BSA protein concentration were prepared. An aliquot (10 μL) of tissue extract was taken and diluted to 1 mL using phosphate buffered saline (PBS). An aliquot (3 mL) of reagent C was added to each of the standard and tissue extract and incubated at room temperature. After 1 h, 0.3 mL diluted Folin-Ciocalteu phenol reagent (1:1 mixed with MilliQ water) was added and mixed vigorously using a vortex. The standards and samples were allowed to stand at room temperature for 45 min before absorbance was measured at 660 nm. A standard curve was drawn with standard concentrations on the X-axis versus absorbance on the Y-axis. An equation was obtained from standard

curve and used to calculate the real concentration of samples. Protein content of rat tissue extract was calculated from the equation.

4.2.5 ACE Inhibition Assay

4.2.5.1 Materials

Rat tissue extract was obtained from step 4.2.3. ACE and hippuryl-histidinyl-leucine (HHL) were purchased from Sigma (Sigma Chemical, St. Louise, MO., U.S.A.).

4.2.5.2 Methods

ACE activity was determined according to Cushman and Cheung (Cushman & Cheung, 1971) with modifications. HHL was prepared at concentration of 4.15 mM using 0.1 M sodium borate buffer containing 0.3 M NaCl (pH 8.3). At 37°C, 100 μL rat tissue extract was pre-incubated for 10 min. For control, rat tissue extract was replaced with 100 μL of PBS containing 10 mU commercial ACE. HHL was pre-incubated at 37°C for 3 min before an aliquot of 150 μL was added into animal tissue extract (sample) or PBS (control) and incubated at 37°C. The reaction was terminated after 30 min by adding 250 μL of 1 M HCl. Ethyl acetate (500 μL) was then added to the reaction mixture and vortexed vigorously for 1 min to extract hippuric acid (HA) liberated by ACE. After centrifugation at 5,000xg for 10 min, 200 μL of the upper layer was transferred into a clean tube and dried by nitrogen flow. Then 1 mL MilliQ water was added into the tube to dissolve the residue and absorbance at 228 nm was measured. Absorbance of animal tissue

extract was compared with that of commercial ACE, and relative ACE activity in the tissue (per mg protein) was calculated.

4.2.6 Angiotensin II Assay

4.2.6.1 Materials

Rat tissue extract was obtained from step 4.2.3. HPLC grade acetonitrile and trichloroacetic acid (TCA) were purchased from Fisher (Fisher Scientific, Ottawa, ON, Canada). Angiotensin II and HPLC grade trifluoroacetic acid (TFA) was purchased from Sigma (Sigma Chemical, St. Louise, MO., U.S.A.).

4.2.6.2 Methods

Rat tissue extract was first heated in boiling water for 5 min and cooled to room temperature. An aliquot of animal tissue extract was combined with an equal volume of 50 g/L TCA solution, centrifuged at 10,000xg for 10 min and the supernatant was analyzed for angiotensin II level by an HPLC method with modifications as follows (Takai *et al.*, 2001).

Detection of angiotensin II level in the animal tissues was conducted using a HPLC method. 1) After injecting 10 μL of 450 μM commercial angiotensin II or 200 μL of rat tissue extract into a Symmetry® 3.0 × 150 mm 5 μm C18 column which was coupled to a Waters HPLC system (Waters, Mississauga, ON), elution was carried out with a linear gradient of acetonitrile (20-40% over 20 min containing 0.05% v/v TFA). Intensity was monitored and recorded at 210 nm. Column was washed between each

injection by increasing acetonitrile to 100% and kept for 10 min ensuring no prominent peak was ever detected during the regeneration of the column. 2) Angiotensin II spiking test: About 0.5 mg commercial angiotensin II was added to the rat tissue extract, and then 200 μL was injected and eluted on the column using the same methods as previously described. Retention time and integrated area were obtained using the software (Empower 2) to compare the peaks and do the calculations.

4.2.7 Statistical analysis

Data was analyzed using general linear model of ANOVA followed by Duncan's multiple range test using SAS (SAS, USA). $P < 0.05$ was considered as statistically significant.

4.3 RESULTS

4.3.1 Effect of Pea Protein Hydrolysate on ACE Activity

Results were similar for both kidneys with about 11% lower ACE activities in rats fed with PPH compared with those fed with saline only. There was a significant decrease ($p<0.05$) in ACE activities in brains of rats fed hydrolysate compared to those fed saline, although significant difference was not observed between the two hydrolysate groups (2.68 ± 0.11, 3.05 ± 0.39, and 3.98 ± 0.26 mU/mg protein in 500 mg, 1000 mg, and saline, respectively). ACE activities were decreased by about 28 and 12%, respectively, in 500 mg and 1000 mg treated rats compared with the saline treated rats. ACE activity in lung was still high even after inhibition with the PPH, about twice the activity compared to that in the other organs, which was in accordance with the fact that lung is one of the primary organs where ACE is abundant. Compared with the rats given saline, ACE activities in the lungs of rats given hydrolysate were significantly lower ($p<0.05$) and dose-dependent, with about 18% and 26% lower when fed 500 and 1000 mg hydrolysate (8.29 ± 0.48, 7.51 ± 0.68, and 10.38 ± 0.99 mU/mg protein in 500 mg, 1000 mg, and saline, respectively). ACE activities in the hearts and plasma of rats treated with PPH were not significantly different from those

that received saline. Effects of PPH on ACE activities in each of these organs were shown in Figures 4-9.

Many previous studies demonstrated that food protein hydrolysates/peptides were bioavailable and could exert effects in a relatively short term (Matsui *et al.*, 2004; Fujita *et al.*, 2001; Shin *et al.*, 2001; Suetsuna & Nakano, 2000; Fujita *et al.*, 2000; Saito *et al.*, 2000). Katayama *et al.* (2007) and Qian *et al.* (2007) each reported that after administration of 10 mg/kg bw peptides derived from porcine skeletal muscle and tuna dark muscle to SHR, systolic blood pressure (SBP) was decreased by 24 mmHg and about 18 mmHg, respectively, at 3 h. Sesame protein hydrolysate could reduced SBP by 30 mmHg 8 h after 1 mg/kg bw administration in SHR (Nakaono *et al.*, 2006). A sample of WE80BG derived from whey protein showed a strong hypotensive activity (SBP reduced by 21.2 mmHg after 6 h) at 2 mg/mL (Murakami *et al.*, 2004). Tokunaga *et al.* (2004) reported dose-dependent antihypertensive effect of royal jelly hydrolysate in SHR after 1 h of administration (SBP decreased 0.8, 8.9, and 18.4 mmHg after administration of 10, 50, and 100 mg/kg bw). Antihypertensive effects (mean BP reduced by 19.2 mmHg) showed as early as 8 min after injecting SHR with 5 mg/kg bw wheat germ hydrolysate (Matsui *et al.*, 2000).

Studies detecting ACE activities in animal tissues after administration of bioactive peptides are few. Masuda *et al.* (1996) observed significantly lower ACE activities in lung and aorta extract from SHR given Calpis™ sour milk

than activities from those given saline 6 h after oral administration, but not in the other organs. In normotensive WKY Wistar-Kyoto rats, no difference was detected. In Long-Evans rats in our study, significant differences of ACE activities were only detected in lung and brain tissues, which were organs that have abundant level of ACE. Previous studies showed that aorta was a significant target organ for hypertension by ACE inhibitors (Takai *et al.*, 2004; Masuda *et al.*, 1996). However, we did not collect the rat aorta in this study.

Figure 4. Effects of pea protein hydrolysate on ACE activity in left kidney of Long-Evans rats 2 h after oral gavage.

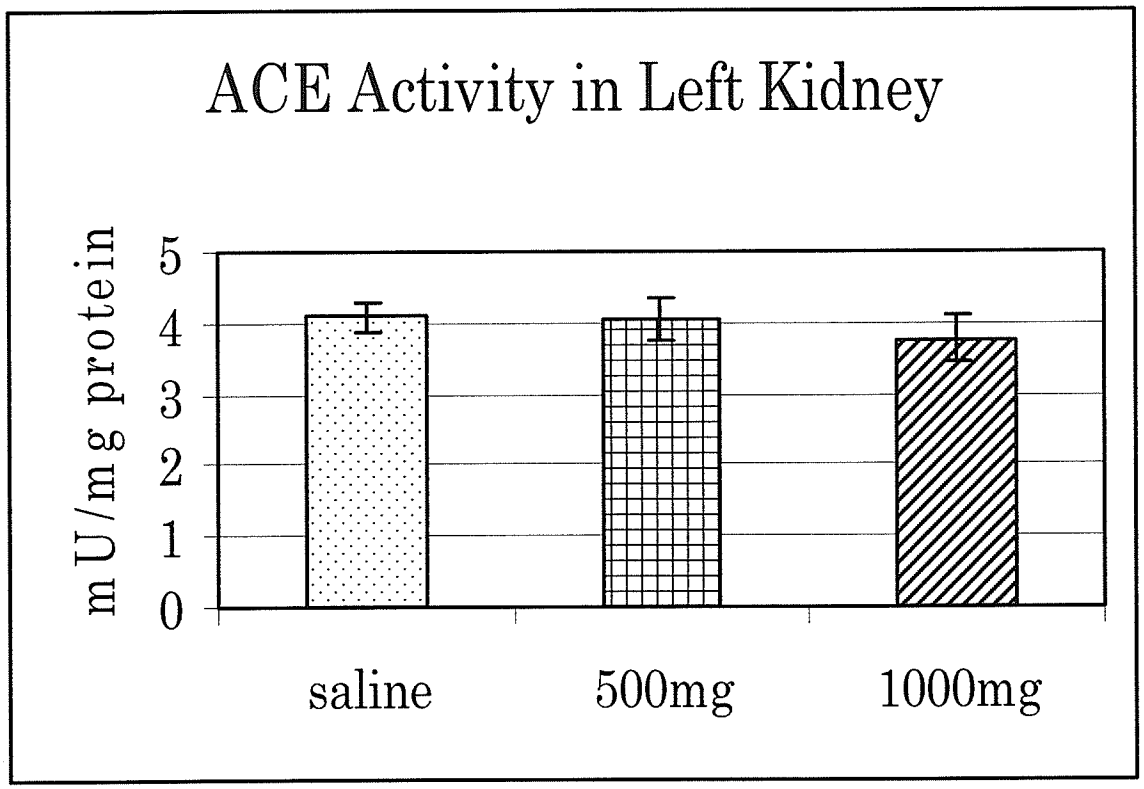


Figure 5. Effects of pea protein hydrolysate on ACE activity in right kidney of Long-Evans rats 2 h after oral gavage.

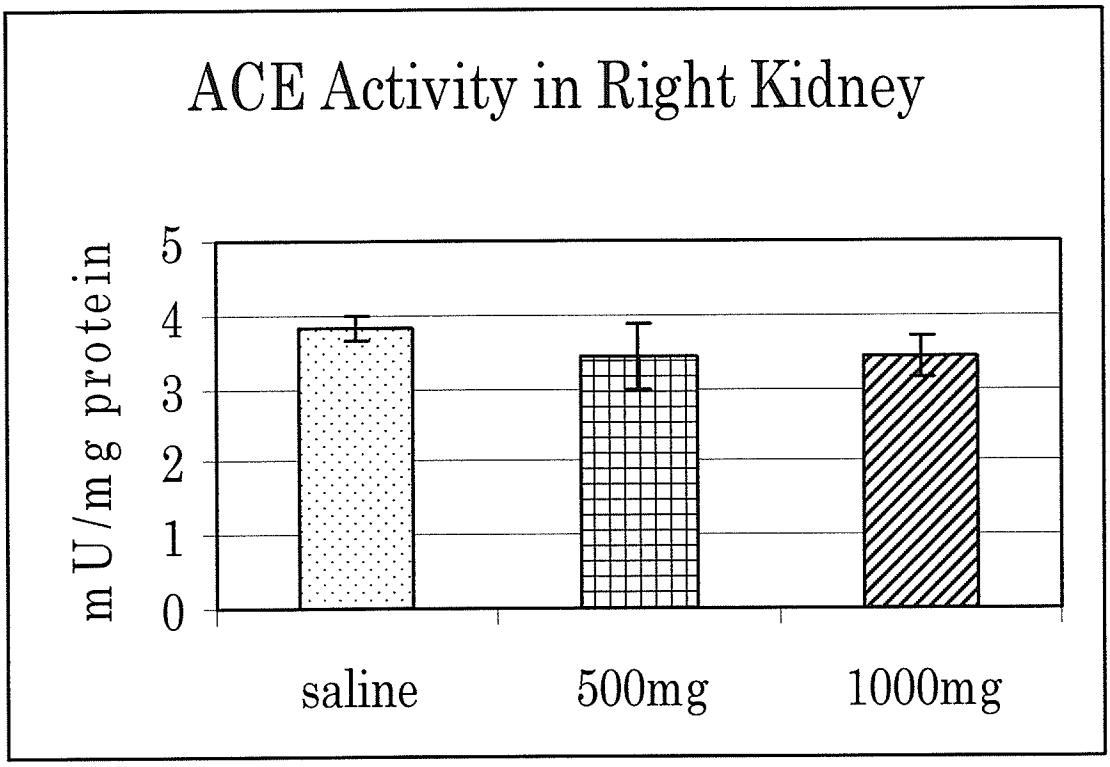


Figure 6. Effects of pea protein hydrolysate on ACE activity in brain of Long-Evans rats 2 h after oral gavage.

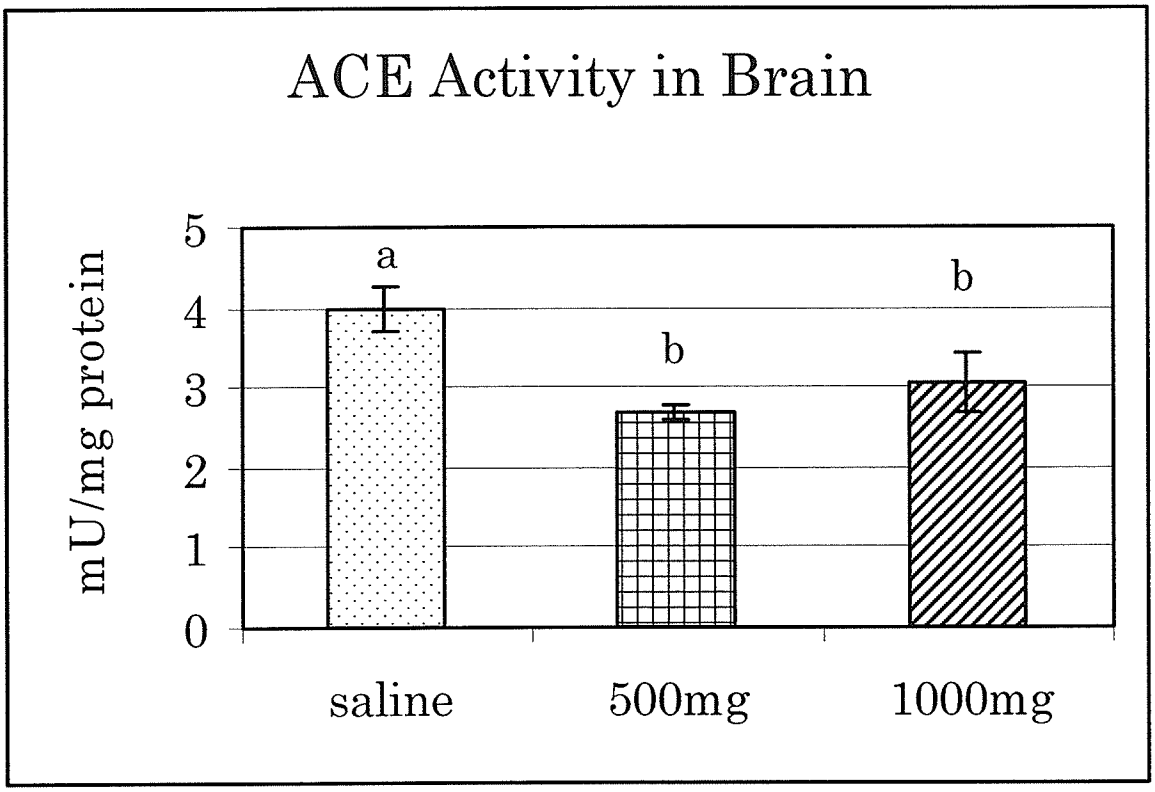


Figure 7. Effects of pea protein hydrolysate on ACE activity in lung of Long-Evans rats 2 h after oral gavage.

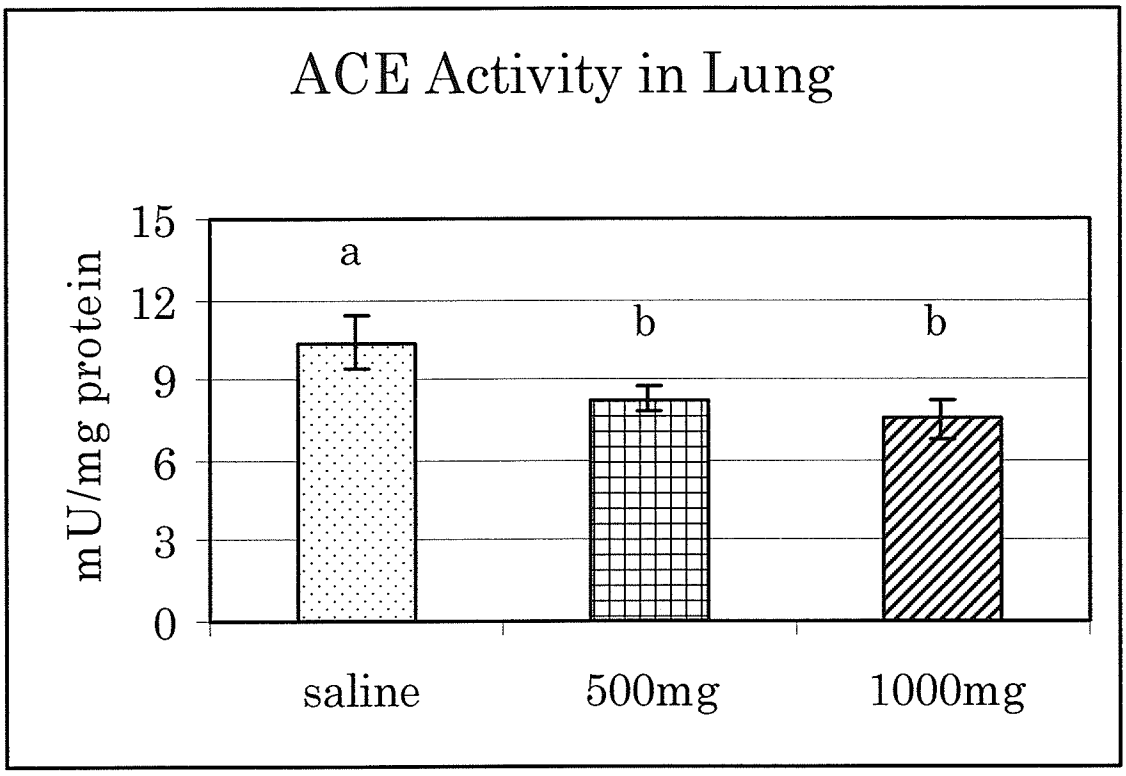


Figure 8. Effects of pea protein hydrolysate on ACE activity in heart of Long-Evans rats 2 h after oral gavage.

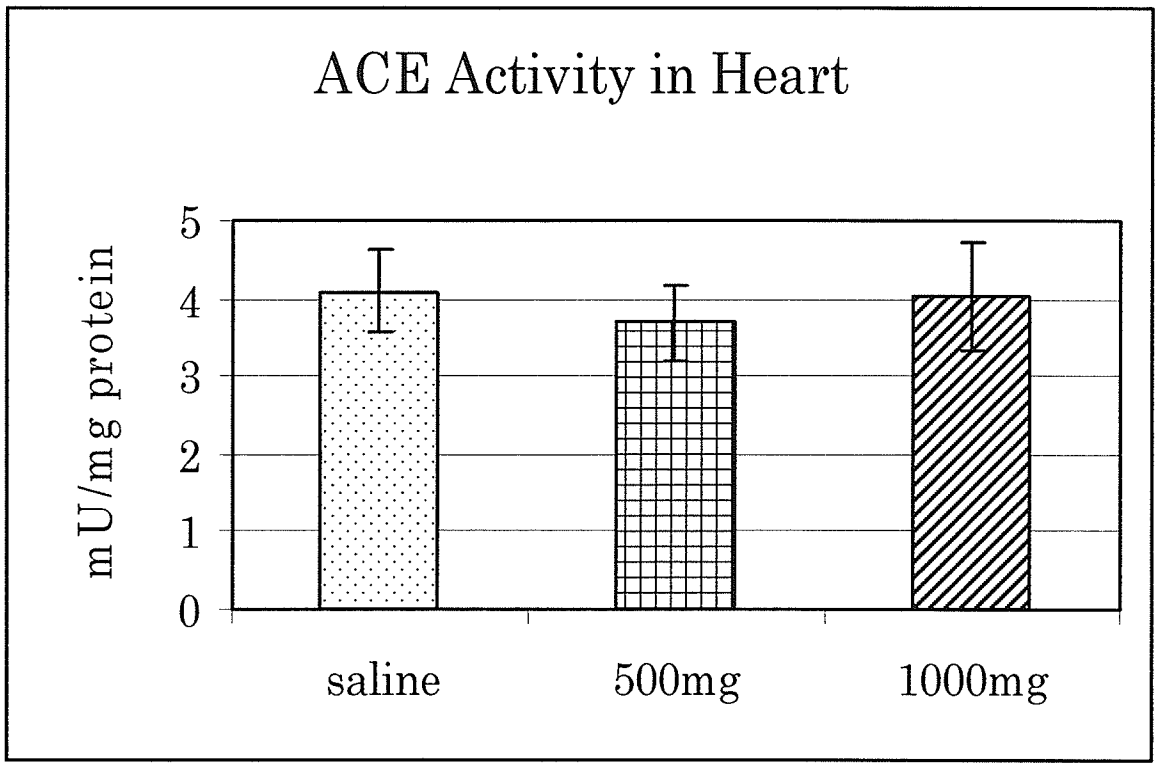
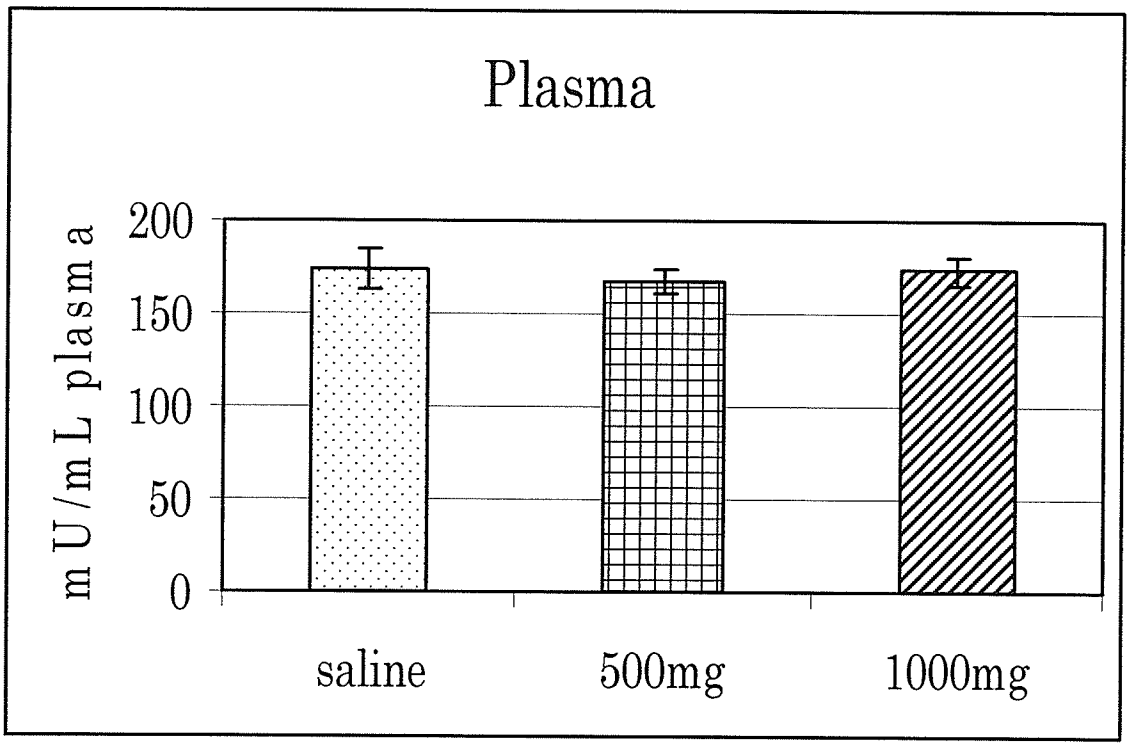


Figure 9. Effects of pea protein hydrolysate on ACE activity in plasma of Long-Evans rats 2 h after oral gavage.



4.3.2 Effect of Pea Protein Hydrolysate on Angiotensin II Level

Figure 10, 11, and 12 shows the HPLC chromatographs of commercial angiotensin II, angiotensin II from rat tissue extract, and rat tissue extract with added commercial angiotensin II (spiked sample), respectively. Retention time of commercial angiotensin II and angiotensin II from rat tissue extract was 6.364 and 5.856 min, respectively, which were very close but not identical. This is because the pure angiotensin has no interfering compounds whereas the rat tissue extracts also contain other peptides which could have shifted the elution time to be slightly different from that of commercial angiotensin II that was dissolved in pure buffer. Result from spiking test showed the same retention time of tissue extract with added commercial angiotensin II as that of pure rat tissue extract. Therefore, it is evident that the peak showing at 5.856 min is angiotensin II. However, spiking with 0.5 mg commercial angiotensin II could overload the peak and caused the peak to be eluted out earlier. Further confirmation of angiotensin II can be performed using LC-MS/MS by looking at the molecular weight and amino acid sequence of the compound.

Angiotensin II level in kidneys, lung, brain, heart, and plasma is shown in Figures 13-18. Generally, angiotensin II level followed similar pattern as that of ACE activity. In kidneys and brains, although 500 and 1000 mg hydrolysate groups showed lower angiotensin II level than that of

saline group, it was not significant. In lung, there was a dose-dependent decrease of angiotensin II level with a significant difference between saline and 1000 mg groups (197.2 ± 11.79, 187.47 ± 11.98, and 255.39 ± 19.82 μmol/mg protein in 500 mg, 1000 mg, and saline groups, respectively). Angiotensin II level in plasma was similar as that in lung, with 4.6% and 17.3% decrease of angiotensin II level in 500 and 1000 mg groups, and the reduction in 1000 mg group was statistically significant (8.78 ± 0.45, 7.61 ± 0.53, and 9.2 ± 0.33 μmol/mL plasma in 500 mg, 1000 mg, and saline groups, respectively). These results agree with previous ACE activity results and the facts that angiotensin II is converted from angiotensin I under the action of ACE, and after production it is released and primarily circulates in blood. Similar studies have provided scanty information on the level of angiotensin II in organs after administration of bioactive peptides, which makes it difficult to compare our results with previous works.

4.4 CONCLUSION

Results from this study showed that 2 h after administration of pea protein hydrolysate, ACE activities in rat lung tissues were dose-dependently inhibited and led to decreased angiotensin II level in both lung and plasma. Interestingly, the PPH was more effective against ACE in the lungs, which is the location where most of the enzyme is synthesized. The PPH seems to be less effective in organs that do not contain high levels of ACE. It is indicated that thermolysin hydrolyzed pea protein contained peptides mixture that was able to escape GIT enzyme-dependent digestion, pass through GI barrier, be absorbed into the circulation, and reach target organs to exert effects. We confirmed that the actions of these peptides could induce *in vivo* ACE inhibition and subsequent reduction of angiotensin II level on a short-term basis. Therefore, the potential therapeutic effects in a disease model were examined in the next series of experiments as documented in Chapter 5 of this thesis.

Figure 10. HPLC chromatogram of 10 µL of 450 µM commercial angiotensin II.

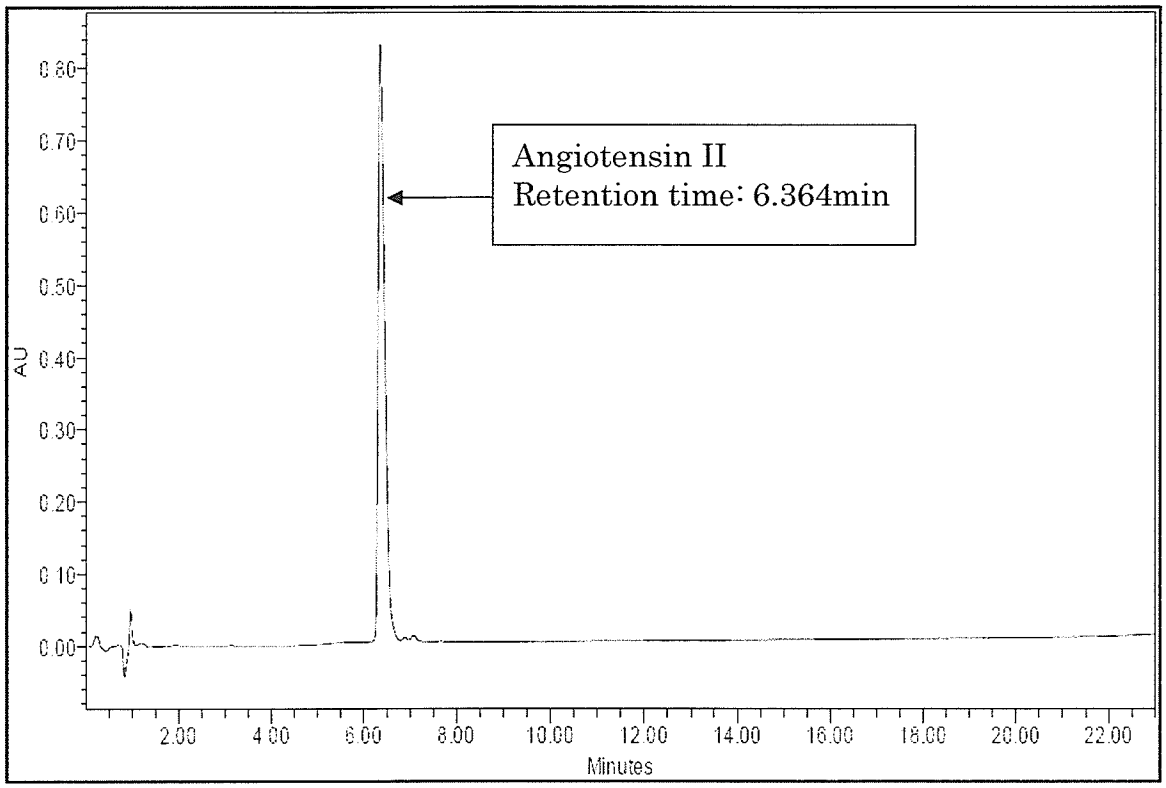


Figure 11. HPLC chromatogram of 200 µL lung tissue extract of Long-Evans rats.

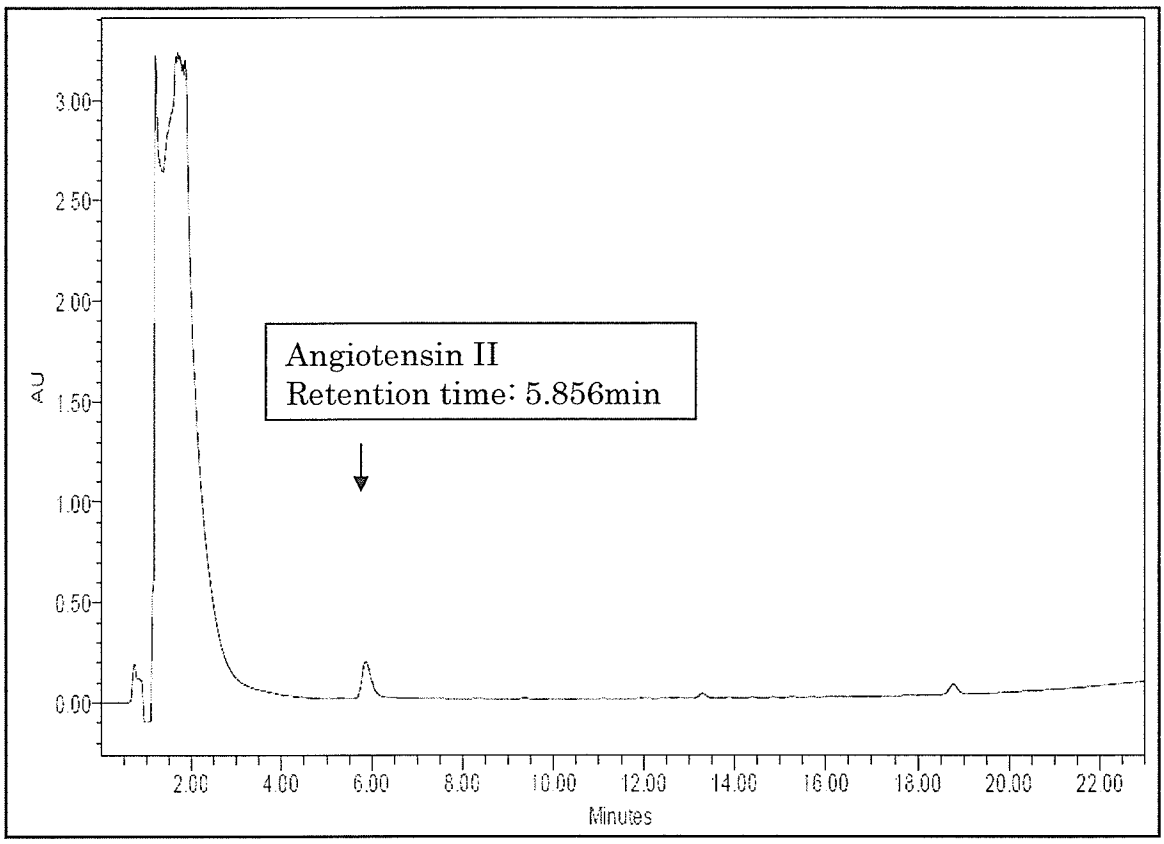


Figure 12. HPLC chromatogram of lung tissue extract of Long-Evans rats with 0.5 mg added commercial angiotensin II.

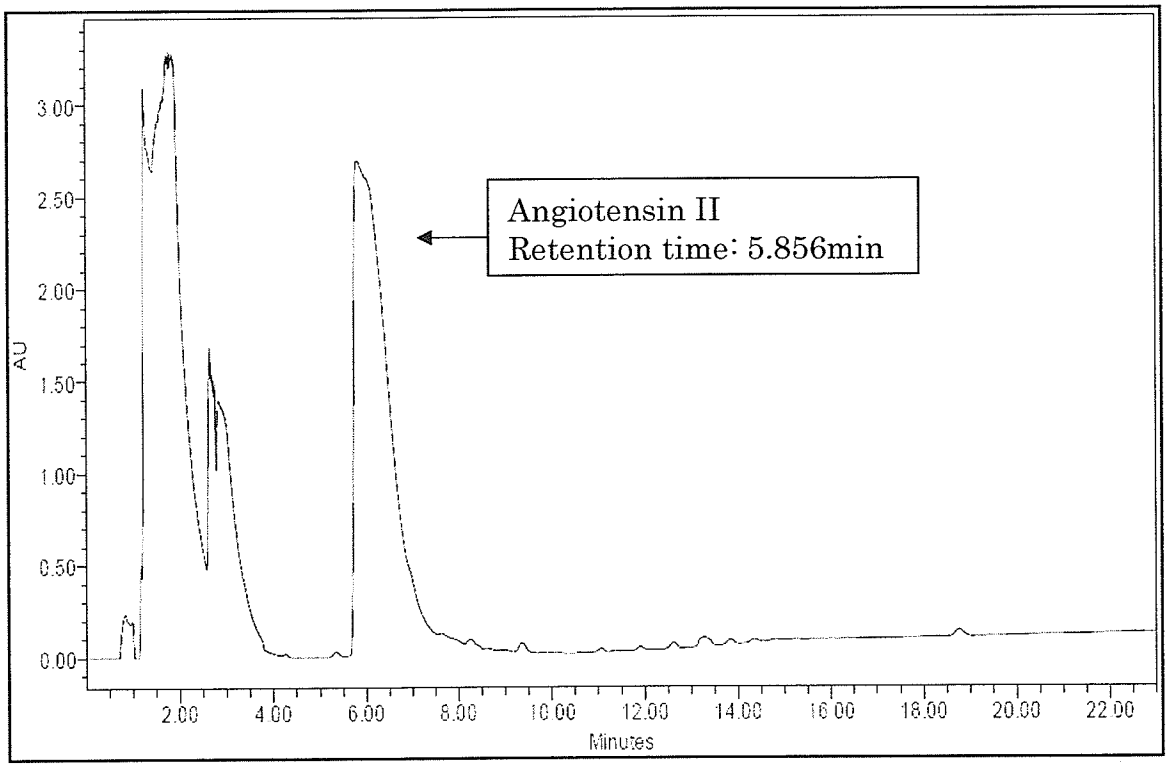


Figure 13. Effects of pea protein hydrolysate on angiotensin II level in left kidney of Long-Evans rats 2 h after oral gavage.

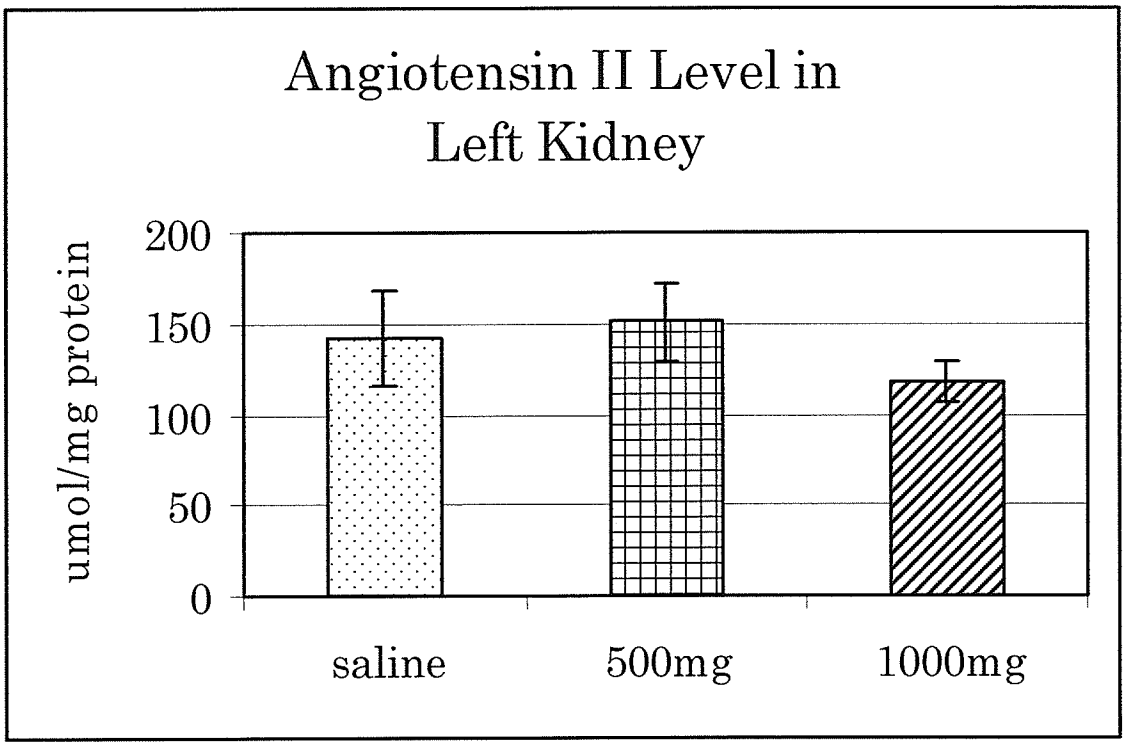


Figure 14. Effects of pea protein hydrolysate on angiotensin II level in right kidney of Long-Evans rats 2 h after oral gavage.

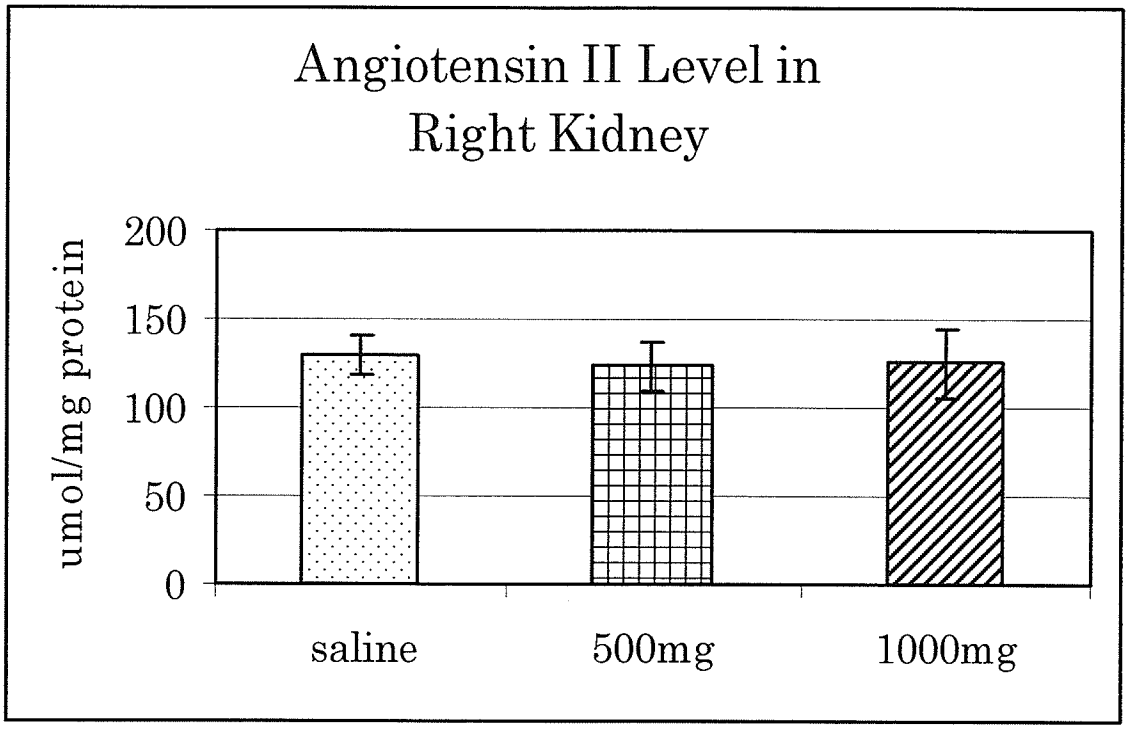


Figure 15. Effects of pea protein hydrolysate on angiotensin II level in brain of Long-Evans rats 2 h after oral gavage.

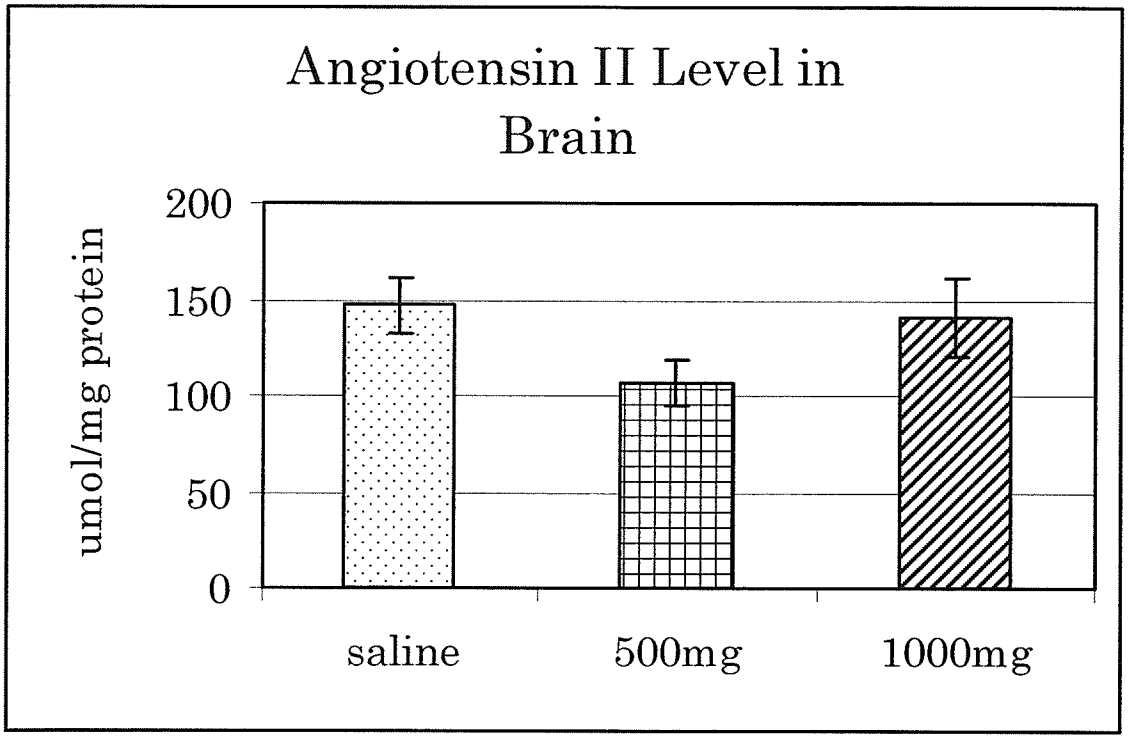


Figure 16. Effects of pea protein hydrolysate on angiotensin II level in lung of Long-Evans rats 2 h after oral gavage.

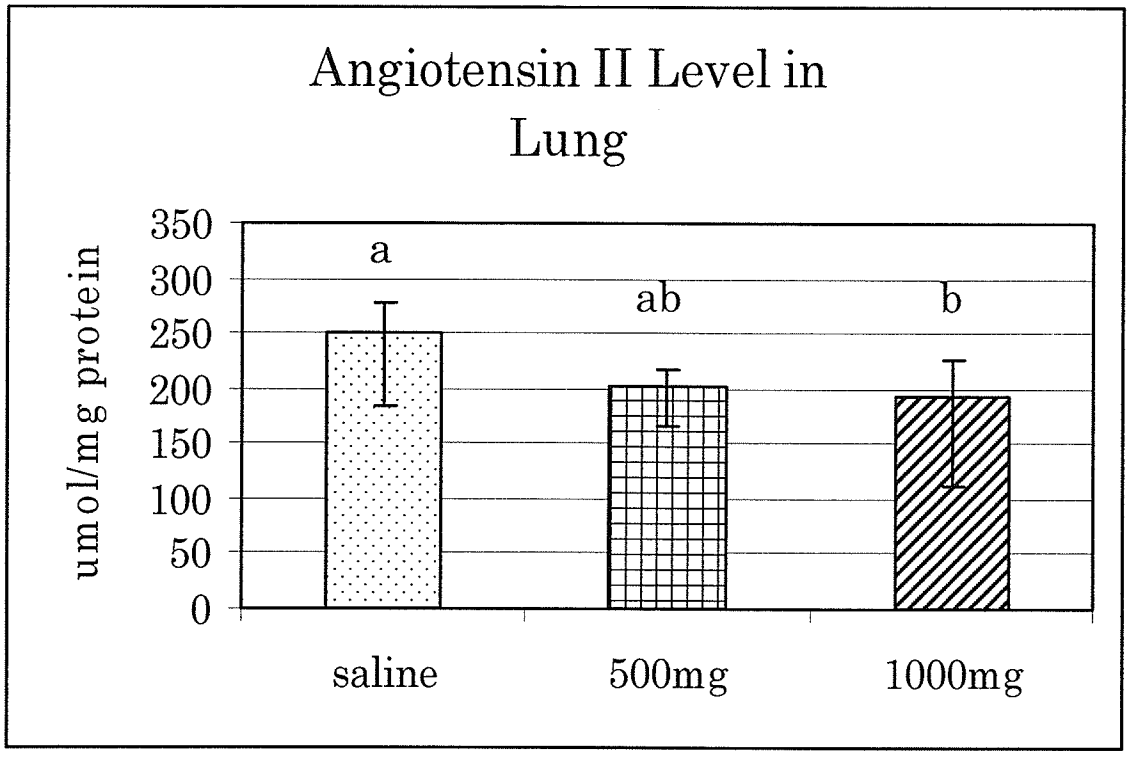


Figure 17. Effects of pea protein hydrolysate on angiotensin II level in plasma of Long-Evans rats 2 h after oral gavage.

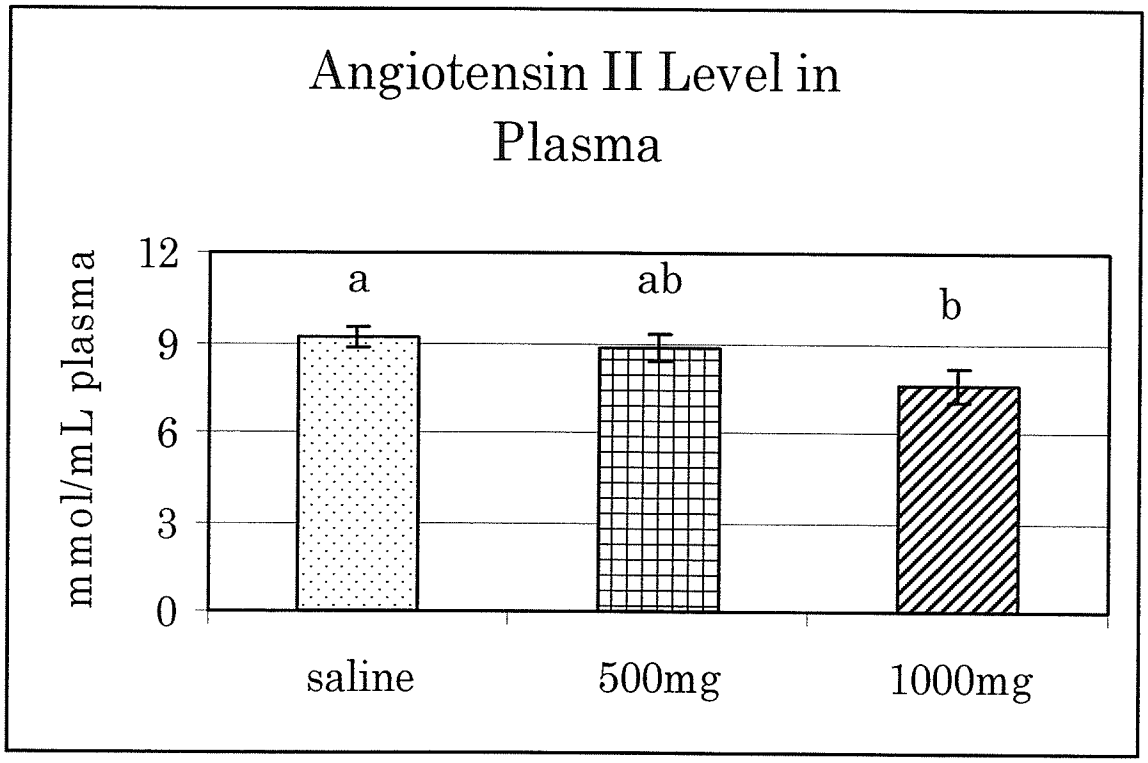
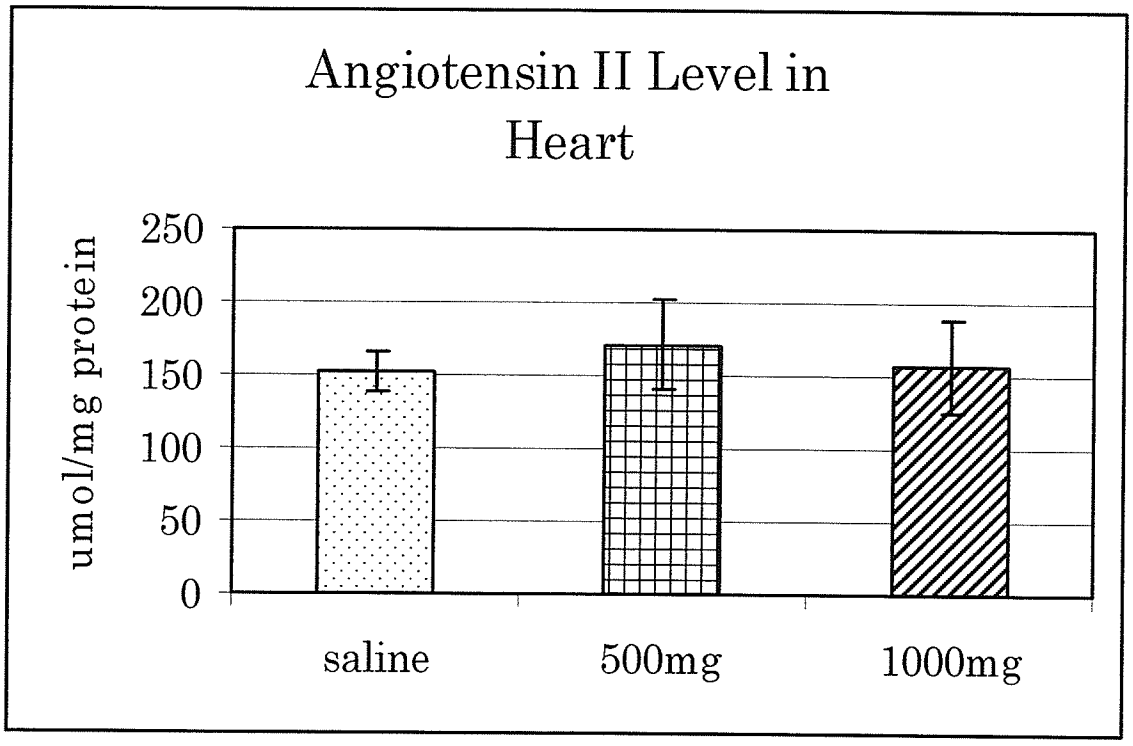


Figure 18. Effects of pea protein hydrolysate on angiotensin II level in heart of Long-Evans rats 2 h after oral gavage.



CHAPTER 5

LONG-TERM ANTIHYPERTENSIVE EFFECTS OF PEA PROTEIN HYDROLYSATE IN POLYCYSTIC KIDNEY DISEASED RATS

5.1 INTRODUCTION

Chronic kidney disease (CKD) is always accompanied by elevated BP and approximately 80% of CKD patients will develop hypertension at some point. From one aspect, fluid retention can be caused by reduced kidney function due to poor control of sodium and fluid. More importantly, injured kidney can lead to activated RAS, which is responsible for BP regulation in human body. As a result more renin and ACE proteins are made, which leads to increased production of angiotensin II and elevated BP.

The Han:SPRD-cy rat is a rat model that genetically develops autosomal dominant polycystic kidney disease (ADPKD) in which heterozygotes develop progressive cystic change, renal interstitial fibrosis, inflammation, hypertension, and uremia in adult life (Cowley *et al.*, 1993). Female rats show much slower progression of histological and functional renal injury than the male rats. Therefore, male rats which are more seriously affected are preferable than females to be studied in a relatively short period of time. Previous studies that investigated antihypertensive activities of bioactive peptides have used SHR. This study is the first one to use PKD rat

model to study the effects of bioactive peptides on hypertension caused by renal disease.

As stated in Chapter 2, several bioactive peptides derived from pea proteins have been shown to possess *in vitro* inhibition of ACE activity. Research from Dr. Aluko's lab has shown renin and ACE inhibitory activities of a PPH obtained from thermolysin-catalyzed hydrolysis of PPI. Thermolysin is a heat-stable microbial proteinase which specifically hydrolyzes peptides on the N-side of hydrophobic residues such as Phe, Leu, Ile, and Val. These hydrophobic amino acids, as substrates or inhibitors and when present at the C-terminal, appear to be preferred by ACE (Cheung *et al.*, 1980). Therefore, thermolysin is widely selected to release antihypertensive peptides from food proteins. Thermolysin hydrolysates from many food proteins have been reported to contain bioactive peptides with *in vivo* antihypertensive effects (Nakashima *et al.*, 2002; Fujita *et al.*, 1995).

The objective was to investigate the long-term antihypertensive effectiveness of PPH. In this study, thermolysin hydrolyzed pea protein was fed to PKD rats for 8 weeks. Blood pressure, ACE, and angiotensin II level in the tissues were examined to determine the hypotensive effects of PPH.

5.2 MATERIALS AND METHODS

5.2.1 Pea Protein Hydrolysate Preparation

PPH was prepared as described in section 3.2.1.

5.2.2 Animal Diet Formulation

Four different rat diets, 'casein', 'PPI', 'hydrolysate 5 (h5)', and 'hydrolysate 10 (h10)', were prepared. All diets contained 39.75% cornstarch, 13.2% dex cornstarch, 10% sucrose, 7% corn oil, 5% fiber, 3.5% mineral mix, 1% vitamin mix, 0.3% L-cystein, 0.25% choline bitart, and 0.0014% tertiary butylhydroquinone (TBHQ). Besides, 'casein', 'PPI', 'h5', and 'h10' diets each contained 20% casein, 20 % PPI, 19.5% casein with 0.5% PPH, and 19% casein with 1% PPH as protein sources, respectively. Diets were formulated based on the AIN (American Institute of Nutrition)-93G guidelines for rodent diets to meet gestation, lactation, and growth requirement. Compositions of the 4 different diets are summarized in Table 13.

Table 13. Diet ingredients (%) of the 4 different diets.

	Diet Group			
Ingredient	Casein	Pea protein isolate	Hydrolysate 5	Hydrolysate 10
Corn starch	39.75%	39.75%	39.75%	39.75%
Casein	20%	–	19.5%	19%
Pea pro. iso.	–	20%	–	–
Hydrolysate	–	–	0.5%	1%
Dex cornstarch	13.2%	13.2%	13.2%	13.2%
Sucrose	10%	10%	10%	10%
Corn oil	7%	7%	7%	7%
Fiber	5%	5%	5%	5%
Min. mix	3.5%	3.5%	3.5%	3.5%
Vit. mix	1%	1%	1%	1%
L-cys	0.3%	0.3%	0.3%	0.3%
Choline bitart	0.25%	0.25%	0.25%	0.25%
TBHQ	0.0014%	0.0014%	0.0014%	0.0014%
Total	100%	100%	100%	100%

5.2.3 Animal Treatment

All animal procedures were approved by the University of Manitoba Committee on Animal Care and were in accordance with the guidelines of the Canadian Council on Animal Care.

All Han:SPRD-cy rats were obtained from the breeding colony maintained by Dr. Harold Aukema, Department of Human Nutritional Sciences, University of Manitoba, Winnipeg. At 3 weeks of age, 72 weanling male Han:SPRD-cy rats were randomly divided into 4 groups with 18 rats in each group (5 normal, 13 diseased) and fed one of the 4 diets for 8 weeks.

Rats were housed individually in hanging transparent plastic cages, and kept in a controlled environment with temperature at 21-23°C, 55% humidity, and 14 h light:10 h dark cycle. Food and water were provided *ad libitum*.

All rats were weighed weekly. From week 4 to 8, 8 rats per group (3 normal, 5 diseased) were randomly selected to measure BP using the tail cuff method (IITC Model 29ssp, IITC Life Science, Woodland Hills, CA., U.S.A.). All rats used for BP measurement were trained 3 times within 1 week before the experiment to get used to the experimental conditions. Ten rats per group were randomly selected and placed in metabolic cage for 24 h at week 4 and 7. Food and water intake were recorded, urine and feces were collected for other analysis. At the end of 8 weeks, rats were anesthetized with a mixture of

katemine and xylazine (9:0.9 mg/per 100 g bw) and blood was collected into heparinized tubes through heart puncture. Lungs, kidneys, and livers were collected, weighed and stored in -80°C freezer for analysis.

5.2.4 Preparation of Tissue Extract

Rat lungs and plasma were obtained as described in section 5.2.3. Rat tissue extract was prepared as described in section 4.2.3.

5.2.5 Determination of Protein Content in Animal Tissues

Rat tissue extract was obtained from step 5.2.4. Protein content in rat tissue extract was determined as described in section 4.2.4.

5.2.6 ACE Activity Assay

Rat tissue extract was obtained from step 5.2.4. ACE activity assay was performed as described in section 4.2.5.

5.2.7 Angiotensin II Assay

5.2.7.1 Materials

Rat tissue extract was obtained from step 5.2.4. Chemicals, buffers, and HPLC system were the same as described in section 4.2.6.

5.2.7.2 Methods

Rat tissues extract was heated in boiling water for 5 min and cooled to room temperature. An aliquot of animal tissue extract was combined with an equal volume of 50 g/L TCA solution, centrifuged at 10,000xg for 10 min and the supernatant was analyzed for angiotensin II level by an HPLC method with modifications as follows (Takai *et al.*, 2001). After injecting 10 μL of 450 μM commercial angiotensin II or 200 μL of animal tissue extract into a Symmetry® 3.0 × 150 mm 5 μm C18 column which was coupled to Waters HPLC system (Waters, Mississauga, ON), a linear gradient (20-40%) of acetonitrile in water (with 0.05% v/v TFA) was used for elution at flow rate of 1 mL/min. Intensity of eluate was monitored and recorded at 210 nm. Column was washed between each injection by increasing acetonitrile to 100% and holding for 10 min to ensure no prominent peak was detected during regeneration of the column. Integrated areas of animal tissues were compared with that of known concentration of commercial angiotensin II, and used to calculate angiotensin II levels in the animal tissue extracts.

5.2.8 Statistical analysis

Data was analyzed using general linear model of ANOVA followed by Duncan's multiple range test using SAS (SAS, USA). $P<0.05$ was considered as statistically significant.

5.3 RESULTS

5.3.1 Long-term Effects of Pea Protein Hydrolysate on the Development of Blood Pressure of PKD Rats

The rats fed PPI did not gain adequate weight in comparison with those in the other diet groups, and therefore, data was not used for analysis. Possible reason could be that trypsin inhibitors, which exist in raw legume proteins, reduced trypsin and chymotrypsin activity and bioavailability of the pea proteins, and hence, interfered with the essential nutrition absorption. However, trypsin inhibitors can be destroyed by heating and therefore was not a problem in the diets that contained the PPH.

The effect of long-term intake of thermolysin catalyzed PPH on the development of hypertension was compared in PKD rats fed with either casein or hydrolysate diet. Figure 19 is a line chart showing systolic blood pressure (SBP) changes for PKD rats fed casein and hydrolysate groups from week 4 to 8. For both hydrolysate (0.5 and 1%) groups, SBP values increased from week 4 to 5, which is probably due to the fact that the intensity of PKD has started to increase but the hydrolysate had not been ingested long enough to suppress BP increase.

At the beginning of the experiment, all 3 groups displayed similar SBP values (casein 148.8 ± 5.4 mmHg, h5 138 ± 4.2 mmHg, and h10 144 ± 4.8 mmHg). It is obvious that ingested PPH started to suppress BP elevation from the 6th week after administration. At week 6, significantly higher ($p<0.05$) SBP was observed in casein group than that in the hydrolysate groups. This hypotensive effect of pea protein hydrolysate was consistent for another 2 weeks till the end of the study. At week 7 and 8, SBP of both hydrolysate groups kept being significantly lower ($p<0.05$) than that of casein group. At week 8, SBP was 170.4 ± 3, 141 ± 5.4, and 139.8 ± 3 mmHg for casein, h5, and h10 group, respectively. Decreases in SBP of 29.4 and 30.6 mmHg (more than 17% compared to casein group) were observed for group h5 and h10, respectively, at the end of the study. Thus, the thermolysin hydrolyzed PPH was able to mitigate the development of hypertension that is associated with PKD rats.

Figure 20 shows the diastolic blood pressure (DBP) changes in PKD rats belonging to casein and hydrolysate groups from week 4 to 8. DBP changes over this period of time resembled the trend as that for SBP. No significant difference was observed among the 3 groups during the first 2 weeks. However, at week 6, the casein group exhibited significantly higher DBP than h10 group but not h5 group (89.5 ± 10.94, 73.73 ±3.04, and 62.7 ± 1.88 mmHg for casein, h5, and h10, respectively). At week 7 and 8, DBP of

casein group was significantly higher than both h5 and h10 groups (at week 7, DBP was 112.2 ± 8.42, 86.88 ± 6.95, and 85.8 ± 6.38 mmHg for casein, h5, and h10, respectively; at week 8, DBP was 109.5 ± 8.85, 84.8 ± 2.95, 83.34 ± 4.98 mmHg for casein, h5, and h10, respectively). Compared to casein group, hydrolysate groups reduced DBP about 23%.

After SHR were given sour milk for 17 weeks, SBP was reduced by 37 mmHg and DBP was reduced 17 mmHg (Miguel *et al.*, 2005). Sato *et al.* (2002) observed 17 mmHg decrease of SBP after feeding the male SHR either 0.1 or 1% wakame hydrolysate for 10 weeks. Sipola *et al.* (2002) gave male SHR 0.4 mg IPP and 0.6 mg VPP per day for 14 weeks and observed decreased SBP by 21 mmHg. When a mixture of IPP and VPP was given to normal female Waistar rats at 2.5-3.5 mg/kg/d, SBP was detected to be 12 mmHg lower after 12 weeks (Sipola *et al.*, 2001). When female SHRs were fed soy protein hydrolysate at 100 and 1000 mg/kg bw for 1 month, their SBP were lowered by 37.8 and 39 mmHg (Wu & Ding, 2001). Astawan *et al.* (1995) reported that the SBP of SHR was significantly decreased when as much as 5 g of crude peptide from Indonesian dried-salted fish/kg of bw/d was fed orally for 16 d. Though we used a different rat disease model, our results are similar or better than those reported for SHR in terms of BP reduction.

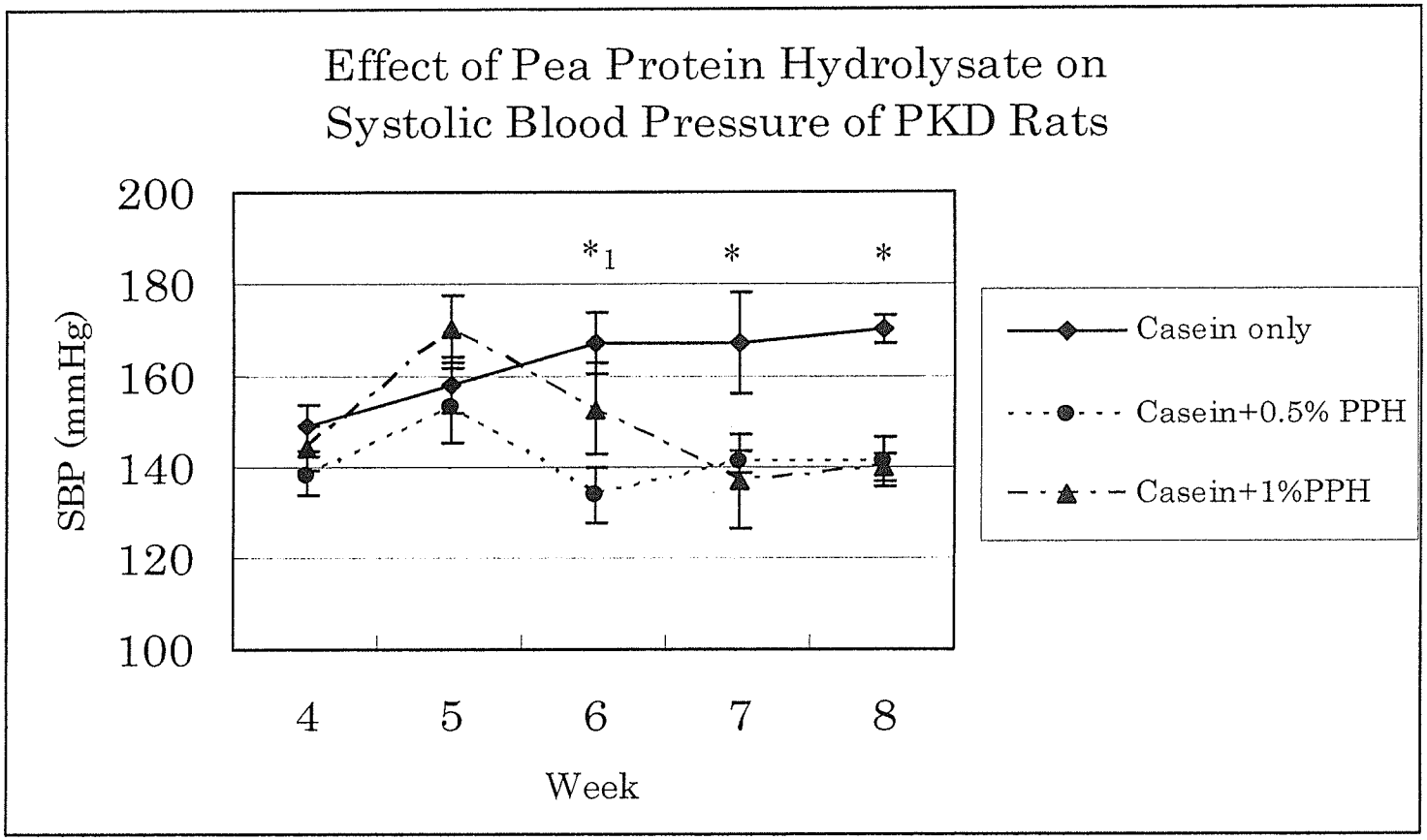
5.3.2 Effect of Pea Protein Hydrolysate on ACE Activities in Tissues

The ACE activities in lung and plasma of PKD rats are shown in Figure 21 and Figure 22. ACE in lung showed much higher activities than that in plasma, and this agrees with the fact that lungs are one of the major tissues that produce ACE protein molecules. In lungs, the casein group showed higher ACE level than the hydrolysate groups, though the differences were statistically not significant ($p>0.05$). In plasma, the h10 group showed highest ACE activity but not significant from the other 2 groups either.

ACE is a key enzyme in the RAS, together with renin, regulating human BP. It was found that renin gene expression was decreased in the kidneys of PKD rats fed PPH compared to those fed casein (Dr. Aluko, personal communication); therefore, it was possible that ACE activities increased to compensate for the down-regulation of the RAS caused by lowered renin expression. It was also observed that ACE gene expression in the kidneys of PKD rats fed hydrolysate was elevated compared with that of the rats fed casein (Dr. Aluko, personal communication), which may be responsible for the elevated plasma ACE level obtained in this work.

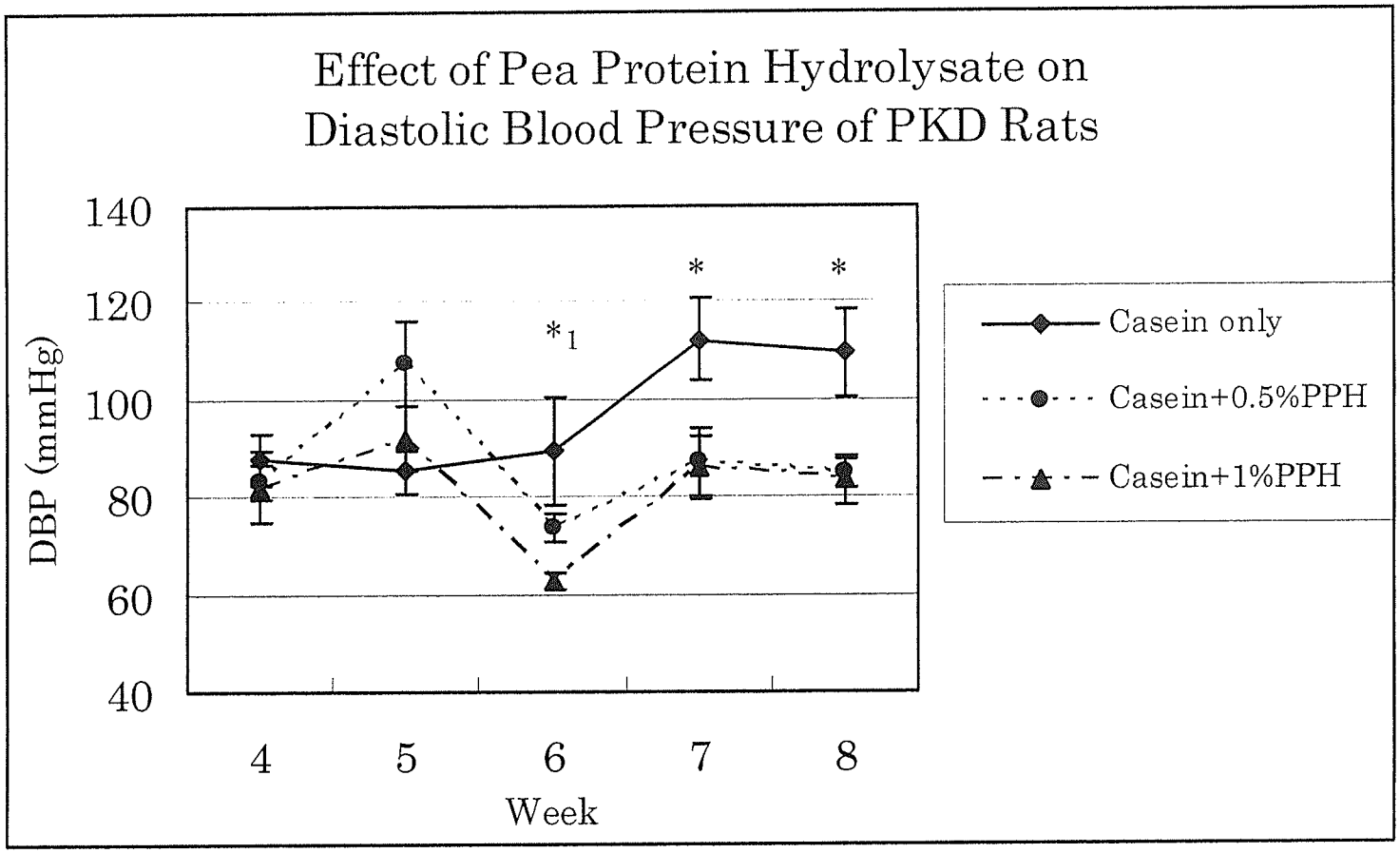
Previous studies have showed inconsistent results regarding ACE activities in different organs after peptides administration (Nakano *et al.*, 2006; Masuda *et al.*, 1996). In addition to the ACE assay method, other factors

Figure 19. Systolic blood pressure of polycystic kidney disease rats fed casein or two levels of pea protein hydrolysate. All diets contained same level (20%) of protein.



*1: P<0.05, caisen compared with casein+0.5%PPH; *: P<0.05, casein compared with both hydrolysate groups.

Figure 20. Diastolic blood pressure of polycystic kidney disease rats fed casein or two levels of pea protein hydrolysate. All diets contained same level (20%) of protein.



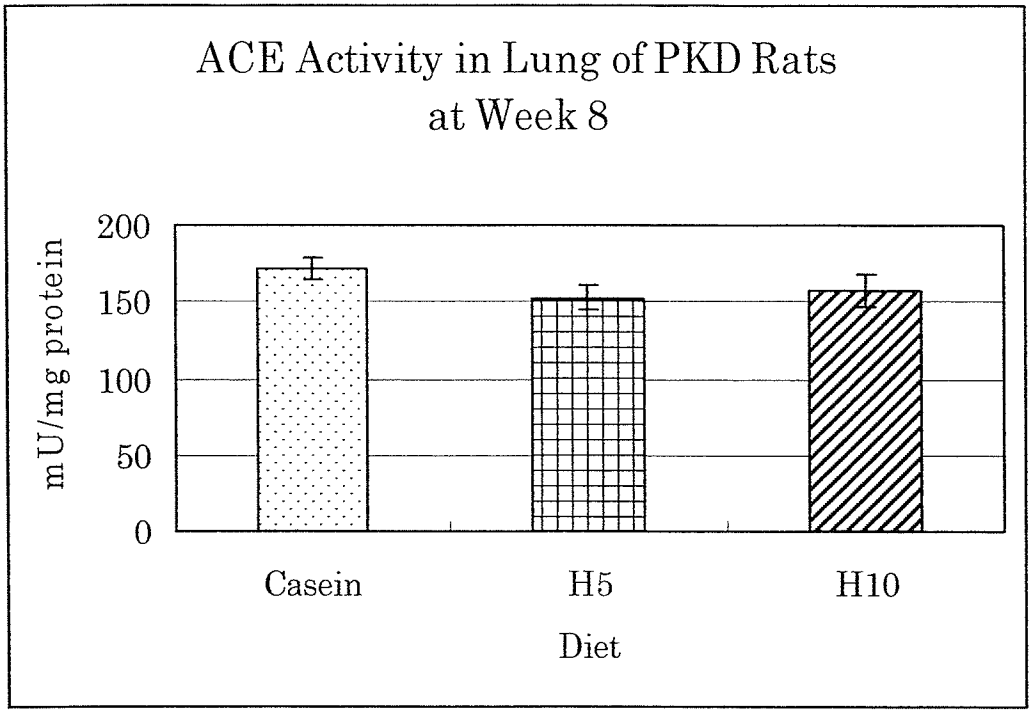
*1: $p<0.05$, casein compared with casein+1%PPH; *: $p<0.05$, casein compared with both hydrolysate groups.

such as rat model, health status of rats, feed dosage, duration of feeding, etc. may also influence the results

5.3.3 Effect of Pea Protein Hydrolysate on Angiotensin II levels in Tissues

Figure 23 shows that angiotensin II level in lungs was higher in PKD rats fed casein than those fed hydrolysate (3.29 ± 0.19 μmol/mg protein in casein vs. 2.57 ± 0.25 and 3.08 ± 0.19 μmol/mg protein in h5 and h10, respectively), though the differences were statistically not significant ($p>0.05$). However, in the plasma, PKD rats fed casein diet had significantly higher ($p<0.05$) angiotensin II level than the hydrolysate groups but there was no difference between the two hydrolysate groups (14.64 ± 0.45, 8.66 ± 0.24, 8.21 ± 0.36 μmol/mg protein in casein, h5, and h10 groups, respectively, Figure 24). As a vasoconstrictor, angiotensin II circulates in the blood and its level is directly related to the BP level. The lower levels of angiotensin II in the PPH groups agree with the SBP results that showed both hydrolysate groups led to significantly lower ($p<0.05$) BP values than the casein group after 8 weeks of diet consumption.

Figure 21. ACE activities in lung of polycystic kidney disease rats at week 8*.

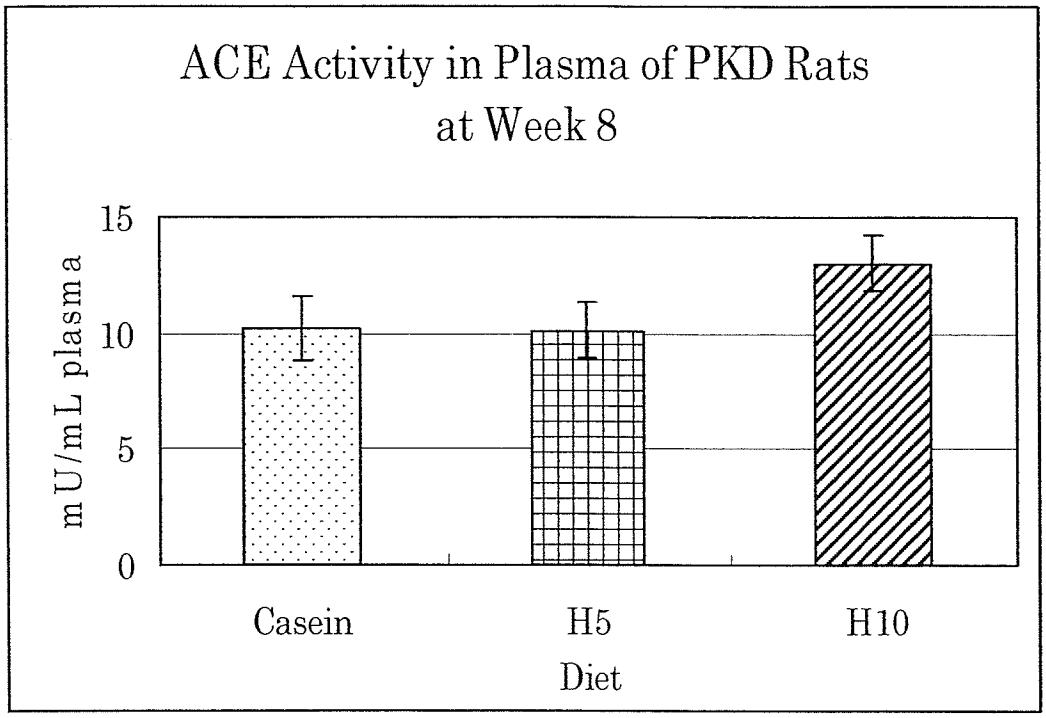


*: Casein, 20% casein

H5, 19.5% casein + 0.5% PPH

H10, 19% casein + 1% PPH

Figure 22. ACE activities in plasma of polycystic kidney disease rats at week 8*.

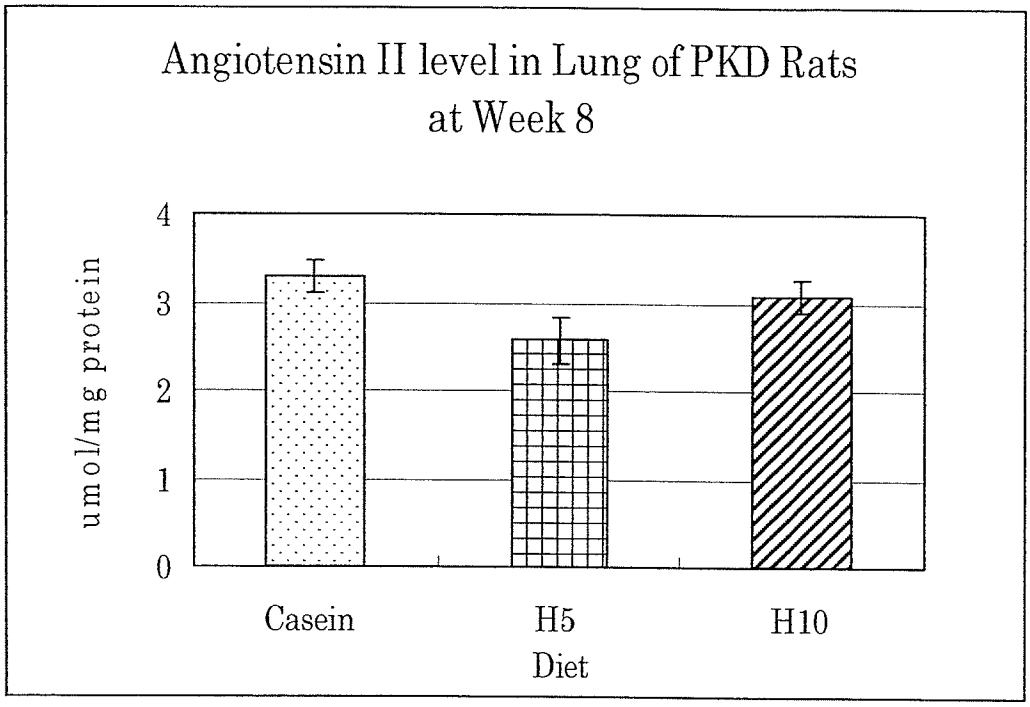


*: Casein, 20% casein

H5, 19.5% casein + 0.5% PPH

H10, 19% casein + 1% PPH

Figure 23. Angiotensin II level in lung of polycystic kidney disease rats at week 8*.

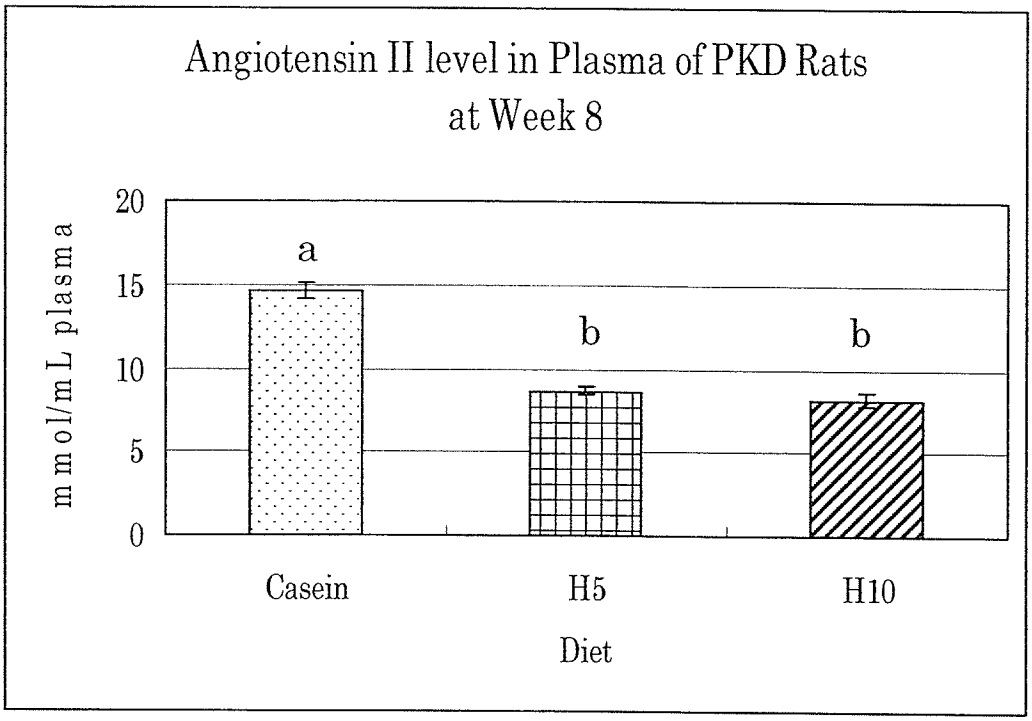


*: Casein, 20% casein

H5, 19.5% casein + 0.5% PPH

H10, 19% casein + 1% PPH

Figure 24. Angiotensin II level in plasma of polycystic kidney disease rats at week 8*.



*: Casein, 20% casein

H5, 19.5% casein + 0.5% PPH

H10, 19% casein + 1% PPH

5.4 CONCLUSION

PPI can be hydrolyzed by thermolysin to produce a protein hydrolysate with anti-hypertensive activities confirmed in a rat model. The development of hypertension in PKD rats was significantly attenuated in both groups receiving the PPH, whereas the casein-only diet did not affect BP. The effect was detectable after 5 weeks of administration, and continued till the end of the study. ACE activities in both lung and plasma were not significantly different among the groups probably because of the compensating effects due to the decreased renin expression. In accordance with the suppression of elevated BP of hydrolysate-fed rats was the decreased angiotensin II level in plasma of the hydrolysate groups in comparison with that of the casein group. However, there was no difference in angiotensin II levels in lung of rats from the 3 experimental groups.

This study indicates that PPH contains peptides mixture that has anti-hypertensive activities by suppressing expression of enzymes involved in the RAS and leading to decreased angiotensin II level. This anti-hypertensive activity is not reduced by GIT enzyme digestion after oral administration, and can decrease BP on a long-term basis. Therefore, this PPH has the potency to be used as a hypotensive therapeutic agent in diseased conditions, such as hypertension and CKD. To the best of our

knowledge this is the first study of its kind to demonstrate hypotensive effects of a food protein-derived hydrolysate in a CKD animal model.

CHAPTER 6

PURIFICATION AND IDENTIFICATION OF BIOACTIVE PEPTIDES FROM PEA PROTEIN HYDROLYSATE

6.1 INTRODUCTION

In addition to their nutritional contributions, food proteins are increasingly being expected to make contributions in the area of functional foods and nutraceuticals. Bioactive functional properties of proteins determine their performance and behavior in the process of influencing human health. Many studies have demonstrated that enzymatic hydrolysis of food proteins can release peptides exhibiting various bioactivities and lead to improved health status (Chan & Li-Chan, 2007; Aihara *et al.*, 2005; Horiguchi *et al.*, 2005; Fujita *et al.*, 2001; Kawasaki *et al.*, 2000; Kagawa *et al.*, 1996; Chabance *et al.*, 1995). Functional properties of proteins can be modified by changing its conformation and structure through physical, chemical or enzymatic treatment. Particular attention has been devoted to enzymatic hydrolysis using selective proteases compared with physical or chemical modifications because of the more moderate hydrolysis conditions as well as less or no undesirable side reactions and by-products associated with enzyme-catalyzed reactions. Furthermore, enzymatic hydrolysis leads to the breakdown of peptide bonds, and resultant peptides have smaller

molecular size and less secondary structures than original intact proteins and are expected to have increased permeability which is crucial for the absorption in GIT. Functional properties depend on both the specificity of the enzyme and the hydrolysis condition(s). Such conditions include pH, temperature, enzyme:substrate ratio, duration of hydrolysis, substrate concentration, and any interactive effects between these parameters. Alcalase, which is produced by a selected strain of *Bacillus licheniformis*, cleaves peptide bonds with broad specificity and was shown to hydrolyze peptides with hydrophobic amino acids such as Phe, Tyr, Trp, Leu, Ile,Val, and Met at the C-terminal (Markland & Smith, 1971). It has been used in several previous studies to produce protein hydrolysates (Seo *et al.*, 2008; Li & Aluko, 2006; Li & Aluko, 2005; Aubes-Dufau & Combes, 1997).

As discussed in Chapter 2, multifunctional peptides are especially important because they can be used to target multiple pathological situations or to offer addictive beneficial effects for one situation. Many studies have been done to compare the effects of single versus dual blockade of RAS using ACE inhibitors and angiotensin II receptor blockers (ARB) in the treatment of diabetic hypertension and nephropathy (Schjoedt *et al.*, 2005; Jacobsen *et al.*, 2004; Jacobsen *et al.*, 2003). It has been shown that this treatment could offer synergistic blockade of RAS and reduce both BP and albuminuria, which was not obtainable with either

monotherapy. In patients with essential hypertension, dual blockade tends to be a safe and more effective treatment to lower BP from both long-term and short-term aspects (Chrysant *et al.*, 2008; Pool *et al.*, 2007; Waeber *et al.*, 2001). Dual blockade treatment was also proved to benefit chronic heart failure (McMurray *et al.*, 2003). In non-diabetic renal disease, superior effects of dual blockade compared to single blockade were reported in some studies (Mori-Takeyama *et al.*, 2008; Campbell *et al.*, 2003; Ruilope *et al.*, 2000). It has been demonstrated that dual blockade can decrease proteinuria more than single blockade, and long-term combined therapy may be more renoprotective than monotherapy. However, it is irrefutable that protection by combination of ACE inhibitor and ARB is still incomplete and can lead to hyperkalemia; therefore, the most recent emergence of combining renin inhibitor and ACE inhibitor (or ARB) may afford a new strategy for RAS blockade (Epstein *et al.*, 2009; Doulton & MacGregor, 2009). Studies from Dr. Aluko's lab showed that a flaxseed protein hydrolysate fraction could inhibit both ACE and renin activities (Udenigwe *et al.*, 2009). However, the current work is the first to report natural food protein-derived peptides with inhibitory activities against ACE, renin, and CaMPDE1.

Mass spectrometry (MS) is a powerful method for the characterization of biological molecules. After early reports of using MS to

obtain sequence information back to 1965, tandem MS (MS/MS) combined with database searching has become an attractive tool to identify amino acid sequence of unknown bioactive peptides derived from proteolytic digestion (Gómez-Ruiz *et al.*, 2008; Quirós *et al.*, 2006; Tessier *et al.*, 2005; Hernádez-Ledesma *et al.*, 2005; Curtis *et al.*, 2002; Barber *et al.*, 1965a; Barber *et al.*, 1965b; Wulfson *et al.*, 1965). Tandem MS provides a way for fragmenting mass-selected ions and measuring the m/z value of the product ions that are produced by the fragmentation (Kinter & Sherman, 2000). However, sequencing is generally limited to low molecular weight peptides. Purification of individual peptide prior to analysis is required due to the presence of large amounts of non-analyte peptides within the protein hydrolysate and contamination with other proteins which may occur in any phase of the experiment. Besides, compared with the other ionization sources, electrospray ionization (ESI) requires considerable sample purification because it is overly sensitive to impurities. Therefore, purification procedures such as membrane filtration and HPLC are necessary. Typically, after digestion and initial separation, the hydrolysate is separated and purified by HPLC and further purification is applied if necessary. Next, the sample is chromatographically separated by liquid chromatography and then directly introduced into the ESI source on a mass spectrometer, which converts the aqueous phase ions to multiple

protonated molecules in a gas phase. The MS records the mass to charge ratio (m/z) value of the ions and allows selected ions to pass to the second MS, where the ions undergo fragmentation to produce daughter ions for sequence information.

Quantitative structure-activity relationship (QSAR) modeling has been extensively used in food science to estimate and predict the activities/properties of peptides and proteins. It is particularly useful for ACE-inhibitory peptides, antimicrobial peptides, and peptides with bitter taste (Foltz *et al.*, 2009; Kim & Li-Chan, 2006; Strøm et al., 2001; Rekdal *et al.*, 1999). Wu *et al.* (2006) performed a QSAR study of ACE-inhibitory peptides using a database constructed of 168 dipeptides and 140 tripeptides, and concluded that amino acid residues with bulky side chains and hydrophobic side chains were preferred for dipeptides. The C-terminal of tripeptides preferred aromatic amino acids while positively charged amino acids were preferred for the middle position and hydrophobic amino acids were preferred at N-terminal. Hydrophobic amino acids at C-terminal and bulky amino acid residues adjacent to the C-terminal were determined to be the major determinants of the intensity of bitterness of di- and tripeptides using QSAR modeling (Wu & Aluko, 2007). Basic idea about QSAR is based on the fact that biological activities/properties are related to molecular variables, such as hydrophobicity, electronic

attributes, steric properties, etc. Therefore, it can translate experimental information on particular peptide and protein properties into more general knowledge about physiochemical or biological mechanisms (Pripp *et al.*, 2005). Molecular structure of peptides and proteins in the database need to be translated into numeric values that characterize the molecules, and identification of amino acid sequences of the peptides and proteins is one of the approaches to provide such useful information. Therefore, identification of peptides and protein sequences is of great value for QSAR modeling not only for estimate, but more importantly to predict and develop more potency peptides and proteins.

Objectives of this study were to, 1) optimize the enzymatic hydrolysis with alcalase to produce low molecular weight peptides (<1000 Da); 2) obtain multi-functional cationic peptide fractions with activities against ACE, renin, and CaMPDE1; 3) purify pea protein-derived bioactive peptides and identify amino acid sequences of the peptides; 4) characterize the inhibitory activities of the purified peptides against ACE, renin, and CaMPDE1.

6.2 MATERIALS AND METHODS

6.2.1 Pea Protein Hydrolysate Preparation

6.2.1.1 Materials

PPI was the same as described in section 3.2.1.1.

6.2.1.2 Methods

PPH was prepared in a reactor with temperature and pH control devices. PPI was dispersed in MilliQ water to obtain a 5% (w/v) protein slurry. After the slurry was heated to 50°C and adjusted to pH 9.0 with 1 M NaOH, 4% alcalase (w/w, on the basis of protein weight) was then added to initiate the hydrolysis. The temperature and pH of the slurry was maintained at constant values for 6 h. The hydrolysis was stopped by adjusting the reaction mixture to pH 4.0. The hydrolysate was then centrifuged at 10,000xg for 25 min. The supernatant was further passed through a 1,000 Da MWCO ultrafiltration membrane, and the resulting permeate was collected and freeze-dried for later use.

6.2.2 Solid Phase Extraction (SPE)

6.2.2.1 Materials

PPH (1 kDa permeate) was prepared as described in section 6.2.1. The cation-exchange system (Bond Elut SCX cartridge, 60 mL column

volume) was purchased from Varian (Varian Canada, Mississauga, ON). The following elution solvents were prepared from analytical reagents: 0.5% (equal amount of methanol and 0.5% ammonium hydroxide), 1.0% (equal amount of methanol and 1.0% ammonium hydroxide), 1.5% (equal amount of methanol and 1.5% ammonium hydroxide), 2.0% (equal amount of methanol and 2.0% ammonium hydroxide), and 5.0% (equal amount of methanol and 5.0% ammonium hydroxide).

6.2.2.2 Methods

SPE methods were provided by Varian (Varian Canada, Mississauga, ON). Ammonium hydroxide concentration in the elution solvents were increased to increase the pH of the solvents so that cationic peptides can be eluted out from the cartridge. PPH freeze-dried powder was prepared into 50 mg/mL in MilliQ water, and mixed with equal amount of 2% phosphoric acid. The cation-exchange cartridge was activated by adding 40 mL of methanol followed by 40 mL of MilliQ water. For peptide separation, first, 40 mL of PPH solution was loaded onto the cartridge, washed with 40 mL 2% formic acid thrice to remove any unbound peptides and then twice with methanol to remove formic acid residues. Secondly, 40 mL of each elution solvent from lowest (0.5%) to highest gradient (5%) was loaded consecutively onto the cartridge to elute the samples; the eluted fraction from each solvent was collected separately. After the highest (5%) gradient,

40 mL of 30% ammonium hydroxide was added to remove any remaining compounds from the cartridge. The eluted fractions were each collected, evaporated, and freeze-dried. Cartridge was re-activated by washings with methanol followed by MilliQ water before next use.

6.2.3 Protein Content Determination of SPE Fractions

6.2.3.1 Materials

Reagent A (2% Na_2CO_3, 0.4% NaOH, 0.16% sodium tartrate, 1% sodium dodecyl sulfate) and reagent B (4% $CuSO_4.5H_2O$) were prepared and stored properly. Reagent C (100 parts reagent A mixed with 1 part reagent B) was prepared on the day of analysis. Bovine serum albumin (BSA) and Folin-Ciocalteu phenol reagent were purchased from Sigma (Sigma Chemicals, St. Louise, MO., U.S.A.).

6.2.3.2 Methods

Standards (BSA) containing 10-100 μg/mL of protein concentration were prepared. Samples containing 10-100 μg/mL of PPH was also prepared. An aliquot (3 mL) of reagent C was added to each of the standard and PPH sample and incubated at room temperature. After 1 h, 0.3 mL diluted Folin-Ciocalteu phenol reagent (1:1 mixed with MilliQ water) was added and mixed vigorously using a vortex. The standards and samples were allowed to stand at room temperature for 45 min before absorbance

was measured at 660 nm. A standard curve was drawn with standard concentrations on the X-axis versus absorbance on the Y-axis. An equation was obtained from standard curve and used to calculate the real concentration of samples. Protein content was calculated as,

$$\text{Protein content \%} = \frac{\text{Real concentration}}{\text{Weighed concentration}} \times 100\%$$

6.2.4 ACE-Inhibitory Activity Assay of SPE Fractions

6.2.4.1 Materials

Six SPE fractions were obtained as described in section 6.2.2. ACE and HHL were purchased from Sigma (Sigma Chemical, St. Louise, MO., U.S.A.).

6.2.4.2 Methods

ACE activity was determined according to Cushman and Cheung (Cushman & Cheung, 1971) with modifications. ACE and HHL were prepared with 0.1 M sodium borate buffer (pH 8.3) containing 0.3 M NaCl into 20 mU/mL and 4.15 mM, respectively. The final reaction mixture contained 1 mg/mL sample, 4 mU ACE, and 3.375 mM HHL. At 37°C, 50 μL of each sample was mixed with 50 μL ACE and pre-incubated for 10 min. For control, 50 μL borate buffer was used instead of sample. HHL was pre-incubated at 37°C for 3 min before 150 μL of it was added into the samples and incubated together at 37°C for 30 min. The reaction was

terminated after 30 min by adding 250 μL 1 M HCl. The mixture was mixed with 500 μL of ethyl acetate and vortexed immediately for 1 min to extract HA liberated by ACE. After centrifuging at 5,000xg for 10 min, 200 μL of the upper solvent layer was transferred into another tube and dried by nitrogen. Then 1 mL MilliQ water was added into the tube to dissolve the residue and absorbance at 228 nm was measured. Percentage of inhibition was calculated as,

$$\text{Inhibition \%} = \frac{\text{Abs. of control} - \text{Abs. of sample}}{\text{Abs. of control}} \times 100\%$$

6.2.5 Renin-Inhibitory Activity Assay of SPE Fractions

6.2.5.1 Materials

Six SPE fractions were obtained as described in section 6.2.2. Renin assay buffer (10X), renin (human recombinant), and 95 μM renin substrate (Dabcyl-gaba-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Thr-EDANS) were provided in the Renin Inhibitor Screening Assay Kit purchased from Cayman Chemical (Cayman Chemical, Michigan, USA). Corning Costar® black polystyrene 96-well microplate was purchased from Fisher (Fisher, Ontario, Canada).

6.2.5.2 Methods

Renin inhibition assay was done according to the instructions provided with the Renin Inhibitor Screening Assay Kit of Cayman. Prior to

assaying, renin assay buffer (10X) was diluted 10 times with MilliQ water to get the final assay buffer which was 50 mM Tris-HCl, pH 8.0, containing 100 mM NaCl. Renin solution was diluted 1:20 with assay buffer before use. Assay buffer was pre-warmed at 37°C right before reaction which was carried out at 37°C. Total volume of reaction solution was 190 μL. Before the reaction, 1) 20 μL of substrate, 160 μL of assay buffer, and 10 μL of MilliQ water were added to the background wells; 2) 20 μL of substrate, 150 μL of assay buffer, and 10 μL of MilliQ water were added to the control wells; 3) 20 μL of substrate, 150 μL of assay buffer, and 10 μL of peptide fractions were added to the corresponding inhibitor wells. Final concentration of peptide samples in the reaction solution was 1 mg/mL. Reactions were then initiated by adding 10 μL of renin each to the control and inhibitor wells. Microplate was carefully shaken for 10 sec to mix, incubated at 37°C for 15 min, and then the fluorescence intensity (FI) was recorded using excitation wavelength of 340 nm and emission wavelength of 490 nm on a spectrofluorimeter (Spectra MAX Gemini, Molecular Devices, Sunnywale, CA). Percentage of inhibition was expressed as,

$$\text{Inhibition \%} = \frac{\text{FI of control well} - \text{FI of inhibitor well}}{\text{FI of control well}} \times 100\%$$

6.2.6 CaMPDE1-Inhibitory Activity Assay of SPE Fractions

6.2.6.1 Materials

Six SPE fractions were obtained as described in section 6.2.2. CaMPDE1 assay buffer (0.36 M Tris-HCl, 0.36 M imidazole, and 0.045 M magnesium acetate, pH 7.5), 4.5 mM $CaCl_2$, reducing agent (12% sodium bisulfite, 1.2% sodium sulfite, and 0.25% 1-amino-2-naphthol-4-sulfonic acid), 10 units/mL 5'-nucleotidase in 10 mM Tris-HCl containing 0.5 mM magnesium acetate (pH 7.0), 10.8 mM cyclic adenosine monophosphate (cAMP, pH 7.0), 0.31 units/mL CaMPDE1, 55% TCA, 0.55% ammonium molybdate in 0.55 M H_2SO_4, and 50 units/mL CaM were prepared and stored appropriately before use.

6.2.6.2 Methods

Before the assay, each of the SPE fractions was mixed with CaM to make a 1 mL solution (CaM-SPE mixture). Final concentration of SPE fraction in the reaction solution was 1 mg/mL. To the assay tube, each of the following reagents was added: 100 µL assay buffer, 20 µL $CaCl_2$, 400 µL MilliQ water, 30 µL 5'-nucleotidase, 50 µL CaMPDE1, and 200 µL CaM-SPE mixture solution. For control, 200 µL CaM at concentration of 50 units/mL was added instead of 200 µL CaM-SPE mixture. The added components were mixed thoroughly and incubated in water bath at 30°C for 10 min. And then 0.1 mL cAMP was added to initiate the reaction.

Reaction was stopped after 90 min by adding 0.1 mL 55% TCA. Protein precipitate was removed by centrifuging at 5,000xg for 10 min. Into a new test tube, 0.5 mL supernatant was pipetted and 0.5 mL ammonium molybdate was added followed by 50 µL reducing agent. All components were mixed thoroughly by vortexing and transferred to a Costar microplate. Absorbance was read at 660 nm using a Varian Cary 50 MPR microplate reader. Percentage of inhibition (inhibition %) was calculated as:

$$\text{Inhibition \%} = \frac{\text{Abs. of control} - \text{Abs. of sample}}{\text{Abs. of control}} \times 100\%$$

6.2.7 HPLC Separation of Fraction SPE 1%

6.2.7.1 Materials

Fraction SPE 1% was prepared as described in section 6.2.2 and used for further purification because it was the most abundant species. Buffer A (MilliQ water with 0.05% TFA) and buffer B (methanol with 0.05% TFA) were prepared from analytical solvents. HPLC system (Waters 717 plus autosampler, Waters 600 controller, Waters 2996 photodiode array detector, and Water fraction collector III) were from Waters (Waters, Milford, MA, U.S.A.).

6.2.7.2 Methods

As will be discussed from section 6.3.1 to 6.3.4, SPE 1% fraction showed moderate inhibitory activities against the enzymes; besides it was

able to be steadily produced with the highest yield and contained the highest protein amount. Considering limited time and intensive labour requirements, it was decided that fraction SPE 1% would be used in the subsequent studies.

SPE 1% fraction sample was dissolved in 0.05% (v/v) TFA water solution at a concentration of 50 mg/mL. The separation was conducted on a Waters Symmetry™ C18 column (5 μm, 19×150 mm, Waters, MA, USA) coupled with Waters 717 plus autosampler, Waters 600 controller, and Waters 2996 photodiode array detector. The injection volume was 2 mL, flow rate was 5 mL/min, and absorbance was monitored at 210 nm. Fraction was automatically collected every minute by Waters fraction collector III. After sample injection, the column was eluted with a gradient of 0-60% methanol containing 0.05% TFA within 90 min, returned to 0% methanol (100% buffer A) within 5 min, and maintained at 100% buffer A for 15 min before the next sample injection. HPLC fractions were collected into different pooled fractions, evaporated and aqueous residue was freeze-dried. Eluate was collected into six pooled fractions that were analyzed for inhibitory activities towards ACE, renin and CaMPDE1. Fraction #3 was the most abundant and most feasible to collect for the purpose of further purification. Therefore, the fraction was named SPE 1%_#3 and used for a second round of HPLC separation.

6.2.8 Protein Content Determination of SPE 1% Fractions

Protein content determination was performed as previously stated in section 6.2.3.

6.2.9 ACE-Inhibitory Activity Assay of SPE 1% Fractions

ACE-inhibitory assay was performed as previously stated in section 6.2.4.

6.2.10 Renin-Inhibitory Activity Assay of SPE 1% Fractions

Renin-inhibitory assay was performed as previously stated in section 6.2.5.

6.2.11 CaMPDE1-Inhibitory Activity Assay of SPE 1% Fractions

CaMPDE1-inhibitory assay was performed as previously stated in section 6.2.6.

6.2.12 HPLC Separation of Fraction SPE 1%_#3

6.2.12.1 Materials

Fraction SPE 1%_#3 was obtained from HPLC separation of fraction SPE 1% as described in section 6.2.7. Buffer A (MilliQ water with 0.05% TFA) and buffer B (methanol with 0.05% TFA) were prepared and stored

properly. HPLC system was from Waters (Waters, Milford, MA, U.S.A.) as described in section 6.2.7.1.

6.2.12.2 Methods

Because limited amount of each fraction obtained from HPLC separation of SPE 1%, fraction SPE 1%-#3, which was the one with largest quantity, was used in the subsequent studies. Fraction SPE 1%_#3 was dissolved in 0.05% (v/v) TFA water solution at a concentration of 10 mg/mL. The separation was conducted on a Water Symmetry™ C_{18} column (5 μm, 19×150 mm, Waters, MA, USA) coupled with Waters 717 plus autosampler, Waters 600 controller, and Waters 2996 photodiode array detector. The injection volume was 2 mL, flow rate was 5 mL/min, and absorbance was monitored at 210 nm. Fraction was automatically collected every minute by Waters fraction collector III. After injection, column was eluted at a gradient from 15-25 % methanol containing 0.05% TFA within 40 min, kept at 25% for 5 min, then returned to 15% methanol within 1 min, and maintained at 15% methanol for another 10 min before next sample injection. After being concentrated, each fraction was evaporated, freeze-dried and ready for further use. This second round of HPLC separation yielded 3 peaks namely SPE 1%_#3-1, SPE 1%_#3-2, and SPE 1%_#3-3, each of which was analyzed for enzyme inhibitory properties

followed by peptide identification and determination of amino acid sequence.

6.2.13 ACE-Inhibitory Activity Assay of SPE 1%_#3 Fractions

ACE-inhibitory assay was performed as previously stated in section 6.2.4.

6.2.14 Renin-Inhibitory Activity Assay of SPE 1%_#3 Fractions

Renin-inhibitory assay was performed as previously stated in section 6.2.5.

6.2.15 CaMPDE1-Inhibitory Activity Assay of SPE 1%_#3 Fractions

PDE-inhibitory assay was performed as previously stated in section 6.2.6.

6.2.16 Peptide Sequence Analysis by Ultra-Performance Liquid Chromatography-Tandem Mass Spectrometry (UPLC-MS/MS)

6.2.16.1 Materials

Peaks SPE 1%_#3-1, SPE 1%_#3-2, SPE 1%_#3-3 were prepared as described in section 6.2.12. Buffer A (MilliQ water) and buffer B (100% methanol) were prepared and stored properly. UPLC-MS/MS system

(Waters Acquity® UPLC, and Waters Quattro micro™ API MS) was from Waters (Waters, Milford, MA, U.S.A.).

6.2.16.2 Methods

Amino acid sequence analysis was done using a Waters Acquity® UPLC system coupled with a Waters Quattro micro™ API MS/MS system. Peaks SPE 1%_#3-1, SPE 1%_#3-2, and SPE 1%_#3-3 were each dissolved in buffer A at 1 mg/mL concentration and eluted at 0.2 mL/min through a Waters Acuity UPLC® HHS T3 1.8 µm column (2.1×100 mm) with gradient of buffer B increased from 10 to 15%, 10 to 20%, and 15 to 25% within 15 min for peak SPE 1%_#3-1, SPE 1%_#3-2, and SPE 1%_#3-3, respectively. Eluate was electrosprayed directly into the mass spectrometer with nitrogen used as collision gas. Capillary voltage was set to 1.5 kV, cone voltage was 40 V, and collision energy was 38 V. Amino acid sequence of each peptide was analyzed from the mass of daughter ions and compared with pea protein sequence using search tools available on the Internet website called Expasy (*www.expasy.ch/tools/*).

6.2.17 Peptide Synthesis

Predicted peptide sequences from the MS/MS data of each peak were synthesized by GenScript USA Inc. (GenScript USA Inc, Piscataway, NJ., U.S.A.).

6.2.18 Inhibitory Activity of Synthesized Peptides against ACE, Renin and CaMPDE1

IC_{50} (half maximal inhibitory concentration) was used as a measurement of effectiveness of synthesized peptides. Determination of IC_{50} values of synthesized peptides for ACE and renin was done in the same procedures as that of inhibitory activities assays except samples were prepared to give 4 different final concentrations of 0.25, 0.5, 1, and 2 mg/mL in the ACE assay, and 0.5, 1, 2, and 3 mg/mL in the renin assay. Final concentration referred to sample concentration in the total reaction volume. Because of limited amount of peptides and their low inhibitory activity against CaMPDE1, CaMPDE1-inhibition assay at final concentration of 1 mg/mL of synthesized peptides was conducted instead of IC_{50} determination.

6.2.19 Statistical analysis

Data was analyzed using general linear model of ANOVA followed by Duncan's multiple range test using SAS (SAS, USA). $P<0.05$ was considered as statistically significant.

6.3 RESULTS

6.3.1 Protein Content Determination of SPE Fractions

Six fractions were obtained after SPE using cation exchange cartridge. Fraction SPE 0.5% had the least positively charged peptides and SPE 30% had the most positively charged peptides. Peptide yields were 0.42, 38.17, 3.75, 2.25, 2.83, and 6.58% for fractions eluted with solvents 0.5, 1, 1.5, 2, 5, and 30%, respectively. Compared with either flaxseed protein hydrolysate (<1 kDa) which protein yield was between 5.4% to 18.5% (Udenigwe *et al.*, 2009) or other SPE fractions in current study, fraction SPE 1% had a very high protein yield (38.17%). SPE fractions 0.5, 1, 1.5, 2, 5, and 30% each showed protein content of 22.37, 100, 77.26, 49.17, 55.58, and 22.17%. This is close to the protein content of cation-exchange fast protein liquid chromatography (FPLC) fractions of flaxseed protein hydrolysate (<1 kDa) which contained 70.6-95.5% (Udenigwe *et al.*, 2009). Impurities may include mostly carbohydrates (soluble sugars) and minor polyphenolic compounds that are present in the original protein isolate. Table 14 shows the protein content and yield of the SPE fractions.

6.3.2 ACE-Inhibitory Activity Assay of SPE Fractions

A previous study from Dr. Aluko's lab showed that thermolysin hydrolysis of flaxseed protein exhibited concentration-dependent ACE inhibition with inhibition of 20% and 61% ACE activity at 0.00625 and 0.05 mg protein/mL, respectively (Udenigwe *et al.*, 2009). Cheng *et al.* reported (2008) alcalase hydrolysate of chicken bone protein showed ACE inhibitory activities after 4 and 8 h digestion (IC_{50} of 1.96 and 0.945 mg/mL, respectively). Water extract of caprine kefir and its 3 kDa permeate each showed low IC_{50} of 0.365 and 0.38 mg/mL (Quirós *et al.*, 2005). Mung-bean hydrolysate generated with neutrase displayed ACE inhibitory activity while those obtained with alcalase exhibited high inhibitory activity with IC_{50} value of 0.64 mg/mL (Li et al., 2005). Alcalase hydrolysate of Alaska Pollock skin displayed IC_{50} values of 0.892, 0.84, 0.629, 0.65, and 0.759 mg/mL after digestion of 1, 2, 6, 12, and 24 h, respectively (Byun & Kim, 2001). In the present study, ACE-inhibitory activity was not detected in fraction SPE 0.5%. For the other fractions, inhibition against ACE ranged from 12% to more than 45%. SPE 5% showed highest inhibition of 45.45%, and SPE 30% showed 12.12% inhibition which was the lowest. Compared

Table 14. Protein content and yield of SPE fractions.

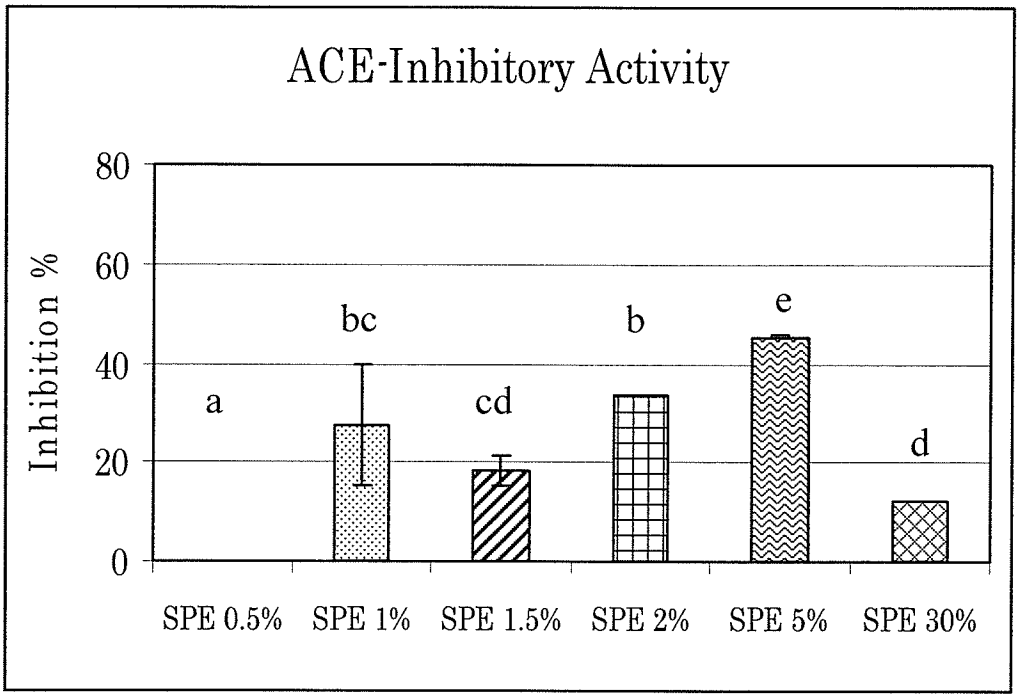
Sample	Protein Content (%)	Yield (%)
SPE 0.5%	22.37	0.42
SPE 1%	100	38.17
SPE1.5%	77.26	3.75
SPE 2%	49.17	2.25
SPE 5%	55.58	2.83
SPE 30%	22.17	6.58

with previous studies, SPE 5% displayed moderate inhibitory activity against ACE, whereas other SPE fractions showed relatively low activities. Figure 25 shows the inhibition percentage of all SPE fractions against ACE. Generally, inhibitory activities of SPE fractions increase with their increase in elution time from the column (higher net positive charges). This is in accordance with the structure-activity study result that positive charges on the side chain of amino acids contribute substantially to the ACE-inhibitory potency of peptides (Ferreira *et al.*, 2007; Meisel, 2003). It has also been postulated that distinct from the catalytic site, positively charged ACE inhibitors can interact with an anionic binding site on the enzyme protein (Vermeirssen *et al.*, 2004; Pihlanto & Korhonen, 2003). However, other than positive charges, other factors may influence the inhibitory activities of peptides, such as binding with the catalytic site resulting in exclusion of the substrate.

6.3.3 Renin-Inhibitory Activity Assay of SPE Fractions

Figure 26 shows the renin inhibition percentage of SPE fractions. Fractions from SPE 0.5% and 2% exhibited no inhibitory activity against renin. Moderate to high activities were observed in the other fractions. SPE 5% showed strongest activity of almost 70% inhibition at 1 mg/mL, while others showed moderate activities. It is difficult to compare with

Figure 25. Percentage inhibition of solid phase extraction fractions against ACE*.



*: Final concentration of each fraction was 1 mg/mL.

other results because there is only one study (from Dr. Aluko's group) reporting renin inhibition from food protein hydrolysate. Results from that study showed lower renin inhibitory activities of flaxseed protein hydrolysate (IC_{50} values ranged from 1.25 to 2.75 mg/mL) compared with fraction SPE 5% and 30%, and similar with other SPE fractions.

6.3.4 CaMPDE1-Inhibitory Activity Assay

SPE fractions exhibited strong to moderate CaMPDE1-inhibitory activities which are shown in Figure 27. Fraction SPE 5% exhibited strongest activity of 62.22% inhibition and lowest inhibition of 19.36% was observed in fraction SPE 1%. There are not many reports of CaMPDE1 inhibition by food protein hydrolysate except Kizawa et al. (1995) who described some peptides isolated from α-casein shown to inhibit CaM-dependent PDE activity. Flavonoids, on the contrary, have been widely described as CaMPDE1 inhibitors. Dioclein, which originated from the plant *Dioclea grandiflora*, strongly inhibited CaMPDE1 with IC_{50} values of 2.47 and 1.44 μM, respectively, in basal- and CaM-activated states (Gonçalves *et al.*, 2009). IC_{50} values of flavones and isoflavones on CaMPDE1 ranged from 14.4 to more than 100 μM (Ko et al., 2004).

It seems that inhibitory activity increased with increase in positive charge intensity of the fractions. It has been well documented that

inhibition of CaMPDE1 can be achieved through two means: direct inhibition of CaMPDE1 by interacting with the catalytic site, and inhibition during activation by interacting with binding site of CaM or Ca^{2+}/CaM complex. Study about CaM revealed that for a compound to inhibit CaM it should carry a positive charge, presumably to interact with the negative charges on the highly acidic CaM (Weiss *et al.*, 1982). We reported before that cationic peptide fractions from pea protein could bind to CaM and inhibited the activity of CaM-dependent protein kinase II (Li & Aluko, 2006; Li & Aluko, 2005). Omoni and Aluko (2006) found that inhibition of CaM-dependent neuronal nitric oxide synthase was correlated with the degree of positive charges of peptide fractions from flaxseed. Hence, whether direct or indirect, a positive relationship between CaMPDE1 inhibition and positive charges does exist.

6.3.5 HPLC Separation of Fraction SPE 1%

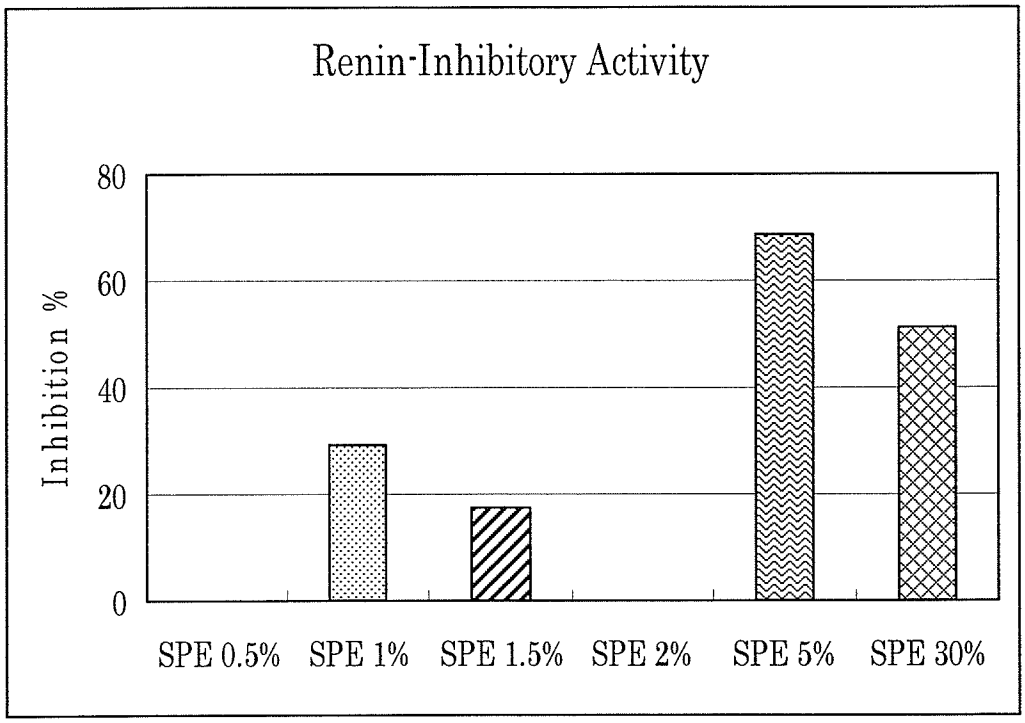
Figure 28 shows the HPLC chromatogram of fraction SPE 1% separation. The chromatogram showed that most peptides were eluted out around 30 min. We further separated fraction SPE 1% into 6 major groups, namely SPE 1%_#1 to #6, collected at 5-9, 10-25, 26-37, 38-48, 49-62, and 63-84 min, respectively. It is clear to see that the third fraction, SPE 1%_#3, was the most abundant, and SPE 1%_#6 was the least. Because the

separation was based on hydrophobicity, the latter the fractions were eluted, the stronger their hydrophobic properties. Therefore, fraction SPE 1%_#6 contained most hydrophobic peptides, whereas SPE 1%_#1 contained most hydrophilic peptides.

6.3.6 Protein Content Determination of SPE 1% Fractions

Table 15 shows the protein content of 6 SPE 1% fractions. Fraction SPE 1%_#5 had the highest protein content which was 100% followed by SPE 1%_#1 which was 94.58%. Protein content in the other fractions ranged between 73% and 87%, which was higher compared with that of SPE fractions and that of flaxseed protein hydrolysate (70.6% to 95.5%) (Udenigwe *et al.*, 2009).

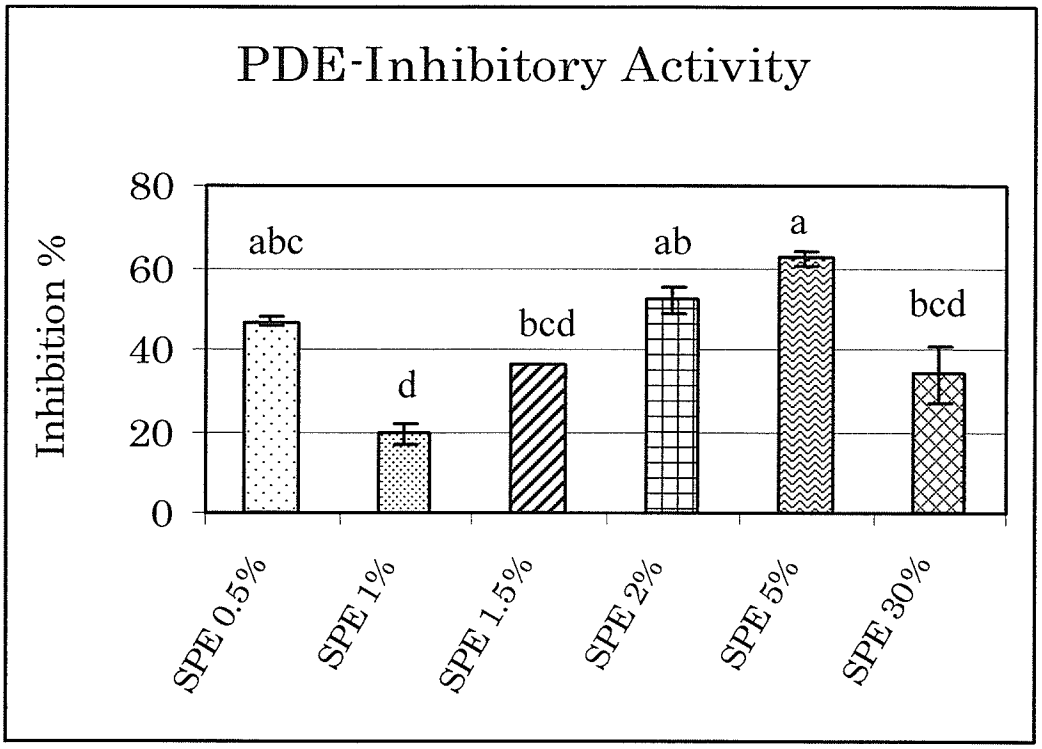
Figure 26. Inhibition percentage of solid phase extraction fractions against renin*.



*: Final concentration of each fraction was 1 mg/mL.

Renin is a temperature sensitive enzyme. Its activity decreases fast even when buried in ice. In this experiment, spectrofluorimeter with only one cuvette holder was used, and loss of renin activity was observed between each measurement. Therefore, we decided that each sample was only measure once to reduce the loss of renin activity. As a result, only one reading was recorded for each sample and no statistical analysis was done.

Figure 27. Inhibition percentage of solid phase extraction fractions against CaMPDE1*.



*: Final concentration of each fraction was 1 mg/mL.

Figure 28. Reverse-phase HPLC chromatogram of fraction SPE 1% separation.

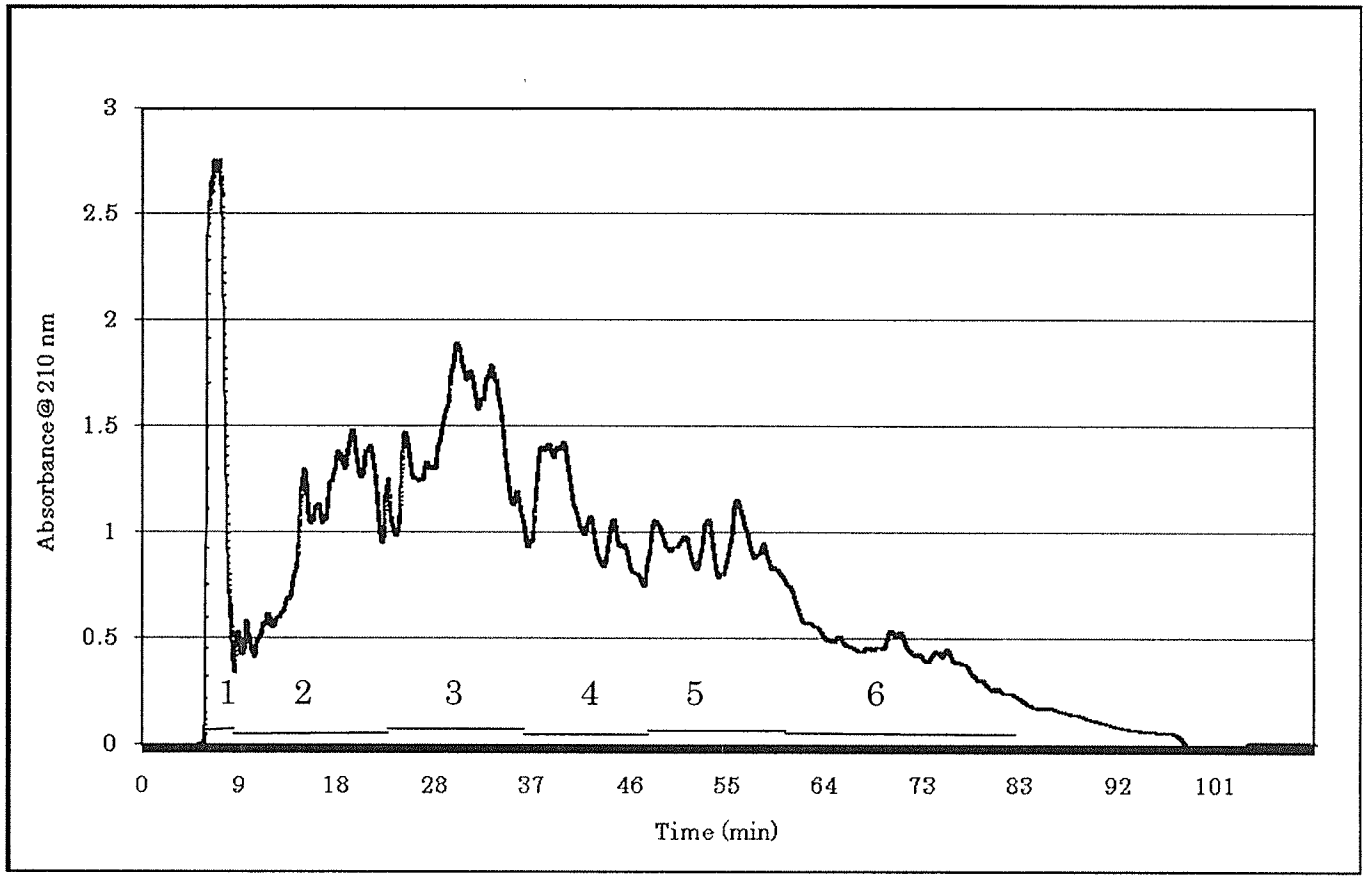


Table 15. Protein content of SPE 1% fractions.

Samples	Protein Content (%)
SPE 1%_#1	94.58
SPE 1%_#2	72.8
SPE 1%_#3	86.89
SPE 1%_#4	85.67
SPE 1%_#5	100
SPE 1%_#6	83.6

6.3.7 ACE-Inhibitory Activity Assay of SPE 1% Fractions

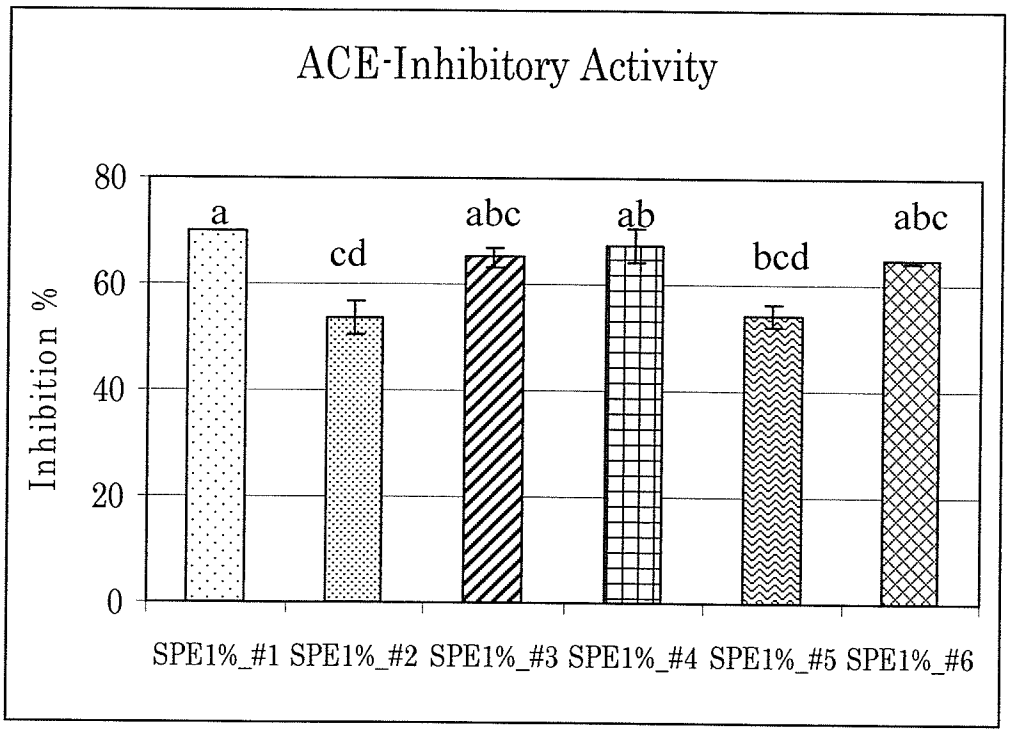
Strong ACE inhibitory activities with more than 50% inhibition of ACE activity were observed in SPE 1% fractions at concentration of 1 mg/mL. SPE 1%_#1 had the highest activity against ACE with 70% inhibition whereas SPE 1%_#2 had the lowest inhibition of 53.66%. Except SPE 1%_#5, which showed 54.25% inhibition, all other fractions exhibited more than 60% inhibition against ACE. Figure 29 shows the inhibition percentage against ACE of each fraction. Similar results were observed in fractions (0.5 mg/mL) separated from mungbean alcalase hydrolysate with 5.36% to 56.19% inhibition of ACE activity (Li *et al.*, 2006). Some other studies showed food protein hydrolysate fractions with higher ACE-inhibitory activities. Guang and Phillips (2009) reported 6 RP-HPLC fractions from peanut flour alcalase hydrolysate and these fractions inhibited ACE activity from less than 5 to about 45% at concentration of 45.5 μg/ml. An HPLC separation of chicken bone protein hydrolysate discovered a number of fractions with ACE inhibitory activities, among which 5 of them exhibited strong activities (IC_{50} values of 0.207, 0.097, 0.123, 0.066, and 0.073 mg/mL, respectively, Cheng *et al.*, 2009). Eight fractions with low ACE-inhibitory activities (IC_{50} values from 21.8 to 416 mg/mL) from caprine kefir were separated by preparative HPLC (Quirós *et al.*, 2005).

Compared with previous results that SPE 1% showed 27.27% inhibition of ACE at concentration of 1 mg/mL, it seems that each fraction from SPE 1% separation on HPLC has stronger ACE-inhibitory activity than when they are mixed together. The results may indicate that increased purity of peptides can contribute to higher activity against target enzymes when compared to their parent protein or crude peptide mixtures.

6.3.8 Renin-Inhibitory Activity Assay of SPE 1% Fractions

This assay utilizes a synthetic peptide substrate which is linked to a fluorophore (EDANS) at one end and to a nonfluorescent chromophore (Dabcyl) at the other (Wang *et al.*, 1993). After cleavage by renin, the product can give fluorescence and be detected at excitation wavelength of 340 nm and emission wavelength of 490 nm. Figure 30 shows the inhibition of renin activity by each of the SPE 1% fractions. All fractions except fraction SPE 1%_#6 showed strong to moderate inhibitory activities with the highest inhibitory activity observed in fraction SPE 1%_#2 (75.73%) and lowest inhibitory activity in fraction SPE 1%_#5 (35.06%). Compared with previous results that SPE 1% inhibited 29.4% of renin activity at the same concentration, each fraction of SPE 1% showed higher inhibitory activities. This may be explained by the reason that each fraction of SPE 1% is more purified than SPE 1% itself, and hence show

Figure 29. Inhibition percentage of SPE 1% fractions against ACE*.



*: Final concentration of each fraction was 1 mg/mL.

stronger activities. Literature about renin inhibition by food protein is scarce and hence difficult to obtain data for comparison with findings from this study.

6.3.9 CaMPDE1-Inhibitory Activity Assay of SPE 1% Fractions

At a final sample concentration of 1 mg/mL, CaMPDE1-inhibitory assay showed no activity from fraction SPE 1%_#1. Fraction SPE 1%_#4 showed highest CaMPDE1-inhibitory activity (36.9%) compared with the others followed by SPE 1%_#2 (31.97%). Fractions SPE 1%_#3 and #5 exhibited relatively low activity against CaMPDE1 which were 20.32% and 20.43%, respectively. Figure 31 shows the CaMPDE1 inhibitory activities of each SPE 1% fractions. Compared with unpublished data from Dr. Aluko's lab that 2 cationic fractions from flaxseed protein hydrolysate inhibited 33.5% and 25% CaMPDE1 activity at 0.1 mg/mL, and the cationic peptide fractions from alcalase hydrolysis of egg white lysozyme showed a little bit higher CaMPDE1 inhibition (26.9% to 43.6%), SPE 1% fractions from pea protein exhibited lower activities. However, different assay systems and CaMPDE1 units were used in these studies.

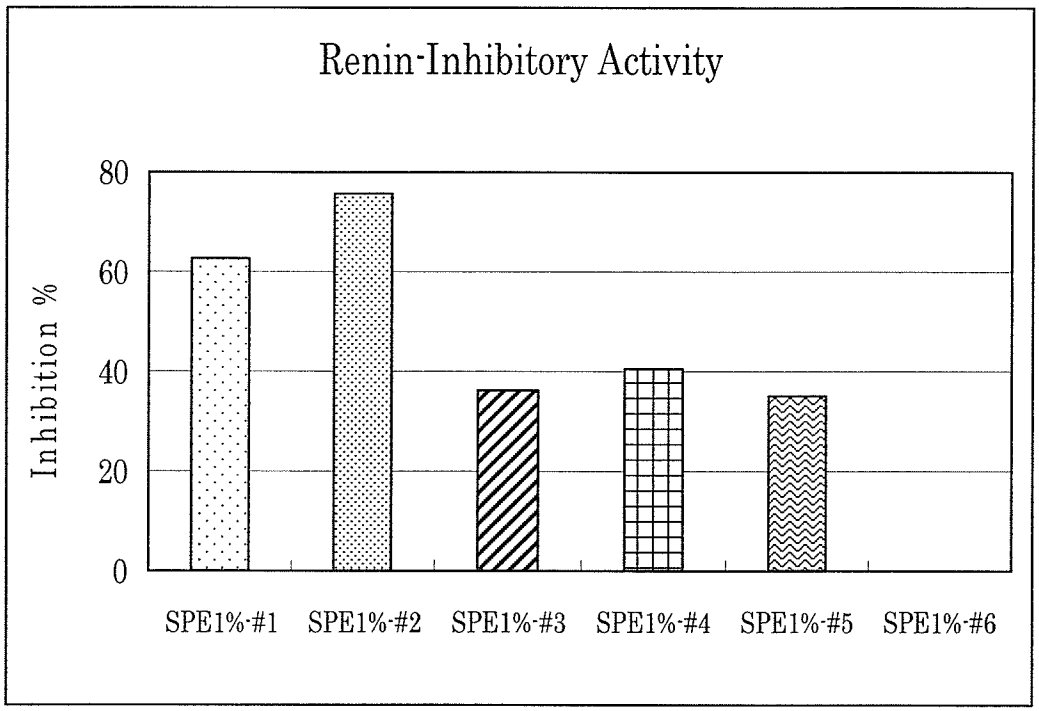
Again, when compared with precursor fraction SPE 1% which showed 19.36% inhibition against CaMPDE1, each SPE 1% fraction exhibited higher activities due to their higher purity. It is observed that

the relationship between cationic character and inhibitory activities disappeared after RP-HPLC separation. This is because HPLC separation is based on hydrophobic properties unlike the SPE fractionation that is based on charge properties.

6.3.10 HPLC Separation of Fraction SPE 1%_#3

Figure 32 shows the HPLC chromatograph of SPE 1%_#3 further separation. There were two relatively small peaks at 18 and 23 min each, and the biggest peak appeared at 28 min. Three peaks were collected separately from 17-21 min, 22-24 min, and 26-31 min, and named SPE 1%_#3-1, SPE 1%_#3-2 and SPE 1%_#3-3. After SPE and 2 steps of HPLC separation, all 3 peaks were presumed to be of very high purity and composed mostly of peptides with little or no contaminating compounds.

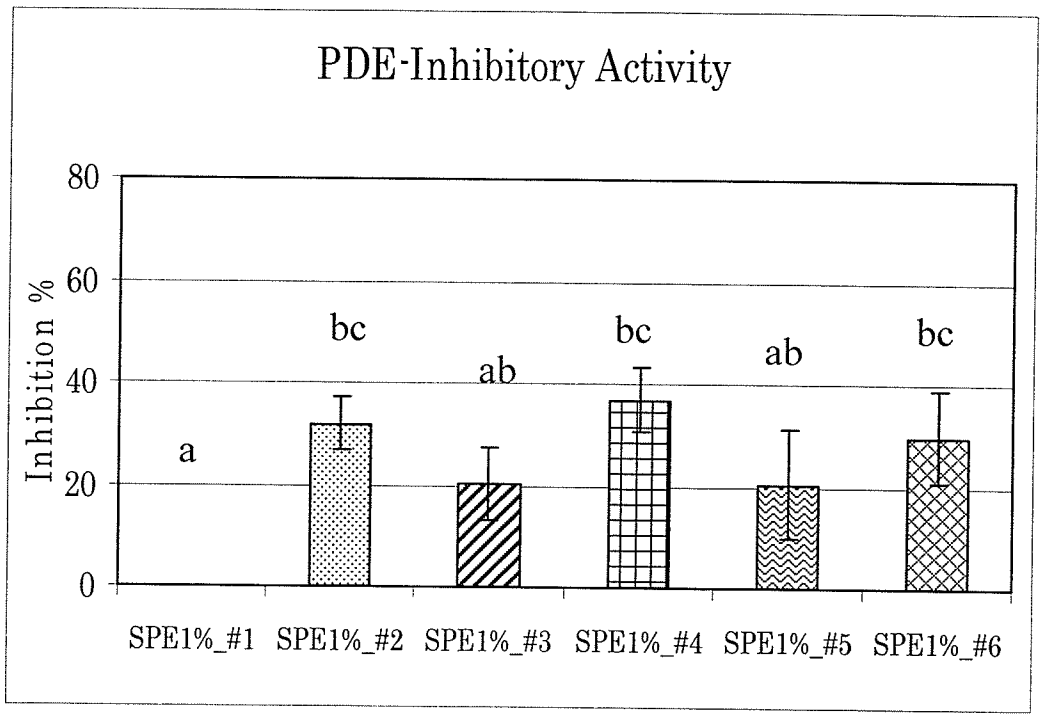
Figure 30. Inhibition percentage of SPE 1% fractions against renin*.



*: Final concentration of each fraction was 1 mg/mL.

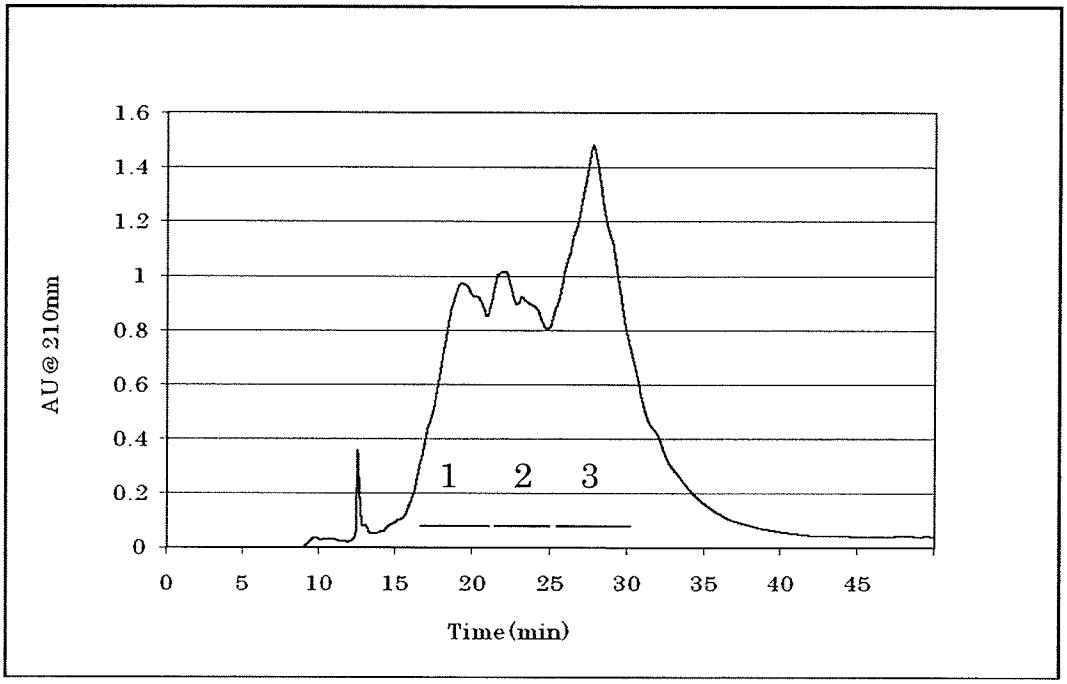
Statistical analysis was not done because of the same reason mentioned before.

Figure 31. Inhibition percentage of SPE 1% fractions against CaMPDE1*.



*: Final concentration of each fraction was 1 mg/mL.

Figure 32. HPLC separation of SPE1%_#3.



6.3.11 ACE-Inhibitory Activities of SPE 1%_#3 Peaks

Figure 33 shows that peak SPE 1%-#3-2 had the highest inhibitory activity with 35.34% inhibition of ACE activity at concentration of 1 mg/mL. Peaks SPE 1%-#3-1 and -3 showed the same inhibition (27.30%). All 3 peaks had lower inhibitory activities in comparison with their precursor fraction, fraction SPE 1%-#3, which exhibited 65.01% inhibition against ACE. A synergistic inhibitory effect of the peak mixture compared to the activity of each individual peak is presumed. In addition, because of limited amount and the purity of these peaks, protein content of each peak was not determined but assumed to be 100% during the assay which may be higher than the actual protein content, and therefore, the inhibition percentage may be a little bit lower than the true value.

Compared with our results, Guang and Phillips (2009) reported 4 peaks with stronger ACE inhibitory activities after secondary RP-HPLC separation of peanut flour hydrolysate with IC_{50} values of 33.1, 27.1, 24.5, and 16.9 μg/mL each. In a study conducted by Li *et al.* (2006), an ACE-inhibitory fraction separated from mung-bean alcalase hydrolysate was further purified by RP-HPLC and 3 subfractions exhibited more than 50% inhibition at concentration of 0.1 mg/mL. Two fractions with potent ACE-inhibitory activities (IC_{50} of 8.1 μg/mL for both) were separated from caprine kefir after 2 steps of preparative HPLC (Quirós *et al.*, 2005). From

Alaska Pollock skin hydrolysate, Byun and Kim (2001) discovered an ACE-inhibitory peptide fraction (IC_{50} of 0.85 μg/mL) after several steps of separation (gel filtration, cationic exchange chromatography, and 2 RP-HPLC). In comparison, all 3 peaks showed relatively low inhibitory activity against ACE.

6.3.12 Renin Inhibitory Activities of SPE 1%_#3 Peaks

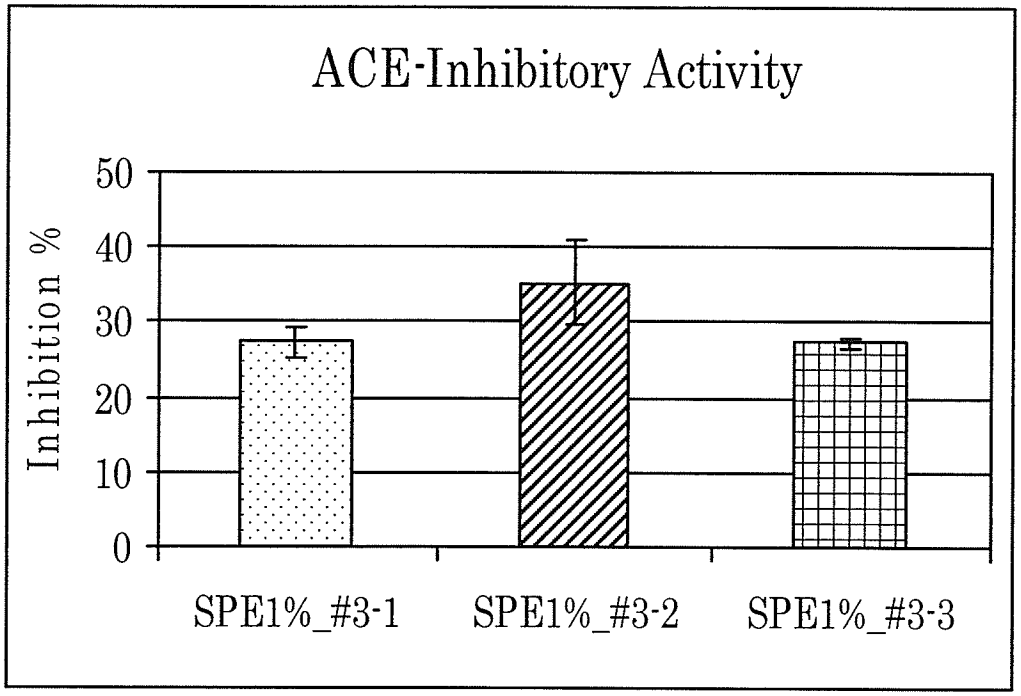
Peak SPE 1%-#3-1 exhibited highest inhibitory activity (40.34% inhibition), and SPE 1%-#3-3 exhibited the lowest activity (33.27% inhibition). Compared with precursor fraction SPE 1%-#3 which showed 36.14% inhibition, it seems that these 3 peaks contain peptides that neither enhance nor weaken each other's inhibitory activities against renin when present as a mixture in the precursor peak. Therefore, renin-inhibitory ability of SPE 1%-#3 comes from the mixed effects of the peptides present in the 3 subsequent peaks. Inhibition percentages of the 3 peaks are shown in Figure 34.

6.3.13 CaMPDE1 Inhibitory Activities of SPE 1%_#3 Peaks

As shown in Figure 35, peak SPE 1%-#3-3 had the highest percentage of inhibition against CaMPDE1 (32.74%), and fractions SPE 1%-#3-1 and -2 had close inhibition percentage (16.5% and 17.35%, respectively). The difference between peak SPE 1%-#3-3 and peaks SPE

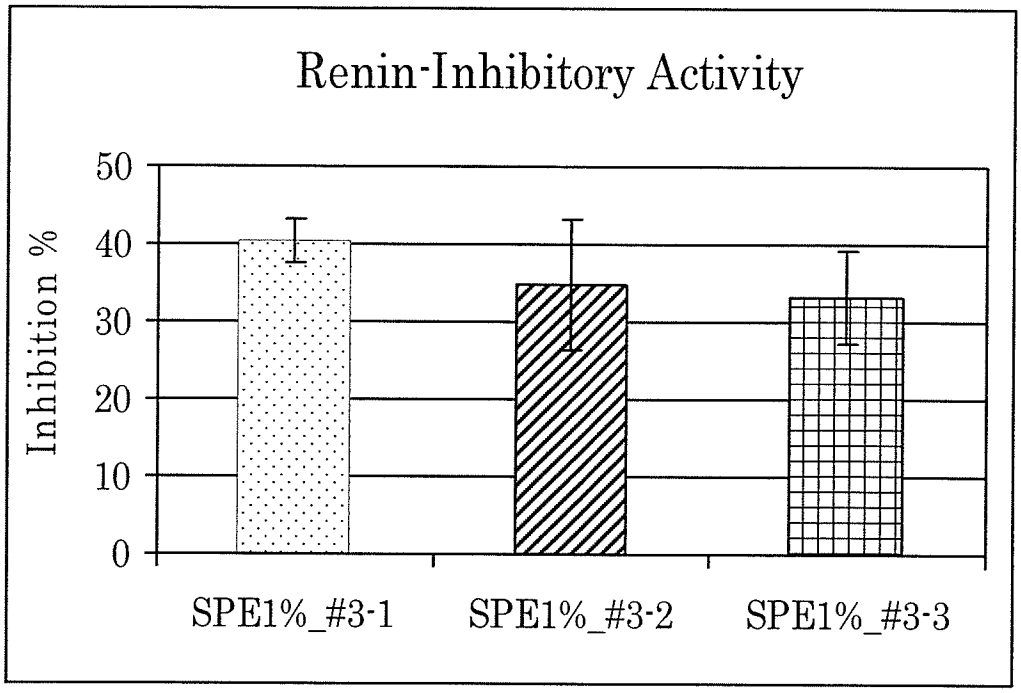
1%-#3-1 and -2 was significant, but no significant difference was observed between peaks SPE 1%-#3-1 and -2. Compared with our results, Ko *et al.* (2004) reported stronger CaMPDE1 inhibition by quercetin and myricetin with IC_{50} values of 27.8 and 24.9 μM, respectively, and moderate inhibition (IC_{50} >100 μM) by 2 flavanones (eriodictyol and hesperetin). Interestingly, in another study hesperetin was found to preferentially inhibit CaMPDE1 isolated from bovine aorta with IC_{50} value of about 74 μM (Orallo *et al.*, 2004). When compared with precursor fraction SPE 1%-#3 which inhibited 20.32% of CaMPDE1 activity, it seems that the effect of peaks SPE 1%-#3-1, -2, and -3 is neither synergistic nor antagonist.

Figure 33. Inhibition percentage of SPE 1%-#3 peaks against ACE*.



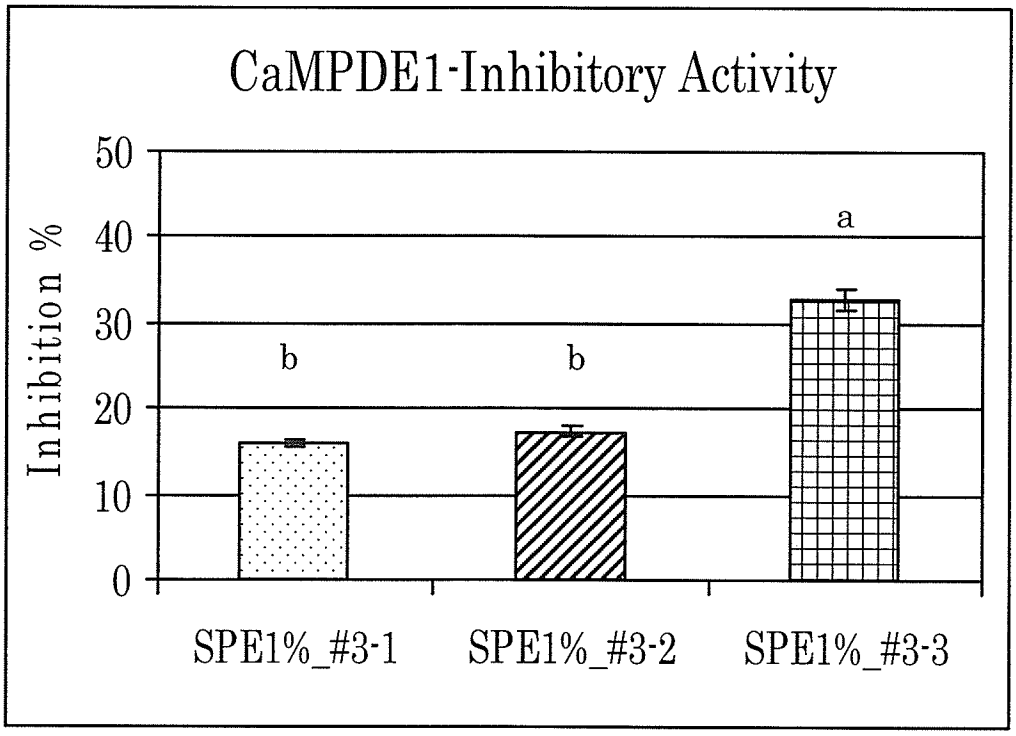
*: Final concentration of each fraction was 1 mg/mL.

Figure 34. Inhibition percentage of SPE 1 %-#3 peaks against renin*.



*: Final concentration of each fraction was 1 mg/mL.

Figure 35. Inhibition percentage of SPE 1%-#3 fractions against CaMPDE1*.



*: Final concentration of each fraction was 1 mg/mL.

6.3.14 Amino Acid Sequence Determination

6.3.14.1 SPE 1% _#3-1

Figure 36 and 37 show the chromatograms of UPLC and MS scan of SPE 1%-#3-1. Major peak of SPE 1%_#3-1 was eluted out at 1.63 min, and high total ion count was detected at 1.63 and 1.93 min, respectively. Figure 38 contains the spectrum acquired at a retention time of 1.724 min in this analysis. This spectrum was evaluated instantaneously by the data system to determine that the two most abundant ions in the spectrum were m/z 85.71, and 288.35. Because m/z 85.71 could not possibly be a peptide of any size, m/z 288.35 was chosen as the expected peptide, and the recorded product ion spectra (MS/MS), which is shown in Figure 39, was subjected for analysis using Expasy. Peptide was identified as IR.

Figure 36. UPLC chromatogram of fraction SPE 1%-#3-1. Absorbance was detected at 210 nm.

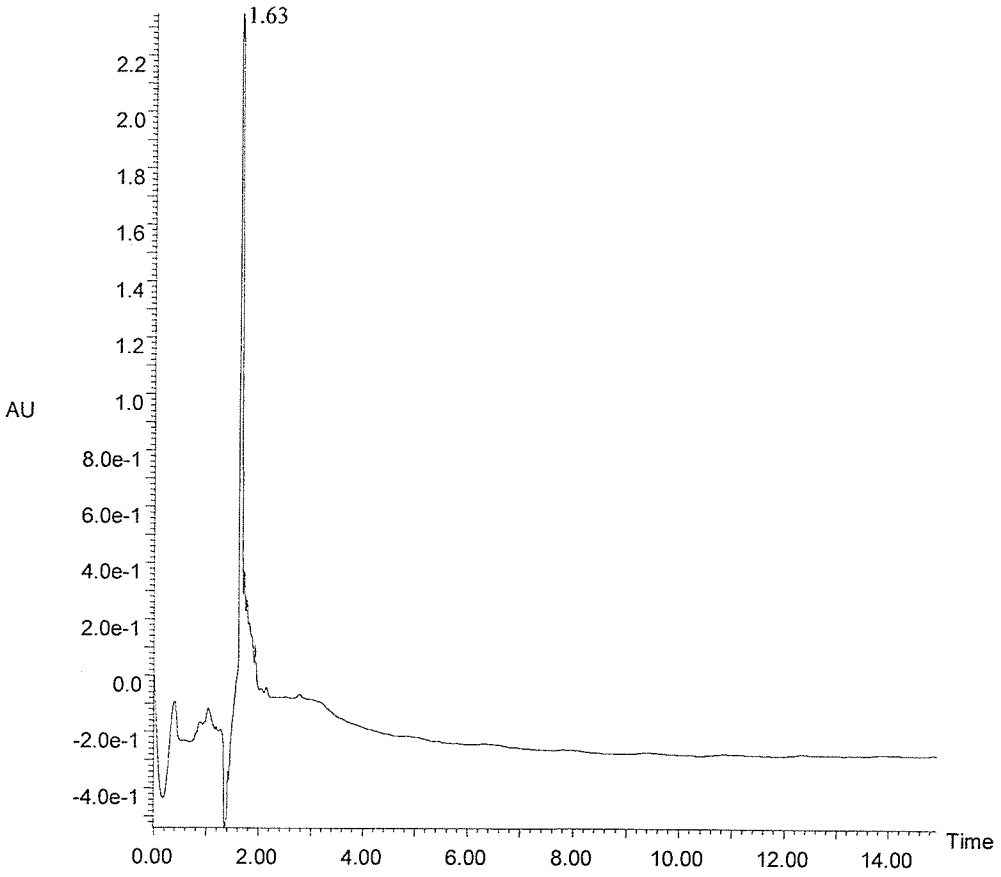


Figure 37. MS scan chromatogram of fraction SPE 1%-#3-1.

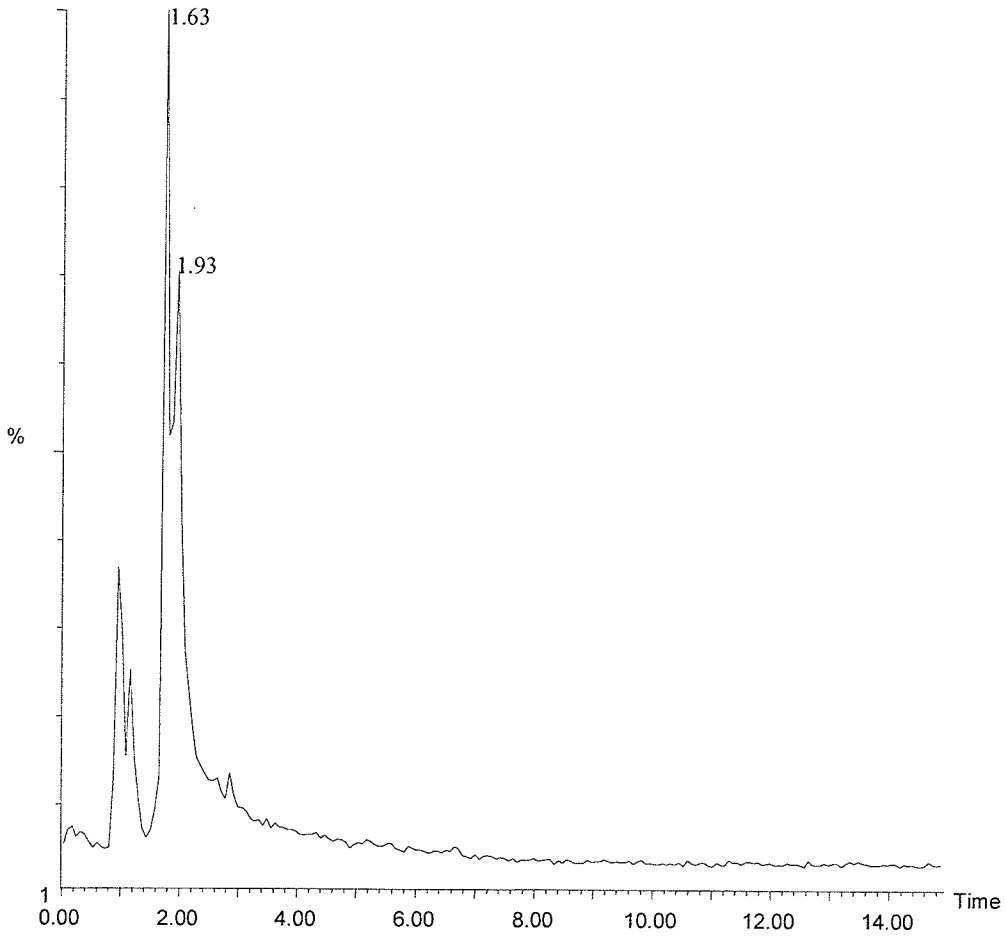


Figure 38. MS spectrum of fraction SPE 1%-#3-1 recorded at retention time of 1.724 min.

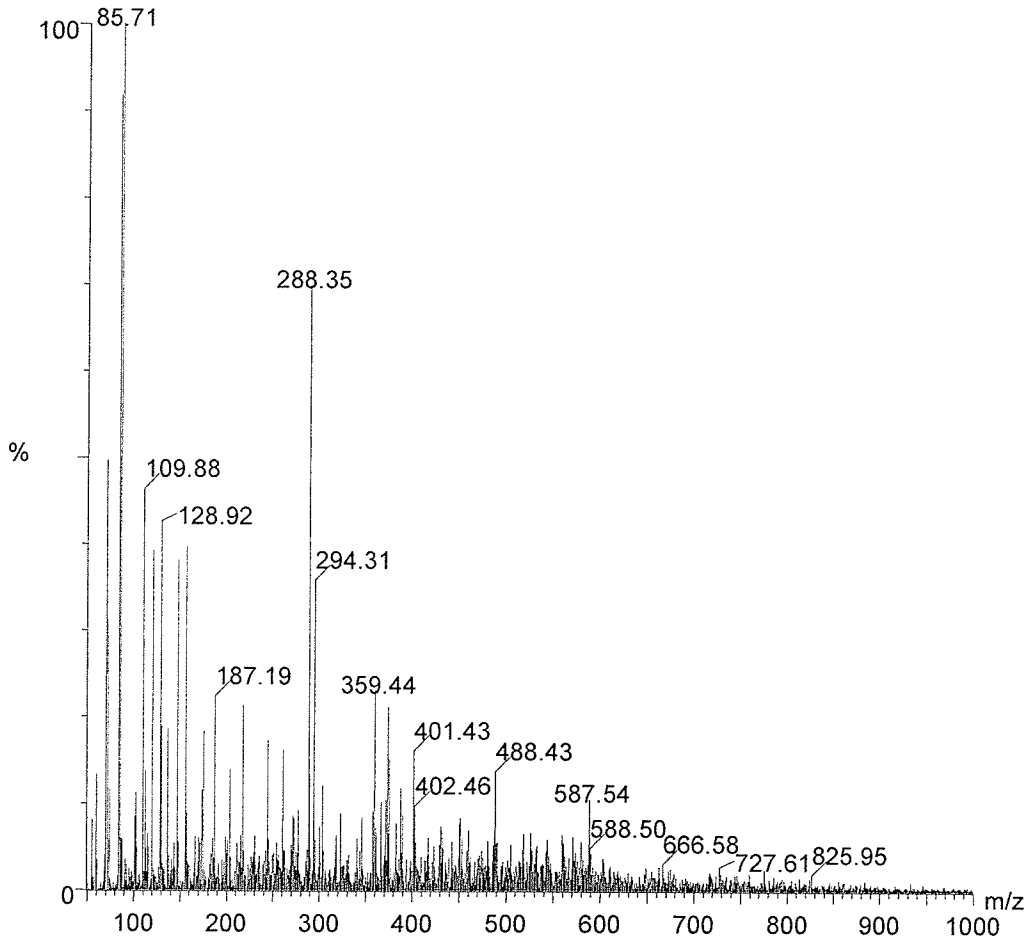
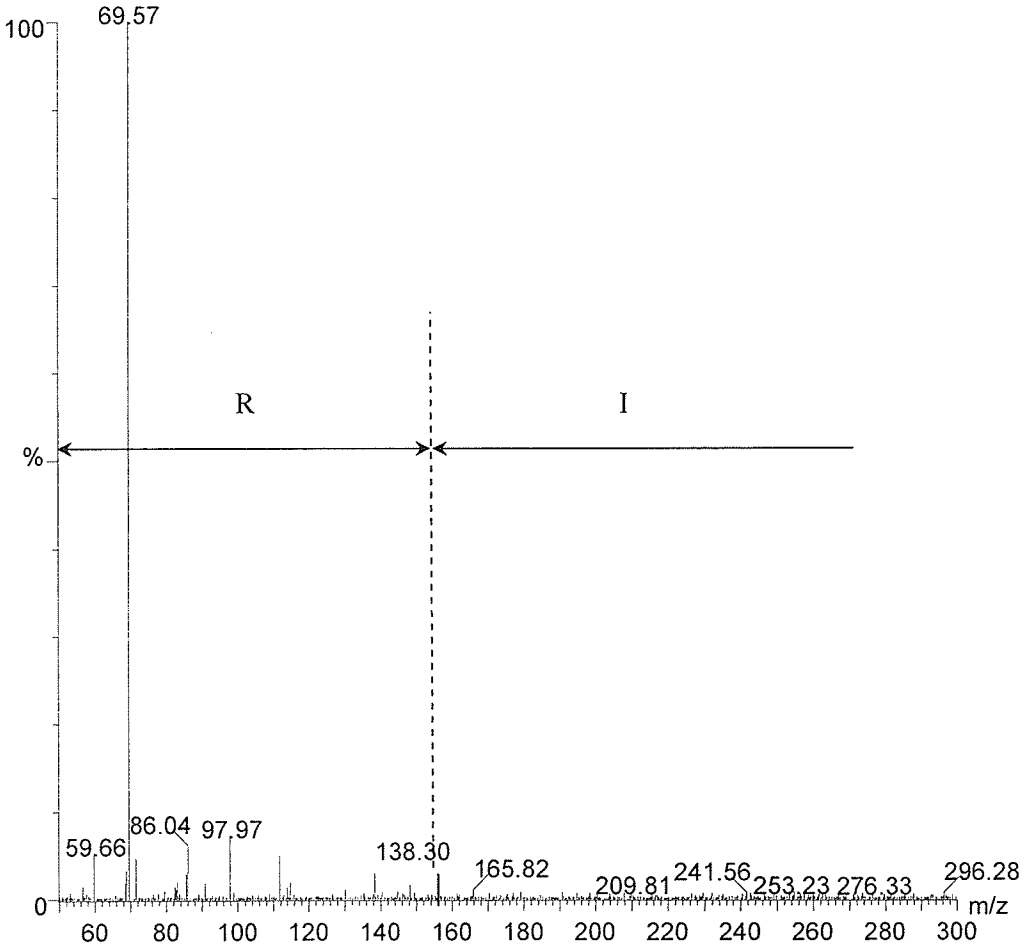


Figure 39. MS/MS spectrum of ion m/z 288.35.



Because the relative intensity of the impurity ion m/z 69.57 was very high, intensity of other ions appeared to be very low and may not be visible in this figure.

6.3.14.2 SPE 1%_#3-2

Figure 40 and 41 show the chromatograms of UPLC and MS scan of SPE 1%-#3-2. Major peak of SPE 1%_#3-2 was eluted out at 2.31 min, and high total ion count was detected at 2.36 and 3.2 min, respectively. Figure 42 contains the spectrum acquired at a retention time of 2.36 min in this analysis. This spectrum was evaluated instantaneously by the data system to determine that an ion m/z 294.31 was the most dominant product. Spectrum of product ion of m/z 294.31 is shown in Figure 43, and the peptide was identified as KF using Expasy.

Figure 40. UPLC chromatogram of fraction SPE 1%-#3-2.

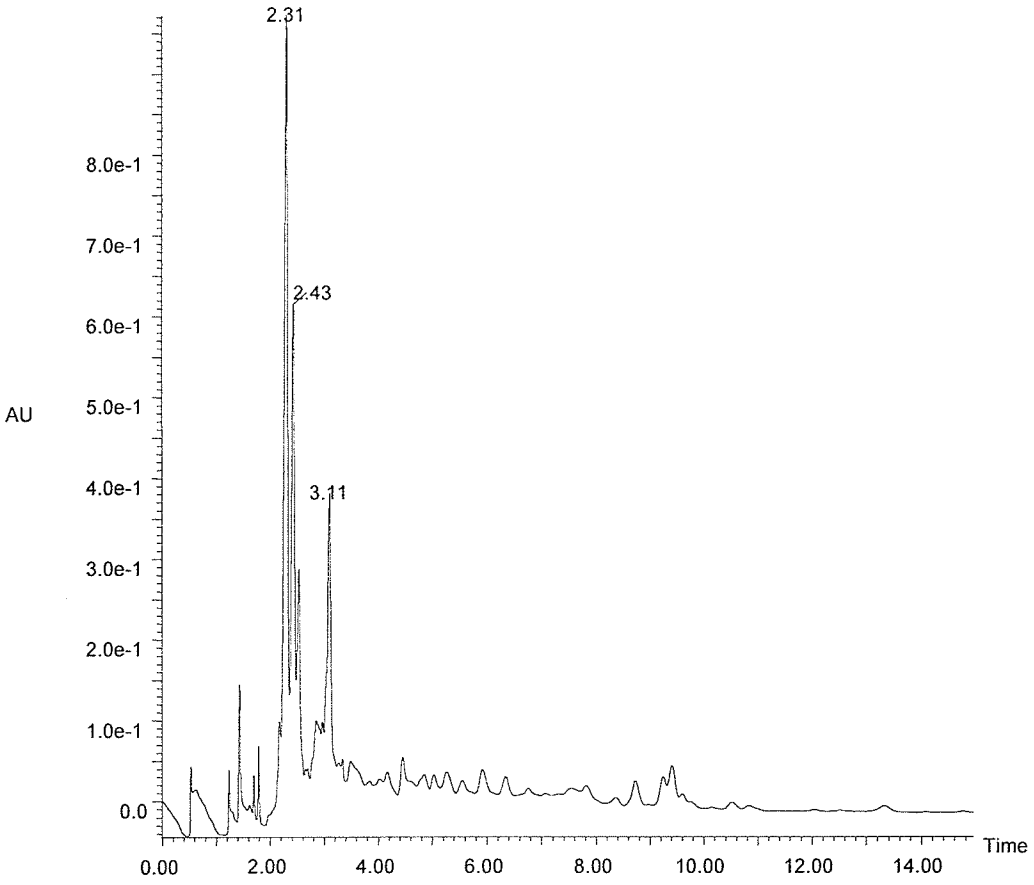


Figure 41. MS scan chromatogram of fraction SPE 1%-#3-2

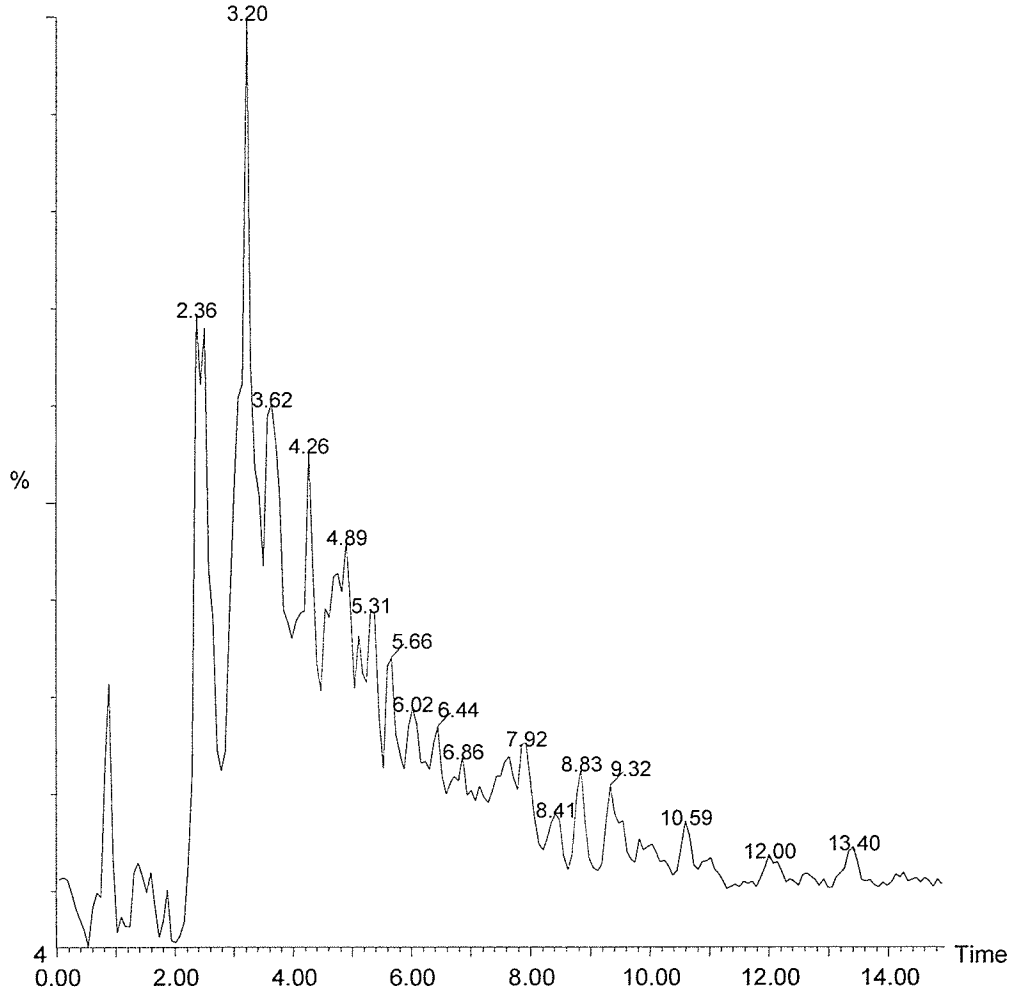


Figure 42. MS spectrum of fraction SPE 1 %-#3-2 at 2.36 min.

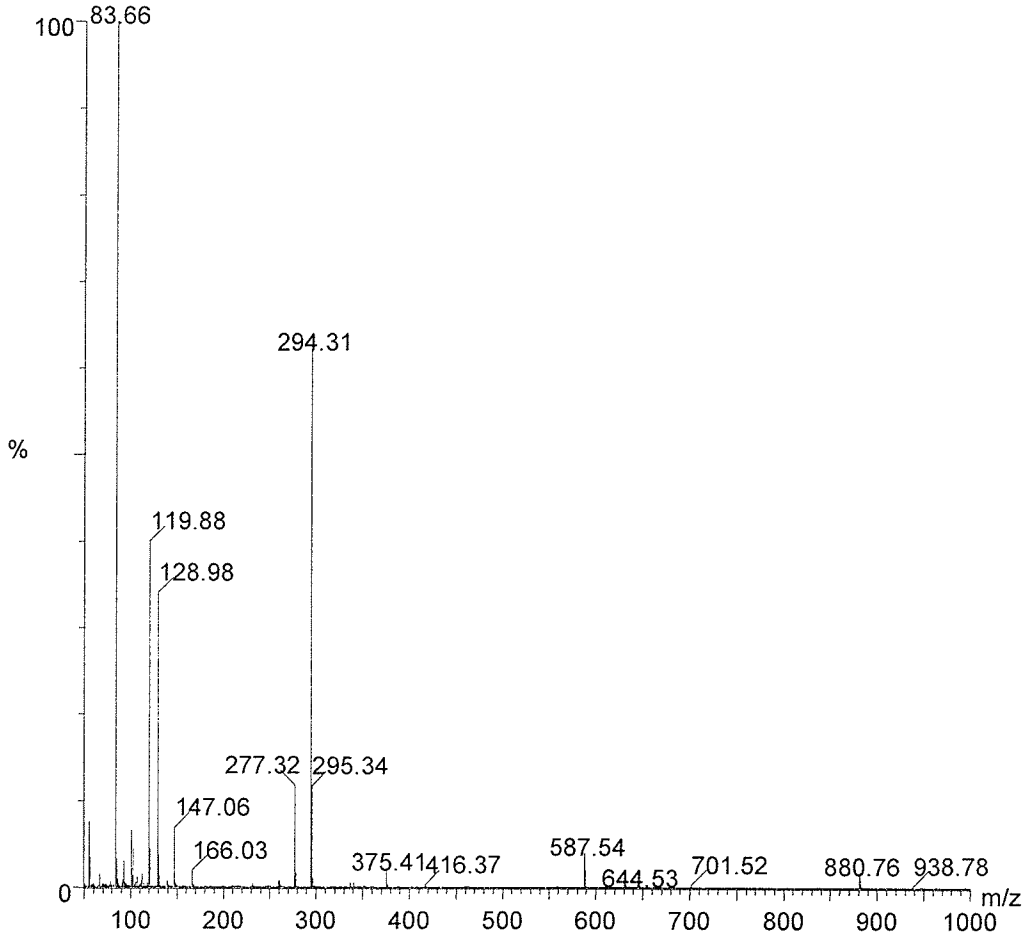
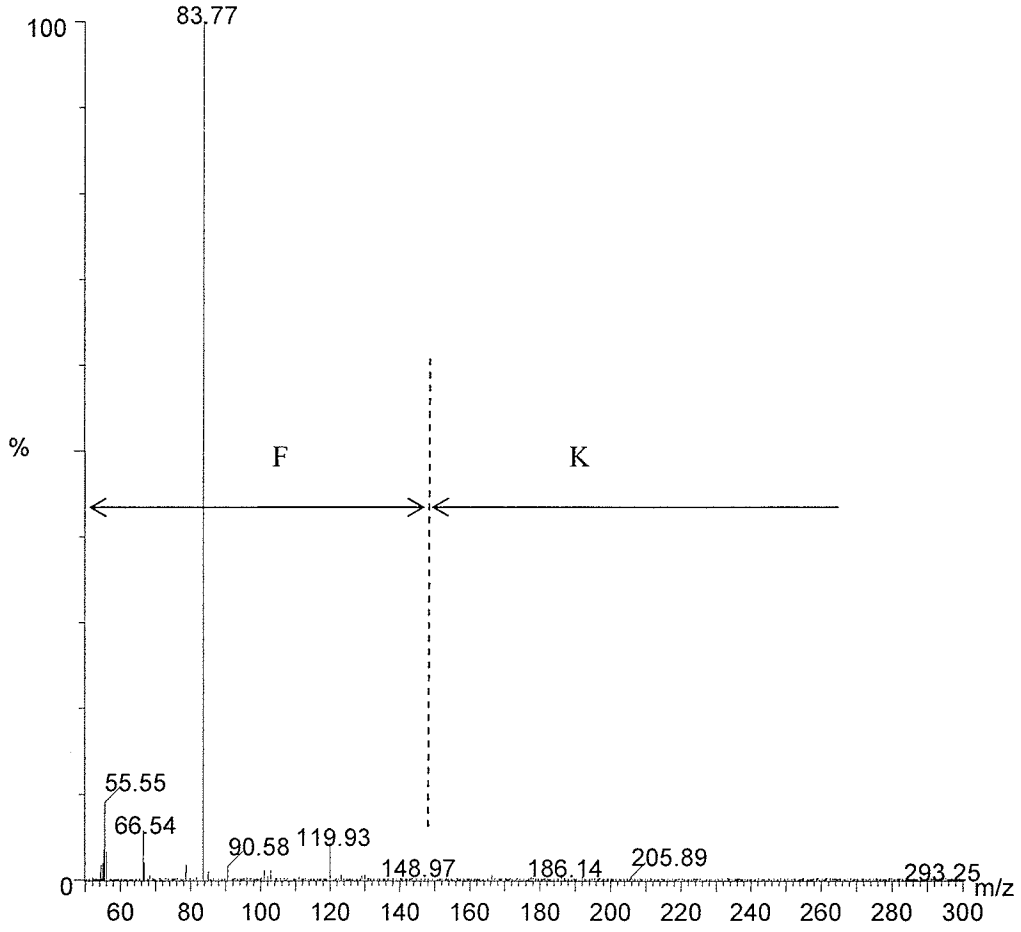


Figure 43. MS/MS spectrum of ion m/z 294.31



Because the relative intensity of the impurity ion m/z 83.77 was very high, intensity of other ions appeared to be very low and may not be visible in this figure.

6.3.14.3 SPE 1%_#3-3

Figure 44 and 45 show the chromatograms of UPLC and MS scan of SPE 1%-#3-3. Major peak of SPE 1%_#3-3 was eluted out at retention time of 3.46 min, and high total ion count was detected around 3.69 min. Figure 46 shows the spectrum acquired at a retention time of 3.96 min in this analysis. This spectrum was evaluated by the data system to determine that an ion with m/z of 295.34 was the most dominant product. Spectrum of product ion of m/z 295.34 is shown in Figure 47, and the peptide was identified as EF using Expasy.

Figure 44. UPLC chromatogram of fraction SPE 1%-#3-3.

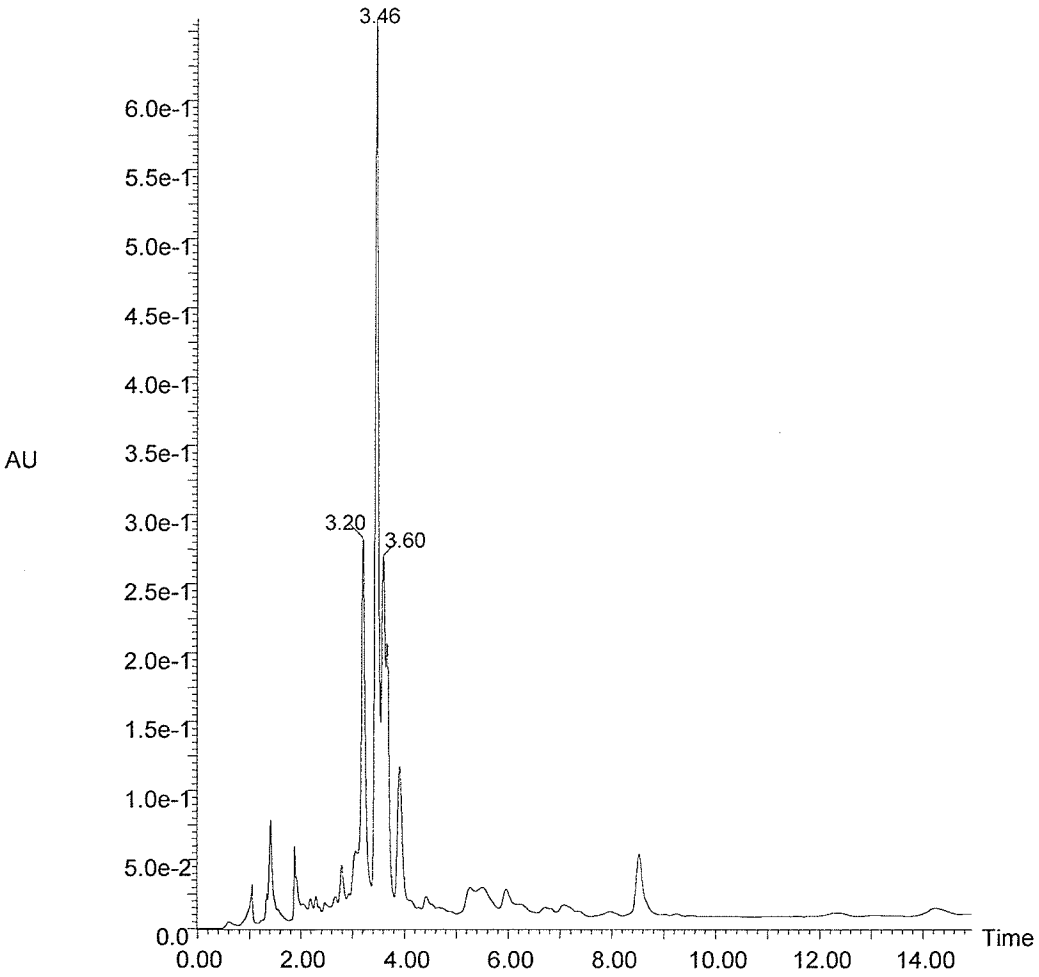


Figure 45. MS scan chromatogram of fraction SPE 1%-#3-3.

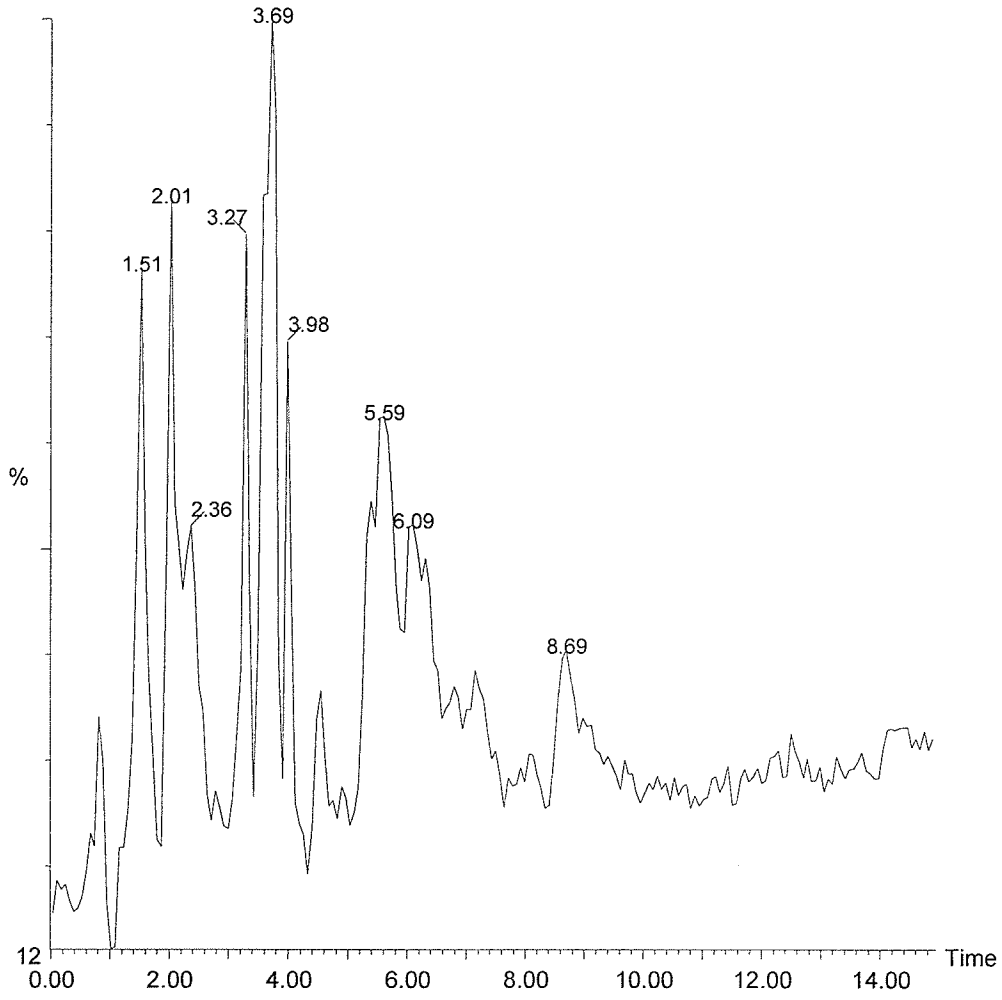


Figure 46. MS spectrum of fraction SPE 1%-#3-3 at 3.976 min.

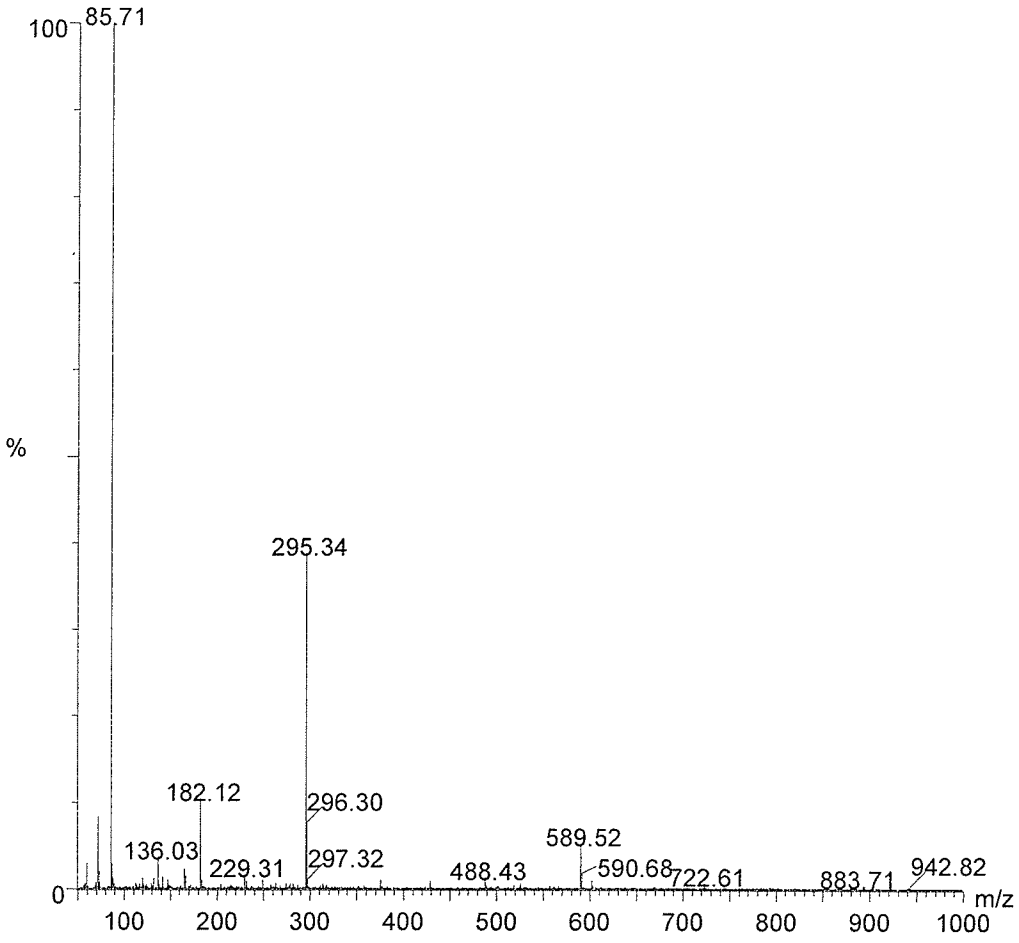
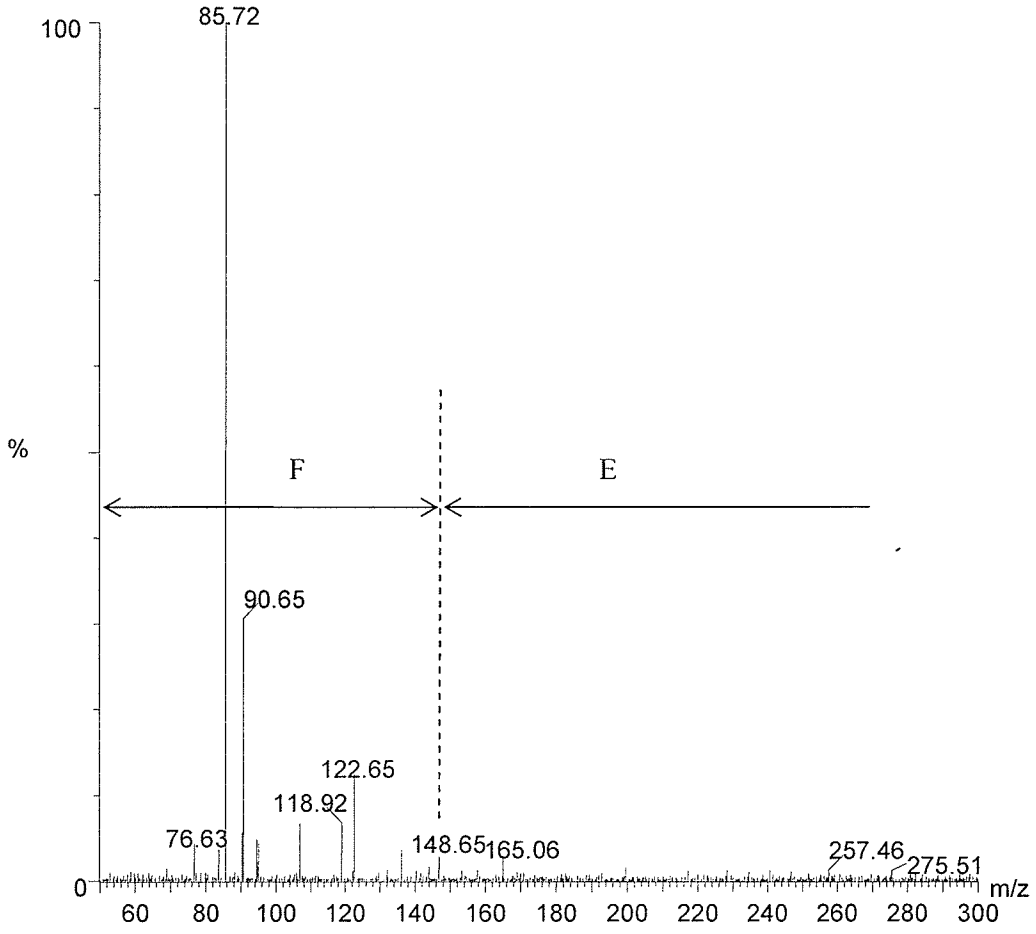


Figure 47. MS/MS spectrum of ion m/z 295.34.



Because the relative intensity of the impurity ion m/z 85.72 was very high, intensity of other ions appeared to be very low and may not be visible in this figure.

6.3.15 Determination of IC_{50} Values of Synthesized Peptides against ACE, Renin, and CaMPDE1

IC_{50} is commonly used to measure the effectiveness of a compound in inhibiting a biological or biochemical function. It is the concentration of a substance that provides 50% inhibition of a certain reaction. Almost all related studies use IC_{50} to represent the effectiveness of the compounds that are studied (Tauzin *et al.*, 2002; Fujita *et al.*, 2000; Maeno *et al.*, 1996; Yamamoto *et al.*, 1994). IC_{50} values of synthesized peptides against ACE and renin are summarized in Table 16. Inhibition percentages of synthesized peptides against ACE, renin, and CaMPDE1 are listed in Table 17.

In ACE assay, IR has the lowest IC_{50} value of 2.25 mM which represents the highest inhibitory activity against ACE. KF, on the contrary, shows the highest IC_{50} and therefore lowest inhibitory activity. Two peptides (KDYRL and KLPAGWLF) derived from mung-bean showed IC_{50} values of 26.5 and 13.4 μM (Li *et al.*, 2006). Byun and Kim (2001) reported 2 peptides (GPM and GPL) isolated from Alaska Pollock with IC_{50} values of 17.13 and 2.65 μM, respectively. Compared with previous studies which have shown a large amount of peptides with IC_{50} values lower than 50 μM, peptides derived from alcalase hydrolyzed PPH displayed low activities against ACE (Yang *et al.*, 2003; Motoi *et al.*, 2003; Sato *et al.*, 2002; Tauzin

et al., 2002; Fujita *et al.*, 2000; Saito *et al.*, 1994). When compared with 168 dipeptides with IC_{50} values ranged from 1.41 to 16982.44 mM collected in a database by Wu *et al.* (2006), IR, EF, and KF have shown strong ACE-inhibitory activities. Compared with 2 KFs in the database with IC_{50} values of 28.18 and 114.82 mM, KF identified from pea protein in this work exhibited lower IC_{50} value (7.23 mM). The dipeptide, IR, with IC_{50} value of 0.696 mM was also identified in pea protein by Vermeirssen (2003); however in that study, the dipeptide and its IC_{50} value were predicted using a database and software. For the first time, current study isolated this dipeptide from pea protein and its *in vitro* IC_{50} value was examined. EF was not observed in previous studies, and therefore was first identified in the current work.

In most cases, determination of the IC_{50} value of the peptide is based on the hydrolysis of the substrate HHL; however, with the use of various modification of the method by Cushman and Cheung (Cushman & Cheung, 1971), and continuously-developed new method, comparison of IC_{50} values among different studies have become more difficult and unreliable. Furthermore, ACE inhibitory activities of peptides vary considerably depending on assay conditions. Murray *et al.* (2004) showed that increasing ACE activity level in the assay from 155 to 211 units/L resulted in a corresponding increase in the IC_{50} value of Captopril® from

9.1 to 39.4 nM, and the same trend was observed for a whey protein hydrolysate sample (IC_{50} value of whey protein hydrolysate ranged from 52.3 to 124.28 mg protein/L when ACE activity level ranged from 114 to 234 units/L). Therefore, careful control of ACE activity during the assay is required in order to obtain comparable and reproducible values for the inhibitory potency of ACE inhibitors. Suetsuna and Nakano (2000) reported an IC_{50} value of 28.3 μM for a fragment, KF, of the tetrapeptide derived from wakame; however, they did not provide details of the amount of ACE units used in their analysis, and therefore, cannot be compared with our results.

IR showed the highest inhibitory activity against renin among the 3 peptides with IC_{50} value of 9.2 mM. KF and EF each showed lower activities compared with IR. There is currently no report of renin-inhibitory food protein-derived peptides that can be used to compare with our results. However, many synthesized peptides have been used as renin inhibitors. Kokubu *et al.* (1968) first described that methyl or ethyl esters of synthesized peptide LLVY and LLVF acted as competitive inhibitor of renin. Peptide PHPFHFFVYK based on hog angiotensinogen sequence inhibited human renin with a micromolar concentration (Corvol *et al.*, 1990). A synthesized peptide, Boc-PF-N(Me)H-Lψ[$CHOHCH_2$]VI-Amp, was reported to inhibit human

renin with IC_{50} value of 0.26 nM (Thaisrivongs *et al.*, 1986). A series of modified forms (incorporation of hydrophilic end groups) of this peptide showed increased solubility and remained strong inhibitory activities against human renin with IC_{50} values ranged from 0.28 to 2.5 nM, and some of these peptides exhibited improved pharmacological efficacy *in vivo* (Thaisrivongs *et al.*, 1991; Bundy *et al.*, 1990). Kokubu *et al.* (1984) synthesized some small peptides with IC_{50} values ranging from 0.08 to 0.32 μM. Compared with the data of synthesized renin inhibitory peptides, peptides derived from pea protein exhibited low activities. When looking at the structure of all 3 synthesized dipeptides, at N-terminal I is a very hydrophobic amino acid while K is partly hydrophobic and E is non-hydrophobic. At C-terminal, both R and F are bulky amino acids. The presence of a hydrophobic amino acid at the N-terminal with a bulky aromatic group at the C-terminal seem to be the most effective arrangement for high potency of dipeptides against renin activity. Such structural character also seems to be preferred by a CaMPDE1 inhibitor. However, such pattern does not exist for ACE inhibitor.

For inhibitory activities against CaMPDE1, results were opposite to those in ACE and renin assays. At 1 mg/mL, EF and KF both showed higher activities than IR, and EF inhibited about 4% more CaMPDE1 activity than KF did. This result is consistent with previous results that

peak SPE 1%-#3-3 had the highest inhibitory activity whereas peak SPE 1%-#3-1 had the lowest activity. Previous studies have shown that a number of natural compounds exhibit CaMPDE1 inhibitory properties such as alkaloids, terpenoids, coumarins, flavonoids, and lignans with similar efficiency as medically used CaMPDE1 inhibitors (Gonçalves *et al.*, 2009; Martínez-Luis *et al.*, 2007; Ko *et al.*, 2004). Some peptides isolated from α-casein pepsin digest were shown to inhibit CaM-dependent PDE activity over the range of 1-50 μM (Kizawa *et al.*, 1995). Several cyclo- and linear peptides derived from Korean herbs *Zizyphus vulharis var spinosus* and *Z. jujube var inermis* showed inhibitory effects on CaMPDE1 with IC_{50} values widely ranged from 4.6 to 140 μM (Martínez-Luis *et al.*, 2007; Hwang *et al.*, 2001; Han *et al.*, 1993). Some polypeptides from insect venom inhibited CaMPDE1 activity through binding calmodulin at the same binding site of CaMPDE1 in a competitive manner (Martínez-Luis et al., 2007; Kataoka *et al.*, 1989; Barnette *et al.*, 1983). Compared with former studies, peptides produced in our study displayed low inhibitory activities against CaMPDE1. From another aspect, comparison between previous studies can be difficult due to the issues such as it is sometimes unclear which of the purified or partially purified PDE1 isozymes were used. For example, ginsenosides, which is a pharmaceutical, inhibits brain PDE1A2 but not brain PDE1B1 (Kakkar *et al.*, 1999).

Table 16. IC_{50} values of synthesized peptides against ACE and renin.

Peptide	ACE (IC_{50}, mM)	Renin (IC_{50}, mM)
IR	2.25	9.2
KF	7.23	17.84
EF	2.98	22.66

Table 17. Inhibition percentage of synthesized peptides against ACE, renin, and CaMPDE1.

Peptide	ACE (Inhibition %)	Renin (Inhibition %)	CaMPDE1 (Inhibition %)
IR	72.26	38.68	3.51
KF	75.34	23.61	9.52
EF	54.74	29.24	13.33

Peptide concentration was 1 mg/mL.

Table 18. Position of IR, KF, and EF in pea protein sequences.

Peptide	Position	Parent protein
IR	f177-178	Legumin A
	f168-169	Legumin A2
	f172-173	Legumin B
	f236-237	Legumin B
	f72-73	Legumin J
	f333-334	Legumin J
	f397-398	Legumin J
	f244-245	Legumin K
	f54-55	Vicilin
	f173-174	Convicilin
	f41-42	Provicilin
	f159-160	Albumin-2
KF	f503-504	Legumin A
	f506-507	Legumin A2
	f42-43	Vicilin
	f59-60	Vicilin
	f269-270	Vicilin
	f55-56	Vicilin 47k
	f262-263	Vicilin 47k
	f265-266	Vicilin 47k
	f57-58	Vicilin 14k
	f161-162	Convicilin
	f277-278	Convicilin
	f382-383	Convicilin
	f89-90	Albumin-1 F
	f82-83	Albumin-2
	f46-47	Provicilin
	f253-254	Provicilin
	f256-257	Provicilin
EF	f190-191	Legumin A
	f191-192	Legumin A2
	f11-12	Legumin B
	f26-27	Legumin J
	f194-195	Legumin J
	f243-244	Legumin J
	f44-45	Legumin K
	f93-94	Legumin K

	f266-267	Vicilin
	f366-367	Convicilin
	f149-150	Lectin
	f156-157	Nodule lectin

Protein primary sequences (position) were obtained from ExPasy proteomics server of the Swiss Institute of Bioinformatics.

6.4 CONCLUSIONS

It is concluded that PPI can be optimally hydrolyzed to produce small peptides with positive charges as evidence by their ability to bind to a cationic SPE matrix. These peptides show various inhibitory activities against ACE, renin, and CaMPDE1. Inhibition of ACE and CaMPDE1 was positively related with electric charges. Two steps of further separation of fraction SPE 1% by RP-HPLC led to the production of 3 purified peaks, and were identified as 3 dipeptides: IR, KF, and EF. Inhibition studies showed that all 3 peptides were multifunctional and could inhibit ACE, renin, and CaMPDE1 activities. It was observed that a hydrophobic residue at N-terminal and bulky amino acid residue at C-terminal was a preferred structural arrangement for renin inhibitor. Dipeptides IR and EF were isolated form food proteins for the first time. These pea protein-derived bioactive peptides will be potential therapeutic agent in the treatment of hypertension and related pathological conditions, and they are superior compared to the single function peptides. This is the first study to date to report such multifunctional peptides derived from pea proteins.

CHAPTER 7

GENERAL DISCUSSION, CONCLUSION, AND FUTURE PERSPECTIVES

7.1 GENERAL DISCUSSION AND CONCLUSION

With the increased cost of health care and further desire to improve quality of life, foods are consumed not only to satisfy hunger and basic nutrient requirement but to prevent nutrition related diseases and provide additional health values. With the increasingly higher life expectancy for consumers, functional foods can play a major role in sustaining healthy living. Global market of functional foods has been estimated to have increased from at least 33 billion US$ in 2000 to nearly 61 billion US$ in 2004 with United States being the largest market segment followed by Japan and Europe (Benkouider, 2004; Hilliam, 2000). Not only food manufacturers, but also the pharmaceutical industry has shown interest in this field. A subcategory of functional foods is functional drinks fortified with functional ingredients, such as cholesterol-lowering drink (with combination of omega-3 and soy), eye-health drinks (with leutein), bone health drinks (with calcium). It was estimated that consumption of functional drinks in Europe could reach 5.1 billion litres by 2009 which corresponds to 23% increase compared with that of 2005 (Siro *et al.*, 2009).

Hypertension is the leading risk for death in North America and a significant public health problem worldwide. Over 5 million Canadian have

hypertension. However, small reduction in BP can significantly decrease the risk of death and stroke (Canadian Hypertension Society). Beside medication, lifestyle modification and diet therapy are also important tools to effectively lower BP. Functional foods containing food protein derived ACE-inhibitory peptides fit very well into the role as dietary therapeutic agents. Some functional foods and beverages containing ACE-inhibitory peptides are already commercialized as described in Chapter 2; however, none of them originated from pea proteins. Pea seed is well established as part of the crop rotation system in Canadian prairie provinces. Pea production in the prairies reached 3.6 million tonnes in 2008 (Statistics Canada). Pea is a well balanced source of amino acids and especially rich in essential amino acid lysine. It has a 98% digestibility as high as milk. Moreover, pea is a low input crop as it is capable of fixing the mast majority of its own N through symbiotic relationship with *Rhizobium* bacteria (Johnston, 2002). Due to the economic importance to the prairie provinces and especially Manitoba, pea was chosen as the source of protein for this research work.

The ultimate goal of this work is to incorporate the bioactive components derived from pea protein into food and drink products and develop functional foods and drinks with hypotensive benefits. Therefore, consumer acceptability is of the most important. However, if sensory characteristics do not fit expectations, there is no doubt that the products will not be welcomed. Peptides released from food protein by enzymatic hydrolysis

tend to give bitter taste; hence we examined the bitterness of PPH. It is indicated that if one wishes to know whether a population or consumer will accept the food, one should take a sample from that same population because a separate sample will not predict the behaviour (O'Mahony, 1991). Potential consumers of PPH-incorporated food and drink products include the population with both essential and secondary hypertension, and those who have a family history of hypertension for a preventive purpose. General population concerning hypertension can also be included. In our study, both male and female University of Manitoba students aged 18 to 24 were recruited as testing panel members because of convenience. This represents not the whole but part of the potential consumer population as hypertension, either primary or secondary, can happen in early life stage and population prevalence of hypertension in the young is increasing (Falkner, 2009; Luma & Spiotta, 2006). Furthermore, taste perception is affected by aging. Studies have shown that significant loss of taste is common in the elderly and is a result of normal aging (Fukunaga *et al.*, 2005; Schiffman, 1997; Murphy, 1993). Therefore, MRT identified by older subjects may be higher than that identified by younger subjects as a result of declined taste threshold sensitivity and be less accurate. While a testing panel can provide the most reliable information, it is still subjective. Electronic tongue (ET), as an instrument measuring and comparing taste quantitatively, can be very objective. Good correlation between human perception of taste and prediction

by ET has been observed (Legin *et al.*, 2004; Uchida *et al.*, 2001). It would be an asset evaluating bitterness of PPH using ET to further confirm and compare with the results obtained from human trial testing. However, the low level of MRT obtained in this work suggests that the PPH can be incorporated into foods at physiologically effective concentrations without imparting negative bitterness properties.

Currently several types of drugs are used to treat hypertension: ACE inhibitors, ARBs, diuretics, calcium-channel blockers, and beta-blockers. When monotherapy is not effective enough, combination therapy using 2 agents are usually used. Using combination therapy always leads to greater efficacy than high-dose monotherapy, and side effects are similar or even fewer in low-dose combination therapy (Neutal, 2006). Hence, both efficacy and safety can be achieved in the management of hypertension. As stated in Chapter 6, studies have shown that treatment with combination of ACE inhibitors and ARBs works more efficiently than either one does alone because it offers dual blockage of RAS (Schjoedt *et al.*, 2005; Jacobsen *et al.*, 2004). However, the blockage is incomplete because an ACE-independent pathway exists to form angiotensin II from angiotensin I via the action of chymase. In Chapter 4, we observed significantly reduced ACE activities in both treated rat groups (500 and 1000 mg); however, significantly decreased angiotensin II level was only detected in the 1000 mg group. This can be explained by the second alternative pathway involving chymase, which may

have only been overcome by the higher dose of PPH. Moreover, in patients with autosomal dominant polycystic kidney disease (ADPKD), plasma renin activity is increased. Compared with ARB which does not alter BP in patients with ADPKD and hypertension, ACE inhibitors not only decreases BP but also more efficient than calcium channel blocker and diuretics (Schrier, 2009; Schrier *et al.*, 2002; Ecder *et al.*, 2001). However, again because angiotensin II generation can occur through chymase pathway in the presence of ACE inhibitors, treatment using ACE inhibitors is not sufficient enough. Beside, ACE inhibitors and ARBs can stimulate an increase in plasma renin activity through a compensatory mechanism. Under such situation, renin inhibitors which directly target the first and rate-limiting step of RAS may provide a better choice by suppressing the effect of renin and leading to a reduced plasma renin activity. Clinical studies reported antihypertensive efficacy of recently approved renin inhibitor, aliskiren, either using alone or combined with other drugs (Sanoski, 2009; Thriller *et al.*, 2008). PPH obtained from thermolysin hydrolysis (<3 kDa) exhibited inhibitory activities against both ACE and renin, and hence are capable of blocking the first and second steps of RAS pathway. Due to the reasons mentioned above, this dual blockade should work better than that by ACE inhibitors and ARBs and be more efficient in patients with ADPKD. Compared with combination treatment using two or multiple drugs, one major advantage of PPH is it offers dual blockade by using only one agent and therefore has less side effect. Besides,

because of its natural character, negative side effect of PPH is yet to be reported when compared to those associated with synthetic drugs. In this work, we demonstrated the bioavailability of PPH using a normotensive rat model while the use of a PKD rat model confirmed the antihypertensive effectiveness *in vivo*.

In the PPH obtained from alcalase hydrolysis (<1 kDa), three multifunctional peptides were identified that displayed inhibitory activities against ACE, renin, and CaMPDE1 *in vitro*. Like thermolysin hydrolyzed PPH, they have the potential to be used to offer addictive effects in the treatment of hypertension or other chronic disease conditions. For the first, we isolated, purified and identified the amino acid sequence of food protein-derived peptides that inhibited renin activity *in vitro*. However, *in vivo* bioavailability and effectiveness of the three peptides were not investigated in this work because of the limited scope of this work.

Major accomplishments of this works include:

- Thermolysin hydrolyzed PPH (<3 kDa) gave moderate bitter taste with MRT of 2.5 mg/mL, and this bitterness was related with relatively high level of hydrophobic amino acid residues in the PPH (Chapter 3);
- Thermolysin hydrolyzed PPH (<3 kDa) was proved to be bioavailable using a normotensive rat model. ACE activity was dose-dependently inhibited in lung of treated rats. Angiotensin II

level was decreased in both lung and plasma of treated rats (Chapter 4);

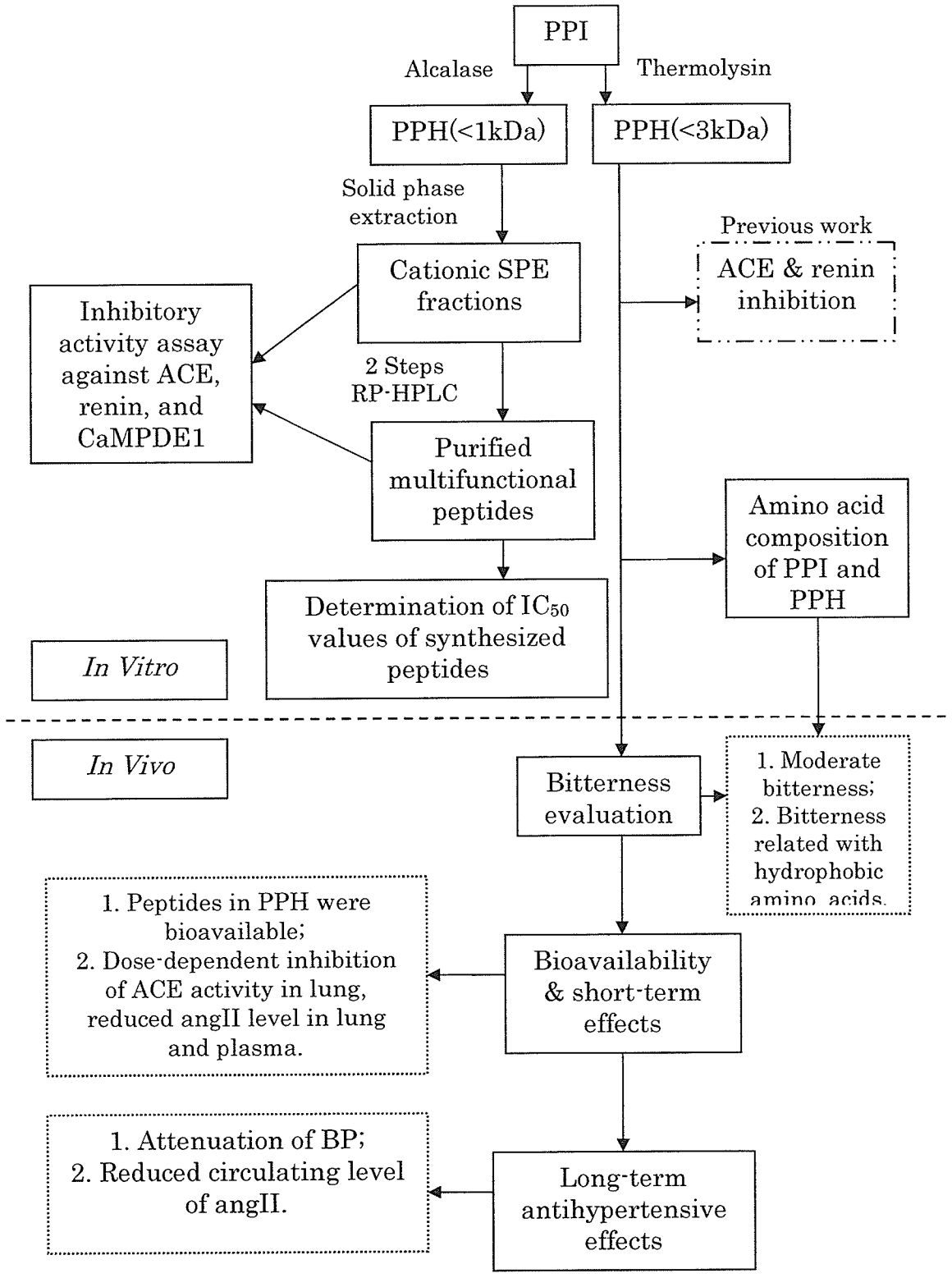
- *In vivo* antihypertensive effectiveness of thermolysin hydrolyzed PPH (<3 kDa) was confirmed in PKD rat model. Development of both SBP and DBP were attenuated by consumption of PPH. Angiotensin II level in plasma was reduced in treated rat groups by the end of the study (Chapter 5);
- PPI could be hydrolyzed by alcalase to produce small molecular weight peptides (<1 kDa). Peptide fractions separated using cation-exchange cartridge and RP-HPLC showed various inhibitory activities against multiple enzymes: ACE, renin, and CaMPDE1. Three multifunctional peptides were purified and identified as IR, KF, and EF, and exhibited inhibitory activities against all three enzymes (Chapter 6).

Figure 48 shows a schematic overview of the major accomplishments of this work.

In conclusion, this work extensively investigated the bioavailability, short-term and long-term effects of thermolysin hydrolyzed PPH. In order to prepare the marketing of the future product containing PPH, sensory characteristic was evaluated. Three peptides were identified from alcalase hydrolyzed PPH, and exhibited multi-functionalities with inhibitory activities against ACE, renin, and CaMPDE1. This study is the first to report bioactive

peptides derived from food proteins with inhibitory activities against multiple enzymes. Bioavailability of food protein hydrolysate was first examined in a normotensive Long-Evans rat model. And for the first time, long-term antihypertensive effectiveness of food protein hydrolysate was investigated in a chronic kidney diseased rat model. Results from this work can contribute to the development of functional foods and nutraceuticals/therapeutic agents using natural food peptides with health beneficial effects.

Figure 48. Schematic overview of the major accomplishments.



7.2 FUTURE PERSPECTIVES

ACE inhibitors have been safely used in the treatment of hypertension and hypertension related diseases such as diabetes, congestive heart failure, chronic kidney diseases, etc. In the last decade, many studies have revealed that ACE inhibitors have more to offer. Moskowitz (2002) presented evidence that ACE deletion/deletion (D/D) genotype was associated with a large amount of common adult diseases, including cardiovascular disease, cancer, and psychiatric diseases. It was also indicated that overactivity of ACE, which was associated with ACE D/D genotype, maybe involved in the pathogenesis of common diseases. Indeed, angII may play a pivotal role in cancer development (Yoshiji *et al.*, 2004). In this region, increased tumor cell apoptosis was observed by treatment using anti-angiogentic agents (Yoshiji *et al.*, 2004). ACE inhibitors, which have anti-angiogenic activity, can suppress experimental tumor growth even at clinically comparable doses (Yoshiji *et al.*, 2002a; Yoshiji *et al.*, 2002b). Therefore, in view of the broad range of actions of ACE inhibitory peptides, the application of food protein-derived ACE inhibitory peptides may increase. Furthermore, multifunctional peptides with both ACE and renin-inhibitory activities can offer addictive anti-angiogeneic effects.

With respect of this work, dose-dependent antihypertensive effect was not observed in the long term. Future studies should be developed to further investigate the dose-response relationship and establish the minimum

effective dose (MED) value, if possible, for humans. PPH (<3 kDa) was used in this work as a whole sample. In the future work, it can be separated and purified to identify the effective peptides. In such way, QSAR can be studied and contribute to the bioactive peptides database and future development of potential bioactive peptides. Even though *in vitro* multi-functionality was observed for alcalase hydrolyzed PPH (<1 kDa), *in vivo* efficacy remained unknown. If adequate amount can be produced in the future, *in vivo* effectiveness can be further investigated and compared with that of thermolysin hydrolysate. With increasing knowledge and improved technologies, influence of the bioactive peptides on genes or the interaction between bioactive peptides and genes (nutrigenomics) can be studied.

CHAPTER 8

REFERENCES

Abubakar, A., Saito, T., Kitazawa, H., Kawai, Y., & Itoh, T. (1998). Structural analysis of new antihypertensive peptides derived from cheese whey protein by proteinase K digestion. *Journal of Dairy Science, 81*, 3131-3138.

Adibi, S. A. (1971). Intestinal transport of dipeptides in man: relative importance of hydrolysis and intact absorption. *The Journal of Clinical Investigation, 50*, 2266-2275.

Adsule, R. N., Lawande, K. M., & Kadam, S. S. (1989). Pea. In D. K. Salunkhe, & S. S. Kadam (Eds.), *CRC handbook of world legumes: Nutritional, chemistry, processing, technology, and utilization. Vol II.* Boca Raton, Florida, USA: CRC Press Inc.

Aihara, K., Kajimoto, O., Hirata, H., Takahashi, R., & Nakamura, Y. (2005). Effect of powdered fermented milk with lactobacillus helveticus on subjects with high-normal blood pressure or mild hypertension. *Journal of the American College of Nutrition, 24*, 257-265.

Arai, S., Yamashita, M., Kato, H., & Fujimaki, M. (1970). Applying proteolytic enzyme on soybean. V. A nondialysable bitter peptide in

peptic hydrolysate of soybean protein and its bitterness relation to the chemical structure. *Agriculture and Biological Chemistry, 34*, 729-738.

Ariyoshi, Y. (1993). Angiotensin-converting enzyme inhibitors derived from food proteins. *Trends in Food Science and Technology, 4*, 139-144.

Astawan, M., Wahyuni, M., Yasuhara, T., Yamada, K., Tadokoro, T., & Maekawa, A. (1995). Effects of angiotensin I-converting enzyme inhibitory substances derived from indonesian dried-salted fish on blood pressure of rats. *Bioscience, Biotechnology, and Biochemistry, 59*, 425-429.

Aubes-Dafau, I., Capdevielle, J., Seris, J. L., & Combes, D. (1995). Bitter peptide from hemoglobin hydrolysate: Isolation and characterization. *FEBS Letters, 364*, 115-119.

Aubes-Dufau, I., & Combes, D. (1997). Effect of different proteases on bitterness of hemoglobin hydrolysates. *Applied Biochemistry and Biotechnology, 67*, 127-138.

Aziz, M., Guyene, T. T., Chatellier, G., & Menard, J. (1994). Blood pressure effects of acute intravenous renin or oral angiotensin converting enzyme inhibition in essential hypertension. *Journal of Hypertension, 12*, 419-427.

Bairoch, A. (2000). The ENZYME database in 2000. *Nucleic Acids Research, 28*, 304-305.

BARBER, M., JOLLES, P., VILKAS, E., & LEDERER, E. (1965). Determination of amino acid sequences in oligopeptides by mass spectrometry. I. the structure of fortuitine, an acylnonapeptide methyl ester. *Biochemical and Biophysical Research Communications, 18*, 469-473.

Barber, M., Wolstenholme, W. A., Guinand, M., Michel, G., Das, B. C., & Lederer, E. (1965). Determination of amino acid sequences in oligopeptides by mass spectrometry. II. The structure of peptidolipin NA. *Tetrahedron Letters, 18*, 1331-1336.

Barnette, M. S., Daly, R., & Weiss, B. (1983). Inhibition of calmodulin activity by insect venom peptides. *Biochemical Pharmacology, 32*, 2929-2933.

Batista, C. V. F., Rosendo da Silva, L., Sebben, A., Scaloni, A., Ferrara, L., Paiva, G. R., et al. (1999). Antimicrobial peptides from the brazilian frog phyllomedusa distincta1. *Peptides, 20*, 679-686.

Bellamy, W., Takase, M., Yamauchi, K., Wakabayashi, H., Kawase, K., & Tomita, M. (1992). Identification of the bactericidal domain of lactoferrin. *Biochimica Et Biophysica Acta, 1121*, 130-136.

Bender, A. T. (2007). Calmodulin-stimulated cyclic nucleotide phosphodiesterase. In J. A. Beavo, S. H. Francis & M. D. Houslay (Eds.), *Cyclic nucleotide phosphodiesterases in health and diseases.* (pp. 35-54). Boca Raton, Florida, USA: CRC Press Inc.

Benkouider, C. (2004). Functional foods: A global overview. *International Food Ingredients, 5*, 66-68.

Bidlingmeyer, B., Cohen, S., Tarvin, T. (1984). Rapid analysis of amino acids using pre-column derivatization. *Journal of Chromatography, 336,* 93-104.

Borel, P., Lairon, D., Termine, E., Grataroli, R., & Lafont, H. (1989). Isolation and properties of lipolysis inhibitory proteins from wheat germ and wheat bran. *Plant Foods for Human Nutrition (Dordrecht, Netherlands), 39*, 339-348.

Bouhallab, S., Leonil, J., & Maubois, J. (1991). Complexation du fer par le phosphopeptide (1-25) de la caséine: Action de l'alcalse et de la phosphotase acide. *Le Lait, 71*, 435-443.

Bounous, G., & Kongshavn, P. A. L. (1985). Differential effect of dietary protein type on the B-cell and T-cell immune responses in mice. *Journal of Nutrition, 115*, 1403-1408.

Brantl, V. (1984). Novel opioid peptides derived from human beta-casein: Human beta-casomorphins. *European Journal of Pharmacology, 106*, 213-214.

Brantl, V., Teschemacher, H., Henschen, A., & Lottspeich, F. (1979). Novel opioid peptides derived from casein (beta-casomorphins). I. Isolation from bovine casein peptone. *Hoppe-Seyler's Zeitschrift Fur Physiologische Chemie, 360*, 1211-1216.

Brickey, D. A., Colbran, R. J., Fong, Y. L., & Soderling, T. R. (1990). Expression and characterization of the alpha-subunit of Ca^{2+}/calmodulin-dependent protein kinase II using the baculovirus expression system. *Biochemical and Biophysical Research Communications, 173*, 578-584.

Brulé, G., & Fauquant, J. (1982). Interactions des protéins du lait et des oligoéléments. *Le Lait, 62*, 323-331.

Bumberger, E., & Belitz, H. (1993). Bitter taste of enzymic hydrolysates of casein. *Zeitschrift für Lebensmitteluntersuchung Und -Forschung A, 197*, 14-19.

Bundy, G. L., Pals, D. T., Lawson, J. A., Couch, S. J., Lipton, M. F., & Mauragis, M. A. (1990). Potent renin inhibitory peptides containing hydrophilic end groups. *Journal of Medicinal Chemistry, 33*, 2276-2283.

Byun, H. G., & Kim, S. K. (2002). Structure and activity of angiotensin I converting enzyme inhibitory peptides derived from alaskan pollack skin. *Journal of Biochemistry and Molecular Biology, 35*, 239-243.

Byun, H., & Kim, S. (2001). Purification and characterization of angiotensin I converting enzyme (ACE) inhibitory peptides from alaska pollack (theragra chalcogramma) skin. *Process Biochemistry, 36*, 1155-1162.

Campbell, R., Sangalli, F., Perticucci, E., Aros, C., Viscarra, C., Perna, A., et al. (2003). Effects of combined ACE inhibitor and angiotensin II antagonist treatment in human chronic nephropathies. *Kidney International, 63*, 1094-1103.

Carr, J. W., Loughheed, T. C., & Baker, B. E. (1965). Studies on protein hydrolysis. IV. Further observations on the taste of enzymic protein hydrolysates. *Journal of the Science of Food and Agriculture, 7*, 629-637.

Cerovsky, V., Hovorka, O., Cvacka, J., Voburka, Z., Bednarova, L., Borovickova, L., et al. (2008). Melectin: A novel antimicrobial peptide from the venom of the cleptoparasitic bee melecta albifrons. *Chembiochem : A European Journal of Chemical Biology, 9*, 2815-2821.

Chabance, B., Jolles, P., Izquierdo, C., Mazoyer, E., Francoual, C., Drouet, L., et al. (1995). Characterization of an antithrombotic peptide from kappa-

casein in newborn plasma after milk ingestion. *The British Journal of Nutrition, 73*, 583-590.

Chan, J. C. K., & Li-Chan, E. (2007). Production of lactoferricin and other cationic peptides from food grade bovine lactoferrin with various iron saturation levels. *Journal of Agricultural and Food Chemistry, 55*, 493-501.

Chen, H. M., Muramoto, K., Yamauchi, F., Fujimoto, K., & Nokihara, K. (1998). Antioxidative properties of histidine-containing peptides designed from peptide fragments found in the digests of a soybean protein. *Journal of Agricultural and Food Chemistry, 46*, 49-53.

Cheng, F., Liu, Y., Wan, T., Lin, L., & Sakata, R. (2008). The development of angiotensin I-converting enzyme inhibitor derived from chicken bone protein. *Animal Science Journal, 79*, 122-128.

Cheng, F., Wan, T., Liu, Y., Chen, C., Lin, L., & Sakata, R. (2009). Determination of angiotensin-I converting enzyme inhibitory peptides in chicken leg bone protein hydrolysate with alcalase. *Animal Science Journal, 80*, 91-97.

Cheung, H. S., Wang, F. L., Ondetti, M. A., Sabo, E. F., & Cushman, D. W. (1980). Binding of peptide substrates and inhibitors of angiotensin-

converting enzyme. importance of the COOH-terminal dipeptide sequence. *The Journal of Biological Chemistry, 255*, 401-407.

Cheung, W. Y. (1970). Cyclic 3':5'-nucleotide phosphodiesterase. determination of an activator. *Biochemical and Biophysical Research Communications, 38*, 533-538.

Cheung, W. Y. (1971). Cyclic 3':5'-nucleotide phosphodiesterase. *Journal of Biological Chemistry, 246*, 2859-2869.

Chiba, H., Tani, F., & Yoshikawa, M. (1989). Opioid antagonist peptides derived from kappa-casein. *The Journal of Dairy Research, 56*, 363-366.

Chiba, H., & Yoshikawa, M. (1991). Bioactive peptides derived from food proteins. *Kagaku to Seibutsu, 29*, 454-458.

Cho, S., Juillerat, M. A., & Lee, C. (2008). Identification of LDL-receptor transcription stimulating peptides from soybean hydrolysate in human hepatocytes. *Journal of Agricultural and Food Chemistry, 56*, 4372-4376.

Chrysant, S. G., Murray, A. V., Hoppe, U. C., Dattani, D., Patel, S., Hsu, H., et al. (2008). Long-term safety, tolerability and efficacy of aliskiren in combination with valsartan in patients with hypertension: a 6-month interim analysis. *Current Medical Research and Opinion, 24*, 1039-1047.

Colbran, R. J. (1993). Inactivation of Ca2+/calmodulin-dependent protein kinase II by basal autophosphorylation. *The Journal of Biological Chemistry, 268*, 7163-7170.

Corvol, P., Chauveau, D., Jeunemaitre, X., & Menard, J. (1990). Human renin inhibitor peptides. *Hypertension, 16*, 1-11.

Cowley, B. D.,Jr, Gudapaty, S., Kraybill, A. L., Barash, B. D., Harding, M. A., Calvet, J. P., et al. (1993). Autosomal-dominant polycystic kidney disease in the rat. *Kidney International, 43*, 522-534.

Curtis, J. M., Dennis, D., Waddell, D. S., MacGillivray, T., & Ewart, H. S. (2002). Determination of angiotensin-converting enzyme inhibitory peptide leu-lys-pro-asn-met (LKPNM) in bonito muscle hydrolysates by LC-MS/MS. *Journal of Agricultural and Food Chemistry, 50*, 3919-3925.

Cushman, D. W., & Cheung, H. S. (1971). Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung. *Biochemical Pharmacology, 20*, 1637-1648.

Daniel, H. (2004). Molecular and integrative physiology of intestinal peptide transport. *Annual Review of Physiology, 66*, 361-384.

Dhanaraj, V., Dealwis, C. G., Frazao, C., Badasso, M., Sibanda, B. L., Tickle, I. J., et al. (1992). X-ray analyses of peptide-inhibitor complexes define

the structural basis of specificity for human and mouse renins. *Nature, 357*, 466-472.

Dong, X. Z., Xu, H. B., Huang, K. X., Liou, Q., & Zhou, J. (2002). The preparation and characterization of an antimicrobial polypeptide from the loach, misgurnus anguillicaudatus. *Protein Expression and Purification, 26*, 235-242.

Doulton, T. W. R., & MacGregor, G. A. (2009). Combination renin-angiotensin system blockade with the renin inhibitor aliskiren in hypertension. *Journal of Renin-Angiotensin-Aldosterone System, in print.*

Duvick, J. P., Rood, T., Rao, A. G., & Marshak, D. R. (1992). Purification and characterization of a novel antimicrobial peptide from maize (zea mays L.) kernels. *Journal of Biological Chemistry, 267*, 18814-18820.

Dzau, V. J. (1987). Molecular studies of human renin structure and synthesis using monoclonal antibodies. *Clinical and Experimental Hypertension.Part A, Theory and Practice, 9*, 1291-1304.

Ecder, T., Edelstein, C. L., Fick-Brosnahan, G. M., Johnson, A. M., Chapman, A. B., Gabow, P. A., & Schrier, R. W. (2001). Diuretics versus angiotensin-converting enzyme inhibitors in autosomal dominant polycystic kidney disease. *American Journal of Nephrology, 21*, 98-103.

Edens, L., Dekker, P., van der Hoeven, R., Deen, F., de Roos, A., & Floris, R. (2005). Extracellular prolyl endoprotease from aspergillus niger and its use in the debittering of protein hydrolysates. *Journal of Agricultural and Food Chemistry, 53*, 7950-7957.

Ehlers, M. R., Fox, E. A., Strydom, D. J., & Riordan, J. F. (1989). Molecular cloning of human testicular angiotensin-converting enzyme: the testis isozyme is identical to the C-terminal half of endothelial angiotensin-converting enzyme. *Proceedings of the National Academy of Sciences of the United States of America, 86*, 7741-7745.

Epand, R. M., & Vogel, H. J. (1999). Diversity of antimicrobial peptides and their mechanisms of action. *Biochemica Et Biophysica Acta, 1462*, 11-28.

Epstein, B. J., Smith, S. M., & Choksi, R. (2009). Recent changes in the landscape of combination RASÂ blockade. *Expert Review of Cardiovascular Therapy, 7*, 1373-1384.

Erba, D., Ciappellano, S., & Testolin, G. (2002). Effect of the ratio of casein phosphopeptides to calcium (w/w) on passive calcium transport in the distal small intestine of rats. *Nutrition (Burbank, Los Angeles County, Calif.), 18*, 743-746.

Esther, C. R., Marino, E. M., Howard, T. E., Machaud, A., Corvol, P., Capecchi, M. R., et al. (1997). The critical role of tissue angiotensin-

converting enzyme as revealed by gene targeting in mice. *The Journal of Clinical Investigation, 99*, 2375-2385.

Falkner, B. (2009). Hypertension in children and adolescents: Epidemiology and natural history. *Pediatric Nephrology, in print.*

Fan, M. Z., Sauer, W. C., & Jaikaran, S. (1994). Amino acid and energy digestibility in peas (*pisum sativum*) from white-flowered spring cultivars for growing pigs. *Journal of the Science of Food and Agriculture, 64*, 249-256.

Fang, X. P., Xia, W. S., Sheng, Q. H., & Wang, Y. L. (2007). Purification and characterization of an immunomodulatory peptide from bovine placenta water-soluble extract. *Preparative Biochemistry & Biotechnology, 37*, 173-184.

Fernandes, J. M. O., Saint, N., Kemp, G. D., & Smith, V. J. (2003). *Oncorhyncin III: A potent antimicrobial peptide derived from the non-histone chromosomal protein H6 of rainbow trout, oncorhynchus mykiss. Biochemical Journal, 373,* 621-628.

Ferreira, I. M. P. L. V. O., Pinho, O., Mota, M. V., Tavares, P., Pereira, A., Gonçalves, M. P., et al. (2007). Preparation of ingredients containing an ACE-inhibitory peptide by tryptic hydrolysis of whey protein concentrates. *International Dairy Journal, 17*, 481-487.

Ferreira, S. H. (1965). A bradykinin-potentiating factor (bpf) present in the venom of bothrops jararca. *British Journal of Pharmacology and Chemotherapy, 24*, 163-169.

Ferreira, S. H., Bartelt, D. C., & Greene, L. J. (1970). Isolation of bradykinin-potentiating peptides from bothrops jararaca venom. *Biochemistry, 9*, 2583-2593.

Fiat, A. M., Migliore-Samour, D., Jolles, P., Drouet, L., Bal dit Sollier, C., & Caen, J. (1993). Biologically active peptides from milk proteins with emphasis on two examples concerning antithrombotic and immunomodulating activities. *Journal of Dairy Science, 76*, 301-310.

Fiat, A., Migliore-Samour, D., Jolles, P., Drouet, L., Sollier, C. B. D., & Caen, J. (1993). Biologically active peptides from milk proteins with emphasis on two examples concerning antithrombotic and immunomodulating activities. *Journal of Dairy Science, 76*, 301-310.

Fidock, M., Miller, M., & Lanfear, J. (2002). Isolation and differential tissue distribution of two human cDNAs encoding PDE1 splice variants. *Cellular Signalling, 14*, 53-60.

FitzGerald, R. J., & O'Cuinn, G. (2006). Enzymatic debittering of food protein hydrolysates. *Biotechnology Advances, 24*, 234-237.

Foltz, M., van Buren, L., Klaffke, W., & Duchateau, G. S. M. J. E. (2009). Modeling of the relationship between dipeptide structure and dipeptide stability, permeability, and ACE inhibitory activity. *Journal of Food Science, 74*, H243-H251.

François Mariotti, Maria E Pueyo, Daniel Tomé, Serge Bérot, Robert Benamouzig, and Sylvain Mahé. (2001). The influence of the albumin fraction on the bioavailability and postprandial utilization of pea protein given selectively to humans. *The Journal of Nutrition, 131*, 1706.

Fredrikson, M., Biot, P., Alminger, M. L., Carlsson, N., & Sandberg, A. (2001). Production process for high-quality pea-protein isolate with low content of oligosaccharides and phytate. *Journal of Agricultural and Food Chemistry, 49*, 1208-1212.

Fujita, H., Yokoyama, K., Yasumoto, R., & Yoshikawa, M. (1995). Antihypertensive effect of thermolysin digest of dried bonito in spontaneously hypertensive rat. *Clinical and Experimental Pharmacology and Physiology, 22*, S304-S305.

Fujita, H., Yamagami, T., & Ohshima, K. (2001). Effects of an ace-inhibitory agent, katsuobushi oligopeptide, in the spontaneously hypertensive rat and in borderline and mildly hypertensive subjects. *Nutrition Research, 21*, 1149-1158.

Fujita, H., Yokoyama, K., & Yoshikawa, M. (2000). Classification and antihypertensive activity of angiotensin I-converting enzyme inhibitory peptides derived from food proteins. *Journal of Food Science, 65*, 564-569.

Fujita, H., & Yoshikawa, M. (1999). LKPNM: A prodrug-type ACE-inhibitory peptide derived from fish protein. *Immunopharmacology, 44*, 123-127.

Fukudome, S., & Yoshikawa, M. (1992). Opioid peptides derived from wheat gluten: Their isolation and characterization. *FEBS Letters, 296*, 107-111.

Fukudome, S., & Yoshikawa, M. (1993). Gluten exorphin C. A novel opioid peptide derived from wheat gluten. *FEBS Letters, 316*, 17-19.

Fukunaga, A., Uematsu, H., & Sugimoto, K. (2005). Influences of aging on taste perception and oral somatic sensation. *J Gerontol A Biol Sci Med Sci, 60*, 109-113.

Gómez-Ruiz, J., López-Expósito, I., Pihlanto, A., Ramos, M., & Recio, I. (2008). Antioxidant activity of ovine casein hydrolysates: Identification of active peptides by HPLC-MS/MS. *European Food Research and Technology, 227*, 1061-1067.

Garcia-Olmedo, F., Molina, A., Alamillo, J. M., & Rodriguez-Palenzuela, P. (1998). Plant defense peptides. *Biopolymers, 47*, 479-491.

Gardner, M. L. (1983). Evidence for, and implications of, passage of intact peptides across the intestinal mucosa. *Biochemical Society Transactions, 11*, 810-813.

Gardner, M. L. (1984). Intestinal assimilation of intact peptides and proteins from the diet--a neglected field? *Biological Reviews of the Cambridge Philosophical Society, 59*, 289-331.

Gardner, M. L. G. (1998). Transmucosal passage of intact peptides. In G. K. Grimble, & F. R. C. Backwell (Eds.), *Peptides in mammalian metabolism: Tissue utilization and clinical targeting.* London, UK: Portland Press Ltd.

Gaucheron, F., Famelart, M. H., & Le Graet, Y. (1996). Iron-supplement caseins: Preparation, physiochemical, characterization and stability. *Journal of Dairy Research, 63*, 233-243.

Gehrke, C., Wall, L., Absheer, J., Kaiser, F., Zumwalt, R. (1985). Sample preparation for chromatography of amino acid: acid hydrolysis of proteins. *Association of Official Analytical Chemists Journal, 68,* 811-821.

Gill, H. S., Doull, F., Rutherfurd, K. J., & Cross, M. L. (2000). Immunoregulatory peptides in bovine milk. *The British Journal of Nutrition, 84* , S111-117.

Golik, A., Modai, D., Averbukh, Z., Sheffy, M., Shamis, A., Cohen, N., et al. (1990). Zinc metabolism in patients treated with captopril versus enalapril. *Metabolism: Clinical and Experimental, 39*, 665-667.

Golik, A., Zaidenstein, R., Dishi, V., Blatt, A., Cohen, N., Cotter, G., et al. (1998). Effects of captopril and enalapril on zinc metabolism in hypertensive patients. *Journal of American College of Nutrition, 17*, 75-78.

Gonçalves, R. L., Lugnier, C., Keravis, T., Lopes, M. J., Fantini, F. A., Schmitt, M., et al. (2009). The flavonoid dioclein is a selective inhibitor of cyclic nucleotide phosphodiesterase type 1 (PDE1) and a cGMP-dependent protein kinase (PKG) vasorelaxant in human vascular tissue. *European Journal of Pharmacology, 620*, 78-83.

Gordon, D. F. J., & Speck, M. L. (1965). Bitter peptide isolated from milk cultures of *streptococcus cremoris. Applied Microbiology, 13*, 537-542.

Grimble, G. K. (2000). Mechanisms of peptide and amino acid transport and their regulation. In P. Fürst, & V. Young (Eds.), *Proteins, peptides, and amino acids in enteral nutrition.* (pp. 63-88). Basel, Switzerland: Nestec Ltd.

Guang, C., & Phillips, R. D. (2009). Purification, activity and sequence of angiotensin I converting enzyme inhibitory peptide from alcalase

hydrolysate of peanut flour. *Journal of Agricultural and Food Chemistry, 57*, 10102-10106.

Gueguen, J., & Barbot, J. (1988). Quantitative and qualitative variability of pea (*pisum sativum* L.) protein composition. *Journal of the Science of Food and Agriculture, 42*, 209-224.

Gueguen, J., & Cerletti, P. (1994). Legume seed proteins. In B. J. F. Hudson (Ed.), *New and developing sources of food proteins.* (pp. 145). London, UK: Chapman and Hall.

Hagemann, D., Bohlender, J., Hoch, B., Krause, E. G., & Karczewski, P. (2001). Expression of Ca2+/calmodulin-dependent protein kinase II delta-subunit isoforms in rats with hypertensive cardiac hypertrophy. *Molecular and Cellular Biochemistry, 220*, 69-76.

Han, Y., Kim, G., Hwang, K., & Han, B. (1993). Binding of sanjoinine-A (frangufoline) to calmodulin. *Archives of Pharmacal Research, 16*, 289-294.

Hansen, R. S., & Beavo, J. A. (1986). Differential recognition of calmodulin-enzyme complexes by a conformation-specific anti-calmodulin monoclonal antibody. *The Journal of Biological Chemistry, 261*, 14636-14645.

Hanson, P. I., & Schulman, H. (1992). Inhibitory autophosphorylation of multifunctional Ca2+/calmodulin-dependent protein kinase analyzed by

site-directed mutagenesis. *The Journal of Biological Chemistry, 267*, 17216-17224.

Hartmann, R., & Meisel, H. (2007). Food-derived peptides with biological activity: From research to food applications. *Current Opinion in Biotechnology, 18*, 163-169.

Hata, Y., Yamamoto, M., Ohni, M., Nakajima, K., Nakamura, Y., & Takano, T. (1996). A placebo-controlled study of the effect of sour milk on blood pressure in hypertensive subjects. *The American Journal of Clinical Nutrition, 64*, 767-771.

Hayes, M., Ross, R. P., Fitzgerald, G. F., Hill, C., & Stanton, C. (2006). Casein-derived antimicrobial peptides generated by *lactobacillus acidophilus* DPC6026. *Applied and Environmental Microbiology, 72*, 2260-2264.

Hazato, T., & Kase, R. (1986). Isolation of angiotensin-converting enzyme inhibitor from porcine plasma. *Biochemical and Biophysical Research Communications, 139*, 52-55.

Henschen, A., Lottspeich, F., Brantl, V., & Teschemacher, H. (1979). Novel opioid peptides derived from casein (beta-casomorphins). II. Structure of active components from bovine casein peptone. *Hoppe-Seyler's Zeitschrift Fur Physiologische Chemie, 360*, 1217-1224.

Hernandez-Ledesma, B., Davalos, A., Bartolome, B., & Amigo, L. (2005). Preparation of antioxidant enzymatic hydrolysates from alpha-lactalbumin and beta-lactoglobulin. identification of active peptides by HPLC-MS/MS. *Journal of Agricultural and Food Chemistry, 53*, 588-593.

Hilliam, M. (2000). Functional food--how big is the market. *The World of Food Ingredients, 12*, 50-52.

Hirai, Y., Permyakov, E. A., & Berliner, L. J. (1992). Proteolytic digestion of alpha-lactalbumin: Physiological implications. *Journal of Protein Chemistry, 11*, 51-57.

Hirata, H., Sonoda, S., Agui, S., Yoshida, M., Ohinata, K., & Yoshikawa, M. (2007). Rubiscolin-6, a [delta] opioid peptide derived from spinach rubisco, has anxiolytic effect via activating [sigma]1 and dopamine D1 receptors. *Peptides, 28*, 1998-2003.

Hiroshi Hara, Ryuhei Funabiki, Mitsuo Iwata, and Ken-Ichi Yamazaki. (1984). Portal absorption of small peptides in rats under unrestrained conditions. *Journal of Nutrition, 114*, 1122.

Hook, S. S., & Means, A. R. (2001). Ca(2+)/CaM-dependent kinases: From activation to function. *Annual Review of Pharmacology and Toxicology, 41*, 471-505.

Hori, G., Wang, M. F., Chan, Y. C., Komatsu, T., Wong, Y., Chen, T. H., et al. (2001). Soy protein hydrolyzate with bound phospholipids reduces serum cholesterol levels in hypercholesterolemic adult male volunteers. *Bioscience, Biotechnology, and Biochemistry, 65*, 72-78.

Horiguchi, N., Horiguchi, H., & Suzuki, Y. (2005). Effect of wheat gluten hydrolysate on the immune system in healthy human subjects. *Bioscience, Biotechnology, and Biochemistry, 69*, 2445-2449.

Huang, C. Y., Chau, V., Chock, P. B., Wang, J. H., & Sharma, R. K. (1981). Mechanism of activation of cyclic nucleotide phosphodiesterase: Requirement of the binding of four Ca^{2+} to calmodulin for activation. *Proceedings of the National Academy of Sciences of the United States of America, 78*, 871-874.

Hudmon, A., & Schulman, H. (2002). Neuronal CA2+/calmodulin-dependent protein kinase II: The role of structure and autoregulation in cellular function. *Annual Review of Biochemistry, 71*, 473-510.

Humiski, L. M., & Aluko, R. E. (2007). Physicochemical and bitterness properties of enzymatic pea protein hydrolysates. *Journal of Food Science, 72*, S605-S611.

Hwang, K., Han, Y., & Han, B. (2001). Inhibition of calmodulin-dependent calcium-ATPase and phosphodiesterase by various cyclopeptides and

peptide alkaloids from the zizyphus species. *Archives of Pharmacal Research, 24*, 202-206.

Ichikawa, K., Yamamoto, T., & Fukumoto, J. (1959). Studies on the bitter taste peptides produced by proteinases.I.the formation og bitter taste peptides by "neutral proteinase" of *bacillus subtilis* and isolation of the peptides. *J Agr Chem Soc Japan, 33*, 1044-1048.

Ichimura, M., Eiki, R., Osawa, K., Nakanishi, S., & Kase, H. (1996). KS-505a, an isoform-selective inhibitor of calmodulin-dependent cyclic nucleotide phosphodiesterase. *The Biochemical Journal, 316 (Pt 1)*, 311-316.

Iroyukifujita, H., Eiichiyokoyama, K., & Yoshikawa, M. (2000). Classification and antihypertensive activity of angiotensin I-converting enzyme inhibitory peptides derived from food proteins. *Journal of Food Science, 65*, 564-569.

Ishibashi, N., Ono, I., Kato, K., Shigenaga, T., Shinoda, I., Okai, H., et al. (1988). Role of the hydrophobic amino acid residue in the bitterness of peptides. *Agriculture and Biological Chemistry, 52*, 91-94.

Jacobsen, P., Andersen, S., Jensen, B. R., & Parving, H. (2003). Additive effect of ACE inhibition and angiotensin II receptor blockade in type I diabetic patients with diabetic nephropathy. *J Am Soc Nephrol, 14*, 992-999.

Jacobsen, P., Rossing, K., & Parving, H. (2004). Single versus dual blockade of the renin-angiotensin system (angiotensin-converting enzyme inhibitors and/or angiotensin II receptor blockers) in diabetic nephropathy. *Current Opinion in Nephrology and Hypertension, 13*, 319-324.

Jauhiainen, T., Vapaatalo, H., Poussa, T., Kyronpalo, S., Rasmussen, M., & Korpela, R. (2005). Lactobacillus helveticus fermented milk lowers blood pressure in hypertensive subjects in 24-h ambulatory blood pressure measurement. *American Journal of Hypertension : Journal of the American Society of Hypertension, 18*, 1600-1605.

Je, J. Y., Qian, Z. J., & Kim, S. K. (2007). Antioxidant peptide isolated from muscle protein of bullfrog, rana catesbeiana shaw. *Journal of Medicinal Food, 10*, 401-407.

Je, J. Y., Qian, Z. J., Lee, S. H., Byun, H. G., & Kim, S. K. (2008). Purification and antioxidant properties of bigeye tuna (thunnus obesus) dark muscle peptide on free radical-mediated oxidative systems. *Journal of Medicinal Food, 11*, 629-637.

Jiang, X., Li, J., Paskind, M., & Epstein, P. M. (1996). Inhibition of calmodulin-dependent phosphodiesterase induces apoptosis in human leukemic cells. *Proceedings of the National Academy of Sciences of the United States of America, 93*, 11236-11241.

Johnston, A. (2002). Filed pea responses to phosphorus fertilization. *News & Views, Potash & Phosphate Institue (PPI) and the Potash & Phosphate Institue of Canada (PPIC).*

Johnston, C. I., Iansek, R., Millar, J. A., McGrath, B. P., & Matthews, P. G. (1981). Vasoactive peptides and hypertension: Role of angiotensin converting enzyme. *Australian and New Zealand Journal of Medicine, 11*, S59-63.

Johnston, C. I., Millar, J. A., McGrath, B. P., & Matthews, P. G. (1979). Long-term effects of captopril (SQ14 225) on blood-pressure and hormone levels in essential hypertension. *Lancet, 2*, 493-496.

Jollès, P., Lévy-toledano, S., Fiat, A., Soria, C., Gillessen, D., Thomaidis, A., et al. (1986). Analogy between fibrinogen and casein. *European Journal of Biochemistry, 158*, 379-382.

Jollès, P., Fiat, A. M., Migliore-Samour, D., Drouet, L., & Caen, J. (1992). Peptides from milk protein implicated in antithrombosis and immunomodulation. In B. Renner, & G. Sawatzki (Eds.), *New perspective in infant nutrition, symposium antwerp.* (pp. 160-172). New York, USA: Thieme Medical Publications.

Jollès, P., & Migliore-Samour, D. (1986). *Preparation of immunological agents by treating lipid free bovine casein with proteolytic enzyme and*

fractionating the product. . Patent Assignee: Rhone-Poulenc Sante. WPI Acc No. 86-037423/06, United States Patent 4 851 509, European Patent 170 550.

Jollès, P., Migliore-Samour, D., & Parker, F. (1988). *Immuno stimulant substances derived from bovine casein and compositions containing the same.* Patent Assignee: Rhone-Poulenc Sante. United States Patent 4 777 234.

Juillerat, M. A., Baechler, R., Berrocal, R., Chanton, S., Scherz, J. C., & Jost, R. (1989). Tryptic phospopeptide from whole casein. 1. preparation and analysis by fast protein liquid chromatography. *Journal of Dairy Research, 56*, 603-611.

Jung, S., Murphy, P. A., & Johnson, L. A. (2005). Physicochemical and functional properties of soy protein substrates modified by low levels of protease hydrolysis. *Journal of Food Science, 70*, C180-C187.

Junot, C., Gonzales, M. E., Ezan, E., Cotton, J., Vazeux, G., Michaud, A., et al. (2001). RXP 407, a selective inhibitor of the N-domain of angiotensin I-converting enzyme, blocks in vivo the degradation of hemoregulatory peptide acetyl-ser-asp-lys-pro with no effect on angiotensin I hydrolysis. *Journal of Pharmacology and Experimental Therapeutics, 297*, 606-611.

Kagawa, K., Matsutaka, H., Fukuhama, C., Fujino, H., & Okuda, H. (1998). Suppressive effect of globin digest on postprandial hyperlipidemia in male volunteers. *The Journal of Nutrition, 128*, 56-60.

Kagawa, K., Matsutaka, H., Fukuhama, C., Watanabe, Y., & Fujino, H. (1996). Globin digest, acidic protease hydrolysate, inhibits dietary hypertriglyceridemia and val-val-tyr-pro, one of its constituents, possesses most superior effect. *Life Sciences, 58*, 1745-1755.

Kakiuchi, S., & Yamazaki, R. (1970). Calcium dependent phosphodiesterase activity and its activating factor (PAF) from brain studies on cyclic 3',5'-nucleotide phosphodiesterase (3). *Biochemical and Biophysical Research Communications, 41*, 1104-1110.

Kakiuchi, S., & Yamazaki, R. (1970). Calcium-dependent phosphodiesterase activity and its activating factor (PAF) from brain studies on cyclic 3':5'-nucleotide phosphodiesterase. *Biochemical and Biophysical Research Communications, 41*, 1104-1110.

Kakiuchi, S., Yamazaki, R., & Nakajima, H. (1970). Properties of a heat-stable phosphodiesterase activating factor isolated from brain extract. *Proceedings of the Japan Academy, 46*, 587-592.

Kakkar, R., Raju, R. V., & Sharma, R. K. (1999). Calmodulin-dependent cyclic nucleotide phosphodiesterase (PDE1). *Cellular and Molecular Life Sciences : CMLS, 55*, 1164-1186.

Kanda, N., & Watanabe, S. (2001). Regulatory roles of adenylate cyclase and cyclic nucleotide phosphodiesterases 1 and 4 in interleukin-13 production by activated human T cells. *Biochemical Pharmacology, 62*, 495-507.

Kataoka, M., Head, J. F., Seaton, B. A., & Engelman, D. M. (1989). Melittin binding causes a large calcium-dependent conformational change in calmodulin. *The Proceedings of the National Academy of Sciences Online (USA), 86*, 6944-6948.

Katayama, K., Jamhari, Mori, T., Kawahara, S., Miake, K., Kodama, Y., et al. (2007). Angiotensin-I converting enzyme inhibitory peptide derived from porcine skeletal muscle myosin and its antihypertensive activity in spontaneously hypertensive rats. *Journal of Food Science, 72*, S702-6.

Kato, H., & Suzuki, T. (1971). Bradykinin-potentiating peptides from the venom of agkistrodon halys blomhoffi. isolation of five bradykinin potentiators and the amino acid sequences of two of them, potentiators B and C. *Biochemistry, 10*, 972-980.

Katoh, T., & Fujisawa, H. (1991). Calmodulin-dependent protein kinase II. kinetic studies on the interaction with substrates and calmodulin. *Biochimica Et Biophysica Acta, 1091*, 205-212.

Kawakami, H., Dosako, S., & Nakajima, I. (1993). Effect of lactoferrin on iron solubility under neutral conditions. *Bioscience Biotechnology and Biochemistry, 57*, 1376-1377.

Kawasaki, T., Jun, C. J., Fukushima, Y., Kegai, K., Seki, E., Osajima, K., et al. (2002). Antihypertensive effect and safety evaluation of vegetable drink with peptides derived from sardine protein hydrolysates on mild hypertensive, high-normal and normal blood pressure subjects. *Fukuoka Igaku Zasshi = Fukuoka Acta Medica, 93*, 208-218.

Kawasaki, T., Seki, E., Osajima, K., Yoshida, M., Asada, K., Matsui, T., et al. (2000). Antihypertensive effect of valyl-tyrosine, a short chain peptide derived from sardine muscle hydrolyzate, on mild hypertensive subjects. *Journal of Human Hypertension, 14*, 519-523.

Kayser, H., & Meisel, H. (1996). Stimulation of human peripheral blood lymphocytes by bioactive peptides derived from bovine milk proteins. *FEBS Letters, 383*, 18-20.

Keravis, T. M., Duemler, B. H., & Wells, J. N. (1986). Calmodulin sensitive phosphodiesterase of porcine cerebral cortex: Kinetic behavior,

calmodulin activation, and stability. *Journal of Cyclic Nucleotide and Protein Phosphorylation Research, 11*, 365-372.

Kim, C. H., Kim, M. R., & Lee, C. H. (1992). Effect of type of enzyme on the bitterness of partial hydrolyzates of soybean protein. *Food Biotechnology, 1*, 79-84.

Kim, D., Rybalkin, S. D., Pi, X., Wang, Y., Zhang, C., Munzel, T., et al. (2001). Upregulation of phosphodiesterase 1A1 expression is associated with the development of nitrate tolerance. *Circulation, 104*, 2338-2343.

Kim, H., & Li-Chan, E. (2006). Quantitative structure-activity relationship study of bitter peptides. *Journal of Agricultural and Food Chemistry, 54*, 10102-10111.

Kim, M., Yukio, K., Kim, K. M., & Lee, C. (2008). Tastes and structures of bitter peptide, asparagine-alanine-leucine-proline-glutamate, and its synthetic analogues. *Journal of Agricultural and Food Chemistry, 56*, 5852-5858.

Kim, S. K., Byun, H. G., Park, P. J., & Shahidi, F. (2001). Angiotensin I converting enzyme inhibitory peptides purified from bovine skin gelatin hydrolysate. *Journal of Agricultural and Food Chemistry, 49*, 2992-2997.

Kim, S. K., Kim, Y. T., Byun, H. G., Nam, K. S., Joo, D. S., & Shahidi, F. (2001). Isolation and characterization of antioxidative peptides from

gelatin hydrolysate of alaska pollack skin. *Journal of Agricultural and Food Chemistry, 49*, 1984-1989.

Kim, S. Y., Je, J. Y., & Kim, S. K. (2007). Purification and characterization of antioxidant peptide from hoki (johnius belengerii) frame protein by gastrointestinal digestion. *The Journal of Nutritional Biochemistry, 18*, 31-38.

Kim, S., Kim, Y., Byun, H., Nam, K., Joo, D., & Shahidi, F. (2001). Isolation and characterization of antioxidative peptides from gelatin hydrolysate of alaska pollack skin. *Journal of Agricultural and Food Chemistry, 49*, 1984-1989.

Kim, Y. K., Yoon, S., Yu, D. Y., Lonnerdal, B., & Chung, B. H. (1999). Novel angiotensin-I-converting enzyme inhibitory peptides derived from recombinant human alpha s1-casein expressed in escherichia coli. *The Journal of Dairy Research, 66*, 431-439.

Kinter, M., & Sherman, M. E. (2000). Tandem mass spectrometyre. In M. Kinter, & M. E. Sherman (Eds.), *Protein sequencing and identification using tandem mass spectrometry.* (pp. 17). New York, USA: John Wiley.

Kirchhof, P., Fabritz, L., Kilic, A., Begrow, F., Breithardt, G., & Kuhn, M. (2004). Ventricular arrhythmias, increased cardiac calmodulin kinase II

expression, and altered repolarization kinetics in ANP receptor deficient mice. *Journal of Molecular and Cellular Cardiology, 36*, 691-700.

Kitts, D. D., & Weiler, K. (2003). Bioactive proteins and peptides from food sources. Applications of bioprocesses used in isolation and recovery. *Current Pharmaceutical Design, 9*, 1309.

Kizawa, K., Naganuma, K., & Murakami, U. (1995). Calmodulin-binding peptides isolated from alpha-casein peptone. *The Journal of Dairy Research, 62*, 587-592.

Kizawa, K., Naganuma, K., & Murakami, U. (1995). Calmodulin-binding peptides isolated from alpha-casein peptone. *The Journal of Dairy Research, 62*, 587-592.

Kleinert, H. D., Rosenberg, S. H., Baker, W. R., Stein, H. H., Klinghofer, V., Barlow, J., et al. (1992). Discovery of a peptide-based renin inhibitor with oral bioavailability and efficacy. *Science (New York, N.Y.), 257*, 1940-1943.

Ko, W., Shih, C., Lai, Y., Chen, J., & Huang, H. (2004). Inhibitory effects of flavonoids on phosphodiesterase isozymes from guinea pig and their structure–activity relationships. *Biochemical Pharmacology, 68*, 2087-2094.

Kohama, Y., Matsumoto, S., Oka, H., Teramoto, T., Okabe, M., & Mimura, T. (1988). Isolation of angiotensin-converting enzyme inhibitor from tuna muscle. *Biochemical and Biophysical Research Communications, 155*, 332-337.

Kohmura, M., Nio, N., & Ariyoshi, Y. (1990). Inhibition of angiotensin-converting enzyme by synthetic peptide fragments of various beta-caseins. *Agricultural and Biological Chemistry, 54*, 1101-1102.

Kokubu, T., Hiwada, K., Sato, Y., Iwata, T., Imamura, Y., Matsueda, R., et al. (1984). Highly potent and specific inhibitors of human renin. *Biochemical and Biophysical Research Communications, 118*, 929-933.

Kokubu, T., Ueda, E., Fujimoto, S., Hiwada, K., Kato, A., Akutsu, H., et al. (1968). Peptide inhibitors of the renin-angiotensin system. *Nature, 217*, 456-457.

Korhonen, H., & Pihlanto, A. (2003). Food-derived bioactive peptides - opportunities for designing future foods. *Current Pharmaceutical Design, 9*, 1297-1308.

Korhonen, H., & Pihlanto, A. (2006). Bioactive peptides: production and functionality. *International Dairy Journal, 16*, 945-960.

Kukman, I. L., Zelenik-Blatnik, M., & Abram, V. (1995). Isolation of low-molecular-mass hydrophobic bitter peptides in soybean protein

hydrolysates by reversed-phase high-performance liquid chromatography. *Journal of Chromatography A, 704*, 113-120.

Kwiatkowski, A. P., & King, M. M. (1989). Autophosphorylation of the type II calmodulin-dependent protein kinase is essential for formation of a proteolytic fragment with catalytic activity. implications for long-term synaptic potentiation. *Biochemistry, 28*, 5380-5385.

Lahl, W. J., & Braun, S. D. (1994). Enzymatic production of protein hydrolysates for food use. *Food Technology, 48*, 68-71.

Landry, J., & Delhaye, S. (1992). Simplified procedure for the determination of tryptophan of foods and feedstuffs from barytic hydrolysis. *Journal of Agriculture and Food Chemistry, 40,* 776-779.

Lee, J., Bae, I. Y., Lee, H. G., & Yang, C. (2006). Tyr-pro-lys, an angiotensin I-converting enzyme inhibitory peptide derived from broccoli (brassica oleracea italica). *Food Chemistry, 99*, 143-148.

Legin, A., Rudnitskaya, A., Clapham, D., Seleznev, B., Lord, K., & Vlasov, Y. (2004). Electronic tongue for pharmaceutical analytics: Quantification of tastes and masking effects. *Analytical and Bioanalytical Chemistry, 380*, 36-45.

LeVine, H.,3rd, Sahyoun, N. E., & Cuatrecasas, P. (1985). Calmodulin binding to the cytoskeletal neuronal calmodulin-dependent protein

kinase is regulated by autophosphorylation. *Proceedings of the National Academy of Sciences of the United States of America, 82*, 287-291.

Lewington, S., Clarke, R., Qizilbash, N., Peto, R., Collins, R., & Prospective Studies Collaboration. (2002). Age-specific relevance of usual blood pressure to vascular mortality: a meta-analysis of individual data for one million adults in 61 prospective studies. *Lancet, 360*, 1903-1913.

Li, G. H., Qu, M. R., Wan, J. Z., & You, J. M. (2007). Antihypertensive effect of rice protein hydrolysate with in vitro angiotensin I-converting enzyme inhibitory activity in spontaneously hypertensive rats. *Asia Pacific Journal of Clinical Nutrition, 16*, S275-280.

Li, G. H., Le, G. W., Liu, H., & Shi, Y. H. (2005). Mung-bean protein hydrolysates obtained with alcalase exhibit angiotensin I-converting enzyme inhibitory activity. *Food Science and Technology International, 11*, 281-287.

Li, G., Wan, J., Le, G., & Shi, Y. (2006). Novel angiotensin I-converting enzyme inhibitory peptides isolated from alcalase hydrolysate of mung bean protein. *Journal of Peptide Science, 12*, 509-514.

Li, H., & Aluko, R. E. (2005). Kinetics of the inhibition of calcium/calmodulin-dependent protein kinase II by pea protein-derived peptides. *The Journal of Nutritional Biochemistry, 16*, 656-662.

Li, H., & Aluko, R. E. (2006). Structural modulation of calmodulin and calmodulin-dependent protein kinase II by pea protein hydrolysates. *International Journal of Food Sciences and Nutrition, 57*, 178-189.

Li, H., & Roux, S. J. (1992). Casein kinase II protein kinase is bound to lamina-matrix and phosphorylates lamin-like protein in isolated pea nuclei. *Proc Natl Acad Sci U S A, 89*, 8434-8438.

Li, H., & Aluko, R. E. (2005). Kinetics of the inhibition of calcium/calmodulin-dependent protein kinase II by pea protein-derived peptides. *The Journal of Nutritional Biochemistry, 16*, 656-662.

Lockett, C. J., Reyes, A. J., Leary, W. P., Alcocer, L., & Olhaberry, J. V. (1983). Zinc, angiotensin I-converting enzyme and hypertension. *South African Medical Journal, 64*, 1022-1024.

Loughney, K., Martins, T. J., Harris, E. A., Sadhu, K., Hicks, J. B., Sonnenburg, W. K., et al. (1996). Isolation and characterization of cDNAs corresponding to two human calcium, calmodulin-regulated, 3',5'-cyclic nucleotide phosphodiesterases. *The Journal of Biological Chemistry, 271*, 796-806.

Loukas, S., Varoucha, D., Zioudrou, C., Streaty, R. A., & Klee, W. A. (1983). Opioid activities and structures of alpha-casein-derived exorphins. *Biochemistry, 22*, 4567-4573.

Lovati, M. R., Manzoni, C., Gianazza, E., Arnoldi, A., Kurowska, E., Carroll, K. K., et al. (2000). Soy protein peptides regulate cholesterol homeostasis in hep G2 cells. *Journal of Nutrition, 130*, 2543-2549.

Lugnier, C., Follenius, A., Gerard, D., & Stoclet, J. C. (1984). Bepridil and flunarizine as calmodulin inhibitors. *European Journal of Pharmacology, 98*, 157-158.

Luma, G. B., & Spiotta, R. T. (2006). Hypertension in children and adolescents. *Ameircan Family Physician, 73*, 1158-1168.

Maeno, M., Yamamoto, N., & Takano, T. (1996). Identification of an antihypertensive peptide from casein hydrolysate produced by a proteinase from lactobacillus helveticus CP790. *Journal of Dairy Science, 79*, 1316-1321.

Marczak, E. D., Usui, H., Fujita, H., Yang, Y., Yokoo, M., Lipkowski, A. W., et al. (2003). New antihypertensive peptides isolated from rapeseed. *Peptides, 24*, 791-798.

Markland, F. S., & Smith, E. L. (1971). Subtilisins: Primary structure, chemical and physical properties. In P. D. Boyers (Ed.), *The enzyme, vol 3.* (pp. 561-608). New York, USA: Academic Press.

Martínez-Luis, S., Pérez-Vásquez, A., & Mata, R. (2007). Natural products with calmodulin inhibitor properties. *Phytochemistry, 68*(14), 1882-1903.

Maruyama, S., Mitachi, H., Awaya, J., Kurono, M., Tomizuka, N., & Suzuki, H. (1987). Angiotensin-I-converting enzyme activity of the C-terminal hexapeptide of as alpha1-casein. *Agricultural and Biological Chemistry, 51*, 2557-2561.

Maruyama, S., Mitachi, H., Tanaka, H., Tomizuka, N., & Suzuki, H. (1987). Studies on the active site and antihypertensive activity of angiotensin I-converting enzyme inhibitors derived from casein. *Agriculture and Biological Chemistry, 51*, 1581-1586.

Maruyama, S., Nakagomi, K., & Tomizuka, N. (1985). Angiotensin-I-converting enzyme inhibitor derived from an enzymatic hydrolysate of casein. II. isolation and bradykinin-potentiating activity on the uterus and ileum of rats. *Agricultural and Biological Chemistry, 49*, 1405-1409.

Masuda, O., Nakamura, Y., & Takano, T. (1996). Antihypertensive peptides are present in aorta after oral administration of sour milk containing these peptides to spontaneously hypertensive rats. *The Journal of Nutrition, 126*, 3063-3068.

Matar, C., LeBlance, J. G., Martin, L., & Perdigon, G. (2003). Biologically active peptides released in fermented milk: Role and functions. In E. R. Farnworth (Ed.), *Handbook of fermented functional foods. functional foods and nutraceuticals series.* (pp. 177-201). Boca Raton, Florida, USA: CRC Press LTD.

Matoba, N., Usui, H., Fujita, H., & Yoshikawa, M. (1999). A novel anti-hypertensive peptide derived from ovalbumin induces nitric oxide-mediated vasorelaxation in an isolated SHR mesenteric artery. *FEBS Letters, 452*, 181-184.

Matoba, T., & Hata, H. (1972). Relationship between bitterness of peptides and their chemical structures. *Agricultural and Biological Chemistry, 36*, 1423-1431.

Matoba, T., Hayashi, R., & Hata, T. (1970). Isolation of bitter peptides from tryptic hydrolysate of casein and their chemical structure. *Agricultural and Biological Chemistry, 34*, 1235-1243.

Matsufuji, H., Matsui, T., Ohshige, S., Kawasaki, T., Osajima, K., & Osajima, Y. (1995). Antihypertensive effects of angiotensin fragments in SHR. *Bioscience, Biotechnology, and Biochemistry, 59*, 1398-1401.

Matsui, T., Imamura, M., Oka, H., Osajima, K., Kimoto, K., Kawasaki, T., et al. (2004). Tissue distribution of antihypertensive dipeptide, val-tyr, after its single oral administration to spontaneously hypertensive rats. *Journal of Peptide Science : An Official Publication of the European Peptide Society, 10*, 535-545.

Matsui, T., Li, C. H., Tanaka, T., Maki, T., Osajima, Y., & Matsumoto, K. (2000). Depressor effect of wheat germ hydrolysate and its novel

angiotensin I-converting enzyme inhibitory peptide, ile-val-tyr, and the metabolism in rat and human plasma. *Biological & Pharmaceutical Bulletin, 23*, 427-431.

Matsui, T., Tamaya, K., Seki, E., Osajima, K., Matsumo, K., & Kawasaki, T. (2002). Absorption of val-tyr with in vitro angiotensin I-converting enzyme inhibitory activity into the circulating blood system of mild hypertensive subjects. *Biological & Pharmaceutical Bulletin, 25*, 1228-1230.

Matsui, T., Tamaya, K., Seki, E., Osajima, K., Matsumoto, K., & Kawasaki, T. (2002). Val-tyr as a natural antihypertensive dipeptide can be absorbed into the human circulatory blood system. *Clinical and Experimental Pharmacology & Physiology, 29*, 204-208.

Matsui, T., Yukiyoshi, A., Doi, S., Sugimoto, H., Yamada, H., & Matsumoto, K. (2002). Gastrointestinal enzyme production of bioactive peptides from royal jelly protein and their antihypertensive ability in SHR. *The Journal of Nutritional Biochemistry, 13*, 80-86.

Matsumura, N., Fujii, M., Takeda, Y., Sugita, K., & Shimizu, T. (1993). Angiotensin I-converting enzyme inhibitory peptides derived from bonito bowels autolysate. *Bioscience, Biotechnology, and Biochemistry, 57*, 695-697.

McCann, K. B., Shiell, B. J., Michalski, W. P., Lee, A., Wan, J., Roginski, H., et al. (2006). Isolation and characterisation of a novel antibacterial peptide from bovine α_{S1}-casein. *International Dairy Journal, 16*, 316-323.

McGrath, B. P., Matthews, P. G., & Johnston, C. I. (1980). Acute changes in blood pressure and vasoactive hormones after captopril in hypertensive patients. *Clinical and Experimental Pharmacology & Physiology, 7*, 487-491.

McMahon, E. G., Yang, P. C., Babler, M. A., Bittner, S. E., Suleymanov, O. D., Cain-Janicki, K. J., et al. (1995). Effects of SC-56525, a potent, orally active renin inhibitor, in salt-depleted and renal hypertensive dogs. *Hypertension, 26*, 95-100.

McMurray, J. J., Östergren, J., Swedberg, K., Granger, C. B., Held, P., Michelson, E. L., et al. (2003). Effects of candesartan in patients with chronic heart failure and reduced left-ventricular systolic function taking angiotensin-converting-enzyme inhibitors: The CHARM-added trial. *The Lancet, 362*, 767-771.

Meisel, H. (1993). In K. D. Schwenke, & R. Mothes (Eds.), *Food proteins: Structure and functionality* (pp. 67-75) New York, USA: VCH Weinheim.

Meisel, H. (1997). Biochemical properties of regulatory peptides derived from milk proteins. *Biopolymers, 43*, 119-128.

Meisel, H., & FitzGerald, R. J. (2003). Biofunctional peptides from milk proteins: mineral binding and cytomodulatory effects. *Current Pharmaceutical Design, 9*, 1289-1295.

Mellander, O. (1950). The physiological importance of the casein phosphopeptide calcium salts. II. Peroral calcium dosage of infants. *Acta Societatis Medicorum Upsaliensis, 55*, 247-255.

Mendis, E., Rajapakse, N., & Kim, S. K. (2005). Antioxidant properties of a radical-scavenging peptide purified from enzymatically prepared fish skin gelatin hydrolysate. *Journal of Agricultural and Food Chemistry, 53*, 581-587.

Mendis, E., Rajapakse, N., Byun, H., & Kim, S. (2005). Investigation of jumbo squid (dosidicus gigas) skin gelatin peptides for their in vitro antioxidant effects. *Life Sciences, 77*, 2166-2178.

Michibata, H., Yanaka, N., Kanoh, Y., Okumura, K., & Omori, K. (2001). Human Ca2+/calmodulin-dependent phosphodiesterase PDE1A: Novel splice variants, their specific expression, genomic organization, and chromosomal localization. *Biochimica Et Biophysica Acta (BBA) - Gene Structure and Expression, 1517*, 278-287.

Migliore-Samour, D., Floc'h, F., & Jolles, P. (1989). Biologically active casein peptides implicated in immunomodulation. *The Journal of Dairy Research, 56*, 357-362.

Migliore-Samour, D., & Jolles, P. (1988). Casein, a prohormone with an immunomodulating role for the newborn? *Experientia, 44*, 188-193.

Miguel, M., & Aleixandre, A. (2006). Antihypertensive peptides derived from egg proteins. *The Journal of Nutrition, 136*, 1457-1460.

Miguel, M., Manso, M., Aleixandre, A., Alonso, M. J., Salaices, M., & Lopez-Fandino, R. (2007). Vascular effects, angiotensin I-converting enzyme (ACE)-inhibitory activity, and antihypertensive properties of peptides derived from egg white. *Journal of Agricultural and Food Chemistry, 55*, 10615-10621.

Miguel, M., Muguerza, B., Sanchez, E., Delgado, M. A., Recio, I., Ramos, M., et al. (2005). Changes in arterial blood pressure in hypertensive rats caused by long-term intake of milk fermented by enterococcus faecalis CECT 5728. *The British Journal of Nutrition, 94*, 36-43.

Mine, Y., Ma, F., & Lauriau, S. (2004). Antimicrobial peptides released by enzymatic hydrolysis of hen egg white lysozyme. *Journal of Agricultural and Food Chemistry, 52*, 1088-1094.

Mito, K., Fujii, M., Kuwahara, M., Matsumura, N., Shimizu, T., Sugano, S., et al. (1996). Antihypertensive effect of angiotensin I-converting enzyme inhibitory peptides derived from hemoglobin. *European Journal of Pharmacology, 304*, 93-98.

Miyoshi, S., Ishikawa, H., Kaneko, T., Fukui, F., Tanaka, H., & Maruyama, S. (1991). Structures and activity of angiotensin-converting enzyme inhibitors in an alpha-zein hydrolysate. *Agricultural and Biological Chemistry, 55*, 1313-1318.

Miyoshi, S., Kaneko, T., Ishikawa, H., Tanaka, H., & Maruyama, S. (1995). Production of bioactive peptides from corn endosperm proteins by some proteases. *Annals of the New York Academy of Sciences, 750*, 429-431.

Mizuno, S., Matsuura, K., Gotou, T., Nishimura, S., Kajimoto, O., Yabune, M., et al. (2005). Antihypertensive effect of casein hydrolysate in a placebo-controlled study in subjects with high-normal blood pressure and mild hypertension. *The British Journal of Nutrition, 94*, 84-91.

Mizuno, S., Nishimura, S., Matsuura, K., Gotou, T., & Yamamoto, N. (2004). Release of short and proline-rich antihypertensive peptides from casein hydrolysate with an aspergillus oryzae protease. *Journal of Dairy Science, 87*, 3183-3188.

Mizushima, S., Ohshige, K., Watanabe, J., Kimura, M., Kadowaki, T., Nakamura, Y., et al. (2004). Randomized controlled trial of sour milk on blood pressure in borderline hypertensive men. *American Journal of Hypertension : Journal of the American Society of Hypertension, 17*, 701-706.

Mori-Takeyama, U., Minatoguchi, S., Murata, I., Fujiwara, H., Ozaki, Y., Ohno, M., et al. (2008). Dual blockade of the renninâ€"angiotensin system versus maximal recommended dose of angiotensin II receptor blockade in chronic glomerulonephritis. *Clinical and Experimental Nephrology, 12*, 33-40.

Moskowitz, D. W. (2002). Is angiotensin I-converting enzyme a "master" disease gene? *Diabetes Technology & Therapeutics, 4*, 683-711.

Motoi, H., & Kodama, T. (2003). Isolation and characterization of angiotensin I-converting enzyme inhibitory peptides from wheat gliadin hydrolysate. *Die Nahrung, 47*, 354-358.

Mullally, M. M., Meisel, H., & FitzGerald, R. J. (1997). Identification of a novel angiotensin-I-converting enzyme inhibitory peptide corresponding to a tryptic fragment of bovine beta-lactoglobulin. *FEBS Letters, 402*, 99-101.

Murakami, M., Tonouchi, H., Takahashi, R., Kitazawa, H., Kawai, Y., Negishi, H., et al. (2004). Structural analysis of a new anti-hypertensive peptide (beta-lactosin B) isolated from a commercial whey product. *Journal of Dairy Science, 87*, 1967-1974.

Murphy, C. (1993). Nutrition and chemosensory perception in the elderly. *Critical Reviews in Food Science and Nutrition, 33*, 3-15.

Murray, B. A., Walsh, D. J., & FitzGerald, R. J. (2004). Modification of the furanacryloyl--phenylalanylglycylglycine assay for determination of angiotensin-I-converting enzyme inhibitory activity. *Journal of Biochemical and Biophysical Methods, 59*, 127-137.

Muthalif, M. M., Karzoun, N. A., Benter, I. F., Gaber, L., Ljuca, F., Uddin, M. R., et al. (2002). Functional significance of activation of calcium/calmodulin-dependent protein kinase II in angiotensin II--induced vascular hyperplasia and hypertension. *Hypertension, 39*, 704-709.

Mutus, B., Karuppiah, N., Sharma, R. K., & MacManus, J. P. (1985). The differential stimulation of brain and heart cyclic-AMP phosphodiesterase by oncomodulin. *Biochemical and Biophysical Research Communications, 131*, 500-506.

Nagai T, Suzuki N, Nagashima T. (2006). Antioxidative activities and angiotensin I-converting enzyme inhibitory activities of enzymatic hydrolysates from commercial kamaboko type samples. *Food Science and Technology International, 12*, 335.

Nagaoka, S., Futamura, Y., Miwa, K., Awano, T., Yamauchi, K., Kanamaru, Y., et al. (2001). Identification of novel hypocholesterolemic peptides derived from bovine milk beta-lactoglobulin. *Biochemical and Biophysical Research Communications, 281*, 11-17.

Nagaoka, S., Miwa, K., Eto, M., Kuzuya, Y., Hori, G., & Yamamoto, K. (1999). Soy protein peptic hydrolysate with bound phospholipids decreases micellar solubility and cholesterol absorption in rats and caco-2 cells. *The Journal of Nutrition, 129*, 1725-1730.

Nakade, K., Kaneko, H., Oka, T., Ahhmed, A. M., Muguruma, M., Numata, M., et al. (2009). A cattle heart protein hydrolysate ameliorates hypercholesterolemia accompanied by suppression of the cholesterol absorption in rats and caco-2 cells. *Bioscience, Biotechnology, and Biochemistry, 73*, 607-612.

Nakamura, Y., Kajimoto, O., Kaneko, K., Aihara, K., Mizutani, J., Ikeda, N., et al. (2004). Effects of the liquid yogurts containing 'lactotripeptide (VPP, IPP)' on high-normal blood pressure. *J Nutr Food, 7*, 123-137.

Nakamura, Y., Masuda, O., & Takano, T. (1996). Decrease of tissue angiotensin I-converting enzyme activity upon feeding sour milk in spontaneously hypertensive rats. *Bioscience, Biotechnology, and Biochemistry, 60*, 488-489.

Nakamura, Y., Yamamoto, N., Sakai, K., Okubo, A., Yamazaki, S., & Takano, T. (1995). Purification and characterization of angiotensin I-converting enzyme inhibitors from sour milk. *Journal of Dairy Science, 78*, 777-783.

Nakamura, Y., Yamamoto, N., Sakai, K., & Takano, T. (1995). Antihypertensive effect of sour milk and peptides isolated from it that are inhibitors to angiotensin I-converting enzyme. *Journal of Dairy Science, 78*, 1253-1257.

Nakamura, Y., Yamamoto, N., Sakai, K., & Takano, T. (1995). Antihypertensive effect of sour milk and peptides isolated from it that are inhibitors to angiotensin I-converting enzyme. *Journal of Dairy Science, 78*, 1253-1257.

Nakano, D., Ogura, K., Miyakoshi, M., Ishii, F., Kawanishi, H., Kurumazuka, D., et al. (2006). Antihypertensive effect of angiotensin I-converting enzyme inhibitory peptides from a sesame protein hydrolysate in spontaneously hypertensive rats. *Bioscience, Biotechnology, and Biochemistry, 70*, 1118-1126.

Nakashima, Y., Arihara, K., Sasaki, A., Mio, H., Ishikawa, S., & Itoh, M. (2002). Antihypertensive activities of peptides derived from porcine skeletal muscle myosin in spontaneously hypertensive rats. *Journal of Food Science, 67*, 434-437.

Natesh, R., Schwager, S. L., Sturrock, E. D., & Acharya, K. R. (2003). Crystal structure of the human angiotensin-converting enzyme-lisinopril complex. *Nature, 421*, 551-554.

Neutal, J. M. (2006). The role of combination therapy in the management of hypertension. *Nephrology Dialysis Transplantation, 21*, 1469-1473.

Ney, K. H. (1971). Prediction of bitterness of peptides from their amino acid composition. *Z Lebensm-Unters Forsch, 147*, 64-68.

Nokin, P., Blondiaux, J. P., Schaeffer, P., Jungbluth, L., & Lugnier, C. (1989). Amiodarone is a potent calmodulin antagonist. *Naunyn-Schmiedeberg's Archives of Pharmacology, 339*, 367-373.

Nurminen, M. L., Sipola, M., Kaarto, H., Pihlanto-Leppala, A., Piilola, K., Korpela, R., et al. (2000). Alpha-lactorphin lowers blood pressure measured by radiotelemetry in normotensive and spontaneously hypertensive rats. *Life Sciences, 66*, 1535-1543.

Okai, H., Miyake, I., Fukui, H., Shigenaga, T., Kanehisa, H., & Oka, S. (1979). Relationship between bitterness and chemical structure of bitter

peptide BPla from casein hydrolysate. *American Chemical Society, 177*, 93.

O'MAHONY, M. (1991). Taste perception, food quality and consumer acceptance. *Journal of Food Quality, 14*, 9-31.

Omoni, A., & Aluko, R. (2006). Mechanism of the inhibition of calmodulin-dependent neuronal nitric oxide synthase by flaxseed protein hydrolysates. *Journal of the American Oil Chemists' Society, 83*, 335-340.

Omori, K., & Kotera, J. (2007). Overview of PDEs and their regulation. *Circulation Research, 100*, 309-327.

Ondetti, M. A., Rubin, B., & Cushman, D. W. (1977). Design of specific inhibitors of angiotensin-converting enzyme: New class of orally active antihypertensive agents. *Science (New York, N.Y.), 196*, 441-444.

Ondetti, M. A., Williams, N. J., Sabo, E. F., Pluscec, J., Weaver, E. R., & Kocy, O. (1971). Angiotensin-converting enzyme inhibitors from the venom of bothrops jararaca. isolation, elucidation of structure, and synthesis. *Biochemistry, 10*, 4033-4039.

O'Neil, K. T., & DeGrado, W. F. (1990). How calmodulin binds its targets: Sequence independent recognition of amphiphilic alpha-helices. *Trends in Biochemical Sciences, 15*, 59-64.

Orallo, F., Ã lvarez, E., Basaran, H., & Lugnier, C. (2004). Comparative study of the vasorelaxant activity, superoxide-scavenging ability and cyclic nucleotide phosphodiesterase-inhibitory effects of hesperetin and hesperidin. *Naunyn-Schmiedeberg's Archives of Pharmacology, 370*, 452-463.

Oren, Z., & Shai, Y. (1996). A class of highly potent antibacterial peptides derived from pardaxin, a pore-forming peptide isolated from moses sole fish *pardachirus marmoratus*. *European Journal of Biochemistry, 237*, 303-310.

Oshima, G., Shimabukuro, H., & Nagasawa, K. (1979). Peptide inhibitors of angiotensin I-converting enzyme in digests of gelatin by bacterial collagenase. *Biochimica Et Biophysica Acta, 566*, 128-137.

Otani, H., & Hata, I. (1995). Inhibition of proliferative responses of mouse spleen lymphocytes and rabbit Peyer's patch cells by bovine milk caseins and their digests. *Journal of Dairy Research, 62*, 339-348.

Pak, V., Koo, M., Kasymova, T., & Kwon, D. (2005). Isolation and identification of peptides from soy 11S-globulin with hypocholesterolemic activity. *Chemistry of Natural Compounds, 41*, 710-714.

Park, C. B., Lee, J. H., Park, I. Y., Kim, M. S., & Kim, S. C. (1997). A novel antimicrobial peptide from the loach, misgurnus anguillicaudatus. *FEBS Letters, 411*, 173-178.

Paroli, E. (1988). Opioid peptides from food (the exorphins). *World Review of Nutrition and Dietetics, 55*, 58-97.

Picconi, B., Gardoni, F., Centonze, D., Mauceri, D., Cenci, M. A., Bernardi, G., et al. (2004). Abnormal Ca^{2+}-calmodulin-dependent protein kinase II function mediates synaptic and motor deficits in experimental parkinsonism. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience, 24*, 5283-5291.

Pihlanto, A., & Korhonen, H. (2003). Bioactive peptides and proteins. In S. Taylor (Ed.), *Advances in food & nutrition research.* (pp. 213). Burlington, MA, USA: Elsevier Inc.

Pihlanto-Leppälä, A., Antila, P., Mäntsälä, P., & Hellman, J. (1994). Opioid peptides produced by in-vitro proteolysis of bovine caseins. *International Dairy Journal, 4*, 291-301.

Pins, J. J., & Keenan, J. M. (2006). Effects of whey peptides on cardiovascular disease risk factors. *Journal of Clinical Hypertension (Greenwich, Conn.), 8*, 775-782.

Pool, J. L., Schmieder, R. E., Azizi, M., Aldigier, J., Januszewicz, A., Zidek, W., et al. (2007). Aliskiren, an orally effective renin inhibitor, provides antihypertensive efficacy alone and in combination with valsartan[ast]. *American Journal of Hypertension : Journal of the American Society of Hypertension, 20*, 11-20.

Potter, S. M., Pertile, J., & Berber-Jimenez, M. D. (1996). Soy protein concentrate and isolated soy protein similarly lower blood serum cholesterol but differently affect thyroid hormones in hamsters. *The Journal of Nutrition, 126*, 2007-2011.

Pripp, A. H., Isaksson, T., Stepaniak, L., Sørhaug, T., & Ardö, Y. (2005). Quantitative structure activity relationship modelling of peptides and proteins as a tool in food science. *Trends in Food Science & Technology, 16*, 484-494.

Qian, Z. J., Je, J. Y., & Kim, S. K. (2007). Antihypertensive effect of angiotensin i converting enzyme-inhibitory peptide from hydrolysates of bigeye tuna dark muscle, thunnus obesus. *Journal of Agricultural and Food Chemistry, 55*, 8398-8403.

Qian, Z. J., Jung, W. K., Byun, H. G., & Kim, S. K. (2008a). Protective effect of an antioxidative peptide purified from gastrointestinal digests of oyster, crassostrea gigas against free radical induced DNA damage. *Bioresource Technology, 99*, 3365-3371.

Qian, Z. J., Jung, W. K., & Kim, S. K. (2008b). Free radical scavenging activity of a novel antioxidative peptide purified from hydrolysate of bullfrog skin, rana catesbeiana shaw. *Bioresource Technology, 99*, 1690-1698.

Quiros, A., Hernandez-Ledesma, B., Ramos, M., Amigo, L., & Recio, I. (2005). Angiotensin-converting enzyme inhibitory activity of peptides derived from caprine kefir. *Journal of Dairy Science, 88*, 3480-3487.

Quiros, A., Ramos, M., Muguerza, B., Delgado, M. A., Martin-Alvarez, P. J., Aleixandre, A., et al. (2006). Determination of the antihypertensive peptide LHLPLP in fermented milk by high-performance liquid chromatography-mass spectrometry. *Journal of Dairy Science, 89*, 4527-4535.

Rajapakse, N., Mendis, E., Byun, H. G., & Kim, S. K. (2005). Purification and in vitro antioxidative effects of giant squid muscle peptides on free radical-mediated oxidative systems. *The Journal of Nutritional Biochemistry, 16*, 562-569.

Rasmussen, S., Leth, A., Ibsen, H., Damkjaer Nielsen, M., Nielsen, F., & Giese, J. (1985). Converting enzyme inhibition in mild and moderate essential hypertension. I. Acute effects on blood pressure, the renin-angiotensin system and blood bradykinin after a single dose of captopril. *Acta Medica Scandinavica, 218*, 435-442.

Rekdal, Ø., Andersen, J., Vorland, L. H., & Svendsen, J. S. (1999). Construction and synthesis of lactoferricin derivatives with enhanced antibacterial activity. *Journal of Peptide Science, 5*, 32-45.

Reynolds, E., Riley, P. F., & Adamson, N. (1994). A selective precipitation purification procedure for multiple phosphoseryl-containing peptides and methods for their identification. *Analytical Biochemistry, 217*, 277-284.

Riordan, J. F. (2003). Angiotensin-I-converting enzyme and its relatives. *Genome Biology, 4*, 225.

Rival, S. G., Boeriu, C. G., & Wichers, H. J. (2001a). Caseins and casein hydrolysates. 2. Antioxidative properties and relevance to lipoxygenase inhibition. *Journal of Agricultural and Food Chemistry, 49*, 295-302.

Rival, S. G., Fornaroli, S., Boeriu, C. G., & Wichers, H. J. (2001b). Caseins and casein hydrolysates. 1. Lipoxygenase inhibitory properties. *Journal of Agricultural and Food Chemistry, 49*, 287-294.

Rostas, J. A., & Dunkley, P. R. (1992). Multiple forms and distribution of calcium/calmodulin-stimulated protein kinase II in brain. *Journal of Neurochemistry, 59*, 1191-1202.

Ruilope, L. M., Aldigier, J. C., Ponticelli, C., OddouStock, P., Botteri, F., Mann, J. F., et al. (2000). Safety of the combination of valsartan and

benazepril in patients with chronic renal disease. *Journal of Hypertension, 18*, 89-95.

Saito, T., Nakamura, T., Kitazawa, H., Kawai, Y., & Itoh, T. (2000). Isolation and structural analysis of antihypertensive peptides that exist naturally in gouda cheese. *Journal of Dairy Science, 83*, 1434-1440.

Saito, Y., Wanezaki, K., Kawato, A., & Imayasu, S. (1994). Structure and activity of angiotensin I converting enzyme inhibitory peptides from sake and sake lees. *Bioscience, Biotechnology, and Biochemistry, 58*, 1767-1771.

Sano, J., Ohki, K., Higuchi, T., Aihara, K., Mizuno, S., Kajimoto, O., et al. (2005). Effect of casein hydrolysate, prepared with protease derived from aspergillus oryzae, on subjects with high-normal blood pressure or mild hypertension. *Journal of Medicinal Food, 8*, 423-430.

Sanoski, C. A. (2009). Aliskiren: An oral direct renin inhibitor for the treatment of hypertension. *Pharmacotherapy, 29*, 193.

Sarma, A. V., Ramana Rao, M. H., Sarma, J. A., Nagaraj, R., Dutta, A. S., & Kunwar, A. C. (2002). NMR study of cyclic peptides with renin inhibitor activity. *Journal of Biochemical and Biophysical Methods, 51*, 27-45.

Sasaki, T., Kotera, J., Yuasa, K., & Omori, K. (2000). Identification of human PDE7B, a cAMP-specific phosphodiesterase, *Biochemical and Biophysical Research Communications, 271*, 575-583.

Satake, M., Enjoh, M., Nakamura, Y., Takano, T., Kawamura, Y., Arai, S., et al. (2002). Transepithelial transport of the bioactive tripeptide, val-pro-pro, in human intestinal caco-2 cell monolayers. *Bioscience, Biotechnology, and Biochemistry, 66*, 378-384.

Sato, M., Hosokawa, T., Yamaguchi, T., Nakano, T., Muramoto, K., Kahara, T., et al. (2002). Angiotensin I-converting enzyme inhibitory peptides derived from wakame (undaria pinnatifida) and their antihypertensive effect in spontaneously hypertensive rats. *Journal of Agricultural and Food Chemistry, 50*, 6245-6252.

Sato, M., Oba, T., Yamaguchi, T., Nakano, T., Kahara, T., Funayama, K., et al. (2002). Antihypertensive effects of hydrolysates of wakame (undaria pinnatifida) and their angiotensin-I-converting enzyme inhibitory activity. *Annals of Nutrition & Metabolism, 46*, 259-267.

Satouchi, K., Mori, T., & Matsushita, S. (1974). Characterization of inhibitor protein for lipase in soybean seeds. *Agricultural and Biological Chemistry, 38*, 97-101.

Savage, G. P., & Deo, S. (1989). The nutritional value of peas (pisum sativum). A literature review. *Nutrition Abstracts & Reviews, 59*, 65-88.

Schiffman, S. S. (1997). Taste and smell losses in normal aging and disease. *The Journal of American Medical Association, 278*, 1357-1362.

Schjoedt, K. J., Jacobsen, P., Rossing, K., Boomsma, F., & Parving, H. (2005). Dual blockade of the renin-angiotensin-aldosterone system in diabetic nephropathy: The role of aldosterone. *Horm Metab Res, 37*, 4-8.

Schlimme, E., & Meisel, H. (1995). Bioactive peptides derived from milk proteins. structural, physiological and analytical aspects. *Die Nahrung, 39*, 1-20.

Schrezenmeir, J., Korhonen, H., Williams, C., Gill, H. S., & Shah, N. (2000). Forward. *The British Journal of Nutrition, 84*, s1.

Schrier, R. W. (2009). Renal volume, renin-angiotensin-aldosterone system, hypertension, and left ventricular hypertrophy in patients with autosomal dominant polycystic kidney disease. *J Am Soc Nephrol, 20*, 1888-1893.

Schrier, R., McFann, K., Johnson, A., Chapman, A., Edelstein, C., Brosnahan, G., et al. (2002). Cardiac and renal effects of standard versus rigorous blood pressure control in autosomal-dominant polycystic kidney disease:

Results of a seven-year prospective randomized study. *J Am Soc Nephrol, 13*, 1733-1739.

Schroeder, H. E. (1982). Quantitative studies on the cotyledonary proteins in the genus *pisum. Journal of the Science of Food and Agriculture, 33*, 623-633.

Segall, L., Covic, A., & Goldsmith, D. J. A. (2007). Direct renin inhibitors: The dawn of a new era, or just a variation on a theme? *Nephrology Dialysis Transplantation, 22*, 2435-2439.

Segura, A., Moreno, M., Madueño, F., Molina, A., & García-Olmedo, F. (1999). Snakin-1, a peptide from potato that is active against plant pathogens. *Molecular Plant-Microbe Interactions, 12*, 16-23.

Sekiya, S., Kobayashi, Y., Kita, E., Imamura, Y., & Toyama, S. (1992). Antihypertensive effects of tryptic hydrolysate of casein on normotensive and hypertensive volunteers. *Journal of Japanese Spciety of Nutrition and Food Science, 45*, 513-517.

Seo, W. H., Lee, H. G., & Baek, H. H. (2008). Evaluation of bitterness in enzymatic hydrolysates of soy protein isolate by taste dilution analysis. *Journal of Food Science, 73*, S41-S46.

Seppo, L., Jauhiainen, T., Poussa, T., & Korpela, R. (2003). A fermented milk high in bioactive peptides has a blood pressure-lowering effect in

hypertensive subjects. *The American Journal of Clinical Nutrition, 77*, 326-330.

Seppo, L., Kerojoki, O., Suomalainen, T., & Korpela, R. (2002). The effect of *lactobacillis helveticus* LBK-16H fermented milk on hypertension – a pilot study on humans. *Milchwissenschaft, 57*, 326-330.

Shai, Y. (1994). Pardaxin: Channel formation by a shark repellant peptide from fish. *Toxicology, 87*, 109-129.

Sharma, R. K. (1991). Phosphorylation and characterization of bovine heart calmodulin-dependent phosphodiesterase. *Biochemistry, 30*, 5963-5968.

Sharma, R. K., & Kalra, J. (1994). Characterization of calmodulin-dependent cyclic nucleotide phosphodiesterase isoenzymes. *The Biochemical Journal, 299*, 97-100.

Sharma, R. K., & Wang, J. H. (1986). Purification and characterization of bovine lung calmodulin-dependent cyclic nucleotide phosphodiesterase. an enzyme containing calmodulin as a subunit. *The Journal of Biological Chemistry, 261*, 14160-14166.

Sharma, R. K., Tan, Y., & Raju, R. V. S. (1997). Calmodulin-dependent cyclic nucleotide phosphodiesterase from bovine eye: High calmodulin affinity isozyme immunologically related to the brain 60-kDa isozyme. *Archives of Biochemistry and Biophysics, 339*, 40-46.

Sheih, I. C., Wu, T., & Fang, T. J. (2009). Antioxidant properties of a new antioxidative peptide from algae protein waste hydrolysate in different oxidation systems. *Bioresource Technology, 100*, 3419-3425.

Shin, Z. I., Yu, R., Park, S. A., Chung, D. K., Ahn, C. W., Nam, H. S., et al. (2001). His-his-leu, an angiotensin I converting enzyme inhibitory peptide derived from korean soybean paste, exerts antihypertensive activity in vivo. *Journal of Agricultural and Food Chemistry, 49*, 3004-3009.

Sibony, M., Gasc, J. M., Soubrier, F., Alhenc-Gelas, F., & Corvol, P. (1993). Gene expression and tissue localization of the two isoforms of angiotensin I converting enzyme. *Hypertension, 21*, 827-835.

Sipola, M., Finckenberg, P., Korpela, R., Vapaatalo, H., & Nurminen, M. L. (2002). Effect of long-term intake of milk products on blood pressure in hypertensive rats. *The Journal of Dairy Research, 69*, 103-111.

Siró, I., Kápolna, E., Kápolna, B., & Lugasi, A. (2008). Functional food. product development, marketing and consumer acceptance—A review. *Appetite, 51*, 456-467.

Sirtori, C. R., Lovati, M. R., Manzoni, C., Castiglioni, S., Duranti, M., Magni, C., et al. (2004). Proteins of white lupin seed, a naturally isoflavone-poor

legume, reduce cholesterolemia in rats and increase LDL receptor activity in HepG2 cells. *The Journal of Nutrition, 134*, 18-23.

Sonnenburg, W. K., Seger, D., Kwak, K. S., Huang, J., Charbonneau, H., & Beavo, J. A. (1995). Identification of inhibitory and calmodulin-binding domains of the PDE1A1 and PDE1A2 calmodulin-stimulated cyclic nucleotide phosphodiesterases. *The Journal of Biological Chemistry, 270*, 30989-31000.

Sosulski, F. W., & McCurdy, A. R. (1987). Functionality of flours, protein fractions and isolates from field peas and beans. *Journal of Food Science, 52,* 1010-1014.

Spellman, D., O'Cuinn, G., & FitzGerald, R.,J. (2005). Physicochemical and sensory characteristics of whey protein hydrolysates generated at different total solids levels. *Journal of Dairy Research, 72*, 138-143.

Strøm, M. B., Stensen, W., Svendsen, J. S., & Rekdal, Ø. (2001). Increased antibacterial activity of 15-residue murine lactoferricin derivatives. *The Journal of Peptide Research, 57* 127-139.

Sturrock, E. D., Natesh, R., van Rooyen, J. M., & Acharya, K. R. (2004). Structure of angiotensin I-converting enzyme. *Cellular and Molecular Life Sciences : CMLS, 61*, 2677-2686.

Suetsuna, K. (1998). Purification and identification of angiotensin I-converting enzyme inhibitors from the red alga porphyra yezoensis. *Journal of Marine Biotechnology, 6*, 163-167.

Suetsuna, K., Maekawa, K., & Chen, J. R. (2004). Antihypertensive effects of undaria pinnatifida (wakame) peptide on blood pressure in spontaneously hypertensive rats. *The Journal of Nutritional Biochemistry, 15*, 267-272.

Suetsuna, K. (1998). Isolation and characterization of angiotensin I-converting enzyme inhibitor dipeptides derived from allium sativum L (garlic). *The Journal of Nutritional Biochemistry, 9*, 415-419.

Suetsuna, K., & Nakano, T. (2000). Identification of an antihypertensive peptide from peptic digest of wakame (undaria pinnatifida). *The Journal of Nutritional Biochemistry, 11*, 450-454.

Suetsuna, K., Ukeda, H., & Ochi, H. (2000). Isolation and characterization of free radical scavenging activities peptides derived from casein. *The Journal of Nutritional Biochemistry, 11*, 128-131.

Svenning, C., & Vegarud, G. E. Dairy foods in health. IDF Nutrition Week. New Zealand. 12.

Takahashi, K. (2001). The linkage between beta1 integrin and the actin cytoskeleton is differentially regulated by tyrosine and serine/threonine

phosphorylation of beta1 integrin in normal and cancerous human breast cells. *BMC Cell Biology, 2*, 23.

Takahashi, M., Moriguchi, S., Yoshikawa, M., & Sasaki, R. (1994). Isolation and characterization of oryzatensin: A novel bioactive peptide with ileum-contracting and immunomodulating activities derived from rice albumin. *Biochemistry and Molecular Biology International, 33*, 1151-1158.

Takai, S., Jin, D., Sakaguchi, M., & Miyazaki, M. (2004). Significant target organs for hypertension and cardiac hypertrophy by angiotensin-converting enzyme inhibitors. *Hypertension Research : Official Journal of the Japanese Society of Hypertension, 27*, 213-219.

Takai, S., Sakonjo, H., & Miyazaki, M. (2001). Beneficial effect of trandolapril on the lifespan of a severe hypertensive model. *Hypertension Research : Official Journal of the Japanese Society of Hypertension, 24*, 559-564.

Tani, F., Lio, K., Chiba, H., & Yoshikawa, M. (1990). Isolation and characterization of opioid antagonist peptides derived from human lactoferrin. *Agricultural and Biological Chemistry, 54*, 1803-1810.

Tani, H., Ohishi, H., & Watanabe, K. (1994). Purification and characterization of proteinaceous inhibitor of lipase from wheat flour. *Journal of Agricultural and Food Chemistry, 42*, 2382-2385.

Tauzin, J., Miclo, L., & Gaillard, J. L. (2002). Angiotensin-I-converting enzyme inhibitory peptides from tryptic hydrolysate of bovine alphaS2-casein. *FEBS Letters, 531*, 369-374.

Teo, T. S., & Wang, J. H. (1973). Mechanism of activation of a cyclic adenosine 3':5'-monophosphate phosphodiesterase from bovine heart by calcium ions. identification of the protein activator as a Ca^{2+} binding protein. *The Journal of Biological Chemistry, 248*, 5950-5955.

Teschemacher, H. (2003). Opioid receptor ligands derived from food proteins. *Current Pharmaceutical Design, 9*, 1331-1344.

Tessier, B., Schweizer, M., Fournier, F., Framboisier, X., Chevalot, I., Vanderesse, R., et al. (2005). Prediction of the amino acid composition of small peptides contained in a plant protein hydrolysate by LC–MS and CE–MS. *Food Research International, 38*, 577-584.

Thaisrivongs, S., Pals, D. T., DuCharme, D. W., Turner, S. R., DeGraaf, G. L., Lawson, J. A., et al. (1991). Renin inhibitory peptides. incorporation of polar, hydrophilic end groups into an active renin inhibitory peptide template and their evaluation in a human renin-infused rat model and in conscious sodium-depleted monkeys. *Journal of Medicinal Chemistry, 34*, 633-642.

Thaisrivongs, S., Pals, D. T., Harris, D. W., Kati, W. M., & Turner, S. R. (1986). Design and synthesis of a potent and specific renin inhibitor with a prolonged duration of action in vivo. *Journal of Medicinal Chemistry, 29*, 2088-2093.

Tokunaga, K. H., Yoshida, C., Suzuki, K. M., Maruyama, H., Futamura, Y., Araki, Y., et al. (2004). Antihypertensive effect of peptides from royal jelly in spontaneously hypertensive rats. *Biological & Pharmaceutical Bulletin, 27*, 189-192.

Tokunaga, K. H., Yoshida, C., Suzuki, K. M., Maruyama, H., Futamura, Y., Araki, Y., et al. (2004). Antihypertensive effect of peptides from royal jelly in spontaneously hypertensive rats. *Biological & Pharmaceutical Bulletin, 27*, 189-192.

Triller, D., Evang, S., Tadrous, M., & Yoo, B. (2008). First renin inhibitor, aliskiren, for the treatment of hypertension. *Pharmacy World & Science, 30*, 741-749.

Tsutsumi, K., Kawauchi, Y., Kondo, Y., Inoue, Y., Koshitani, O., & Kohri, H. (2000). Water extract of defatted rice bran suppresses visceral fat accumulation in rats. *Journal of Agricultural and Food Chemistry, 48*, 1653-1656.

Tuomilehto, J., Lindstrom, J., Hyyrynen, J., Korpela, R., Karhunen, M. L., Mikkola, L., et al. (2004). Effect of ingesting sour milk fermented using lactobacillus helveticus bacteria producing tripeptides on blood pressure in subjects with mild hypertension. *Journal of Human Hypertension, 18*, 795-802.

Uchida, T., Kobayashi, Y., Miyanaga, Y., Toukubo, R., Ikezaki, H., Taniguchi, A., et al. (2001). A new method for evaluating the bitterness of medicines by semi-continuous measurement of adsorption using a taste sensor. *Chemical & Pharmaceutical Bulletin, 49*, 1336-1339.

Udenigwe, C., Lin, Y. S., Hou, W. C., & Aluko, R. E. (2009). Kinetics of inhibition of renin and angiotensin I-converting enzyme by flaxseed protein hydrolysate fractions. *Journal of Functional Foods, 1*, 119-207.

Van den Meiracker, A. H., Admiraal, P. J., Man in't Veld, A. J., Derkx, F. H., Ritsema van Eck, H. J., Mulder, P., et al. (1990). Prolonged blood pressure reduction by orally active renin inhibitor RO42-5892 in essential hypertension. *BMJ, 301*, 205-210.

Van Esch, J. H., Tom, B., Dive, V., Batenberg, W. W., Georgiadis, D., Yiotakis, A., et al. (2005). Selective angiotensin-converting enzyme C-domain inhibition is sufficient to prevent angiotensin I-induced vasoconstriction. *Hypertension, 45*, 120-125.

Vasan, R. S., Larson, M. G., Leip, E. P., Evans, J. C., O'Donnell, C. J., Kannel, W. B., et al. (2001). Impact of high-normal blood pressure on the risk of cardiovascular disease. *The New England Journal of Medicine, 345*, 1291-1297.

Vegarud, G. E., Langsrud, T., & Svenning, C. (2000). Mineral-binding milk proteins and peptides; occurrence, biochemical and technological characteristics. *British Journal of Nutrition, 84*, S91-98.

Vercruysse, L., Van Camp, J., & Smagghe, G. (2005). ACE inhibitory peptides derived from enzymatic hydrolysates of animal muscle protein: a review. *Journal of Agricultural and Food Chemistry, 53*, 8106-8115.

Vermeirssen, V., John Van, C., & Willy, V. (2004) Bioavailability of angiotensin I converting enzyme inhibitory peptides. *British Journal of Nutrition, 92,* 357-366.

Vermeirssn, V. (2003). Release and activity of ACE inhibitory peptides from pea and whey protein: fermentation in vitro digestion and transport. (Ph.D., Ghent University).

Waeber, B., Aschwanden, R., Sadecky, L., & Ferber, P. (2001). Combination of hydrochlorothiazide or benazepril with valsartan in hypertensive patients unresponsive to valsartan alone. *Journal of Hypertension, 19*, 2097-2104.

Wakabayashi, H., Takase, M., & Tomita, M. (2003). Lactoferricin derived from milk protein lactoferrin. *Current Pharmaceutical Design, 9*, 1277-1287.

Wako, Y., Ishikawa, S., & Muramoto, K. (1996). Angiotensin I-converting enzyme inhibitors in autolysates of squid liver and mantle muscle. *Bioscience, Biotechnology, and Biochemistry, 60*, 1353-1355.

Wang, G. T., Chung, C. C., Holzman, T. F., & Krafft, G. A. (1993). A continuous fluorescence assay of renin activity. *Analytical Biochemistry, 210*, 351-359.

Wang, J. H., Teo, T. S., Ho, H. C., & Stevens, F. C. (1975). Bovine heart protein activator of cyclic nucleotide phosphodiesterase. *Advances in Cyclic Nucleotide Research, 5*, 179-194.

Webb, K. E.,Jr. (1990). Intestinal absorption of protein hydrolysis products: A review. *Journal of Animal Science, 68*, 3011-3022.

Wei, L., Clauser, E., Alhenc-Gelas, F., & Corvol, P. (1992). The two homologous domains of human angiotensin I-converting enzyme interact differently with competitive inhibitors. *The Journal of Biological Chemistry, 267*, 13398-13405.

Weiss, B., Prozialeck, W. C., & Wallace, T. L. (1982). Interaction of drugs with calmodulin. Biochemical, pharmacological and clinical implications. *Biochemical Pharmacology, 31*, 2217-2226.

Wergedahl, H., Liaset, B., Gudbrandsen, O. A., Lied, E., Espe, M., Muna, Z., et al. (2004). Fish protein hydrolysate reduces plasma total cholesterol, increases the proportion of HDL cholesterol, and lowers acyl-CoA:Cholesterol acyltransferase activity in liver of zucker rats. *The Journal of Nutrition, 134*, 1320-1327.

Williams, C. L., Phelps, S. H., & Porter, R. A. (1996). Expression of Ca2+/calmodulin-dependent protein kinase types II and IV, and reduced DNA synthesis due to the Ca^{2+}/calmodulin-dependent protein kinase inhibitor KN-62 (1-[N,O-bis(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenyl piperazine) in small cell lung carcinoma. *Biochemical Pharmacology, 51*, 707-715.

Wu, J., & Ding, X. (2001). Hypotensive and physiological effect of angiotensin converting enzyme inhibitory peptides derived from soy protein on spontaneously hypertensive rats. *Journal of Agricultural and Food Chemistry, 49*, 501-506.

Wu, J., & Ding, X. (2002). Characterization of inhibition and stability of soy-protein-derived angiotensin-I-converting enzyme inhibitory peptides. *Food Research International, 35*, 367-375.

Wu, J., & Aluko, R. E. (2007). Quantitative structure-activity relationship study of bitter di- and tri-peptides including relationship with angiotensin I-converting enzyme inhibitory activity. *Journal of Peptide Science, 13*, 63-69.

Wu, J., Aluko, R. E., & Nakai, S. (2006). Structural requirements of angiotensin I-converting enzyme inhibitory peptides: quantitative structure-activity relationship study of di- and tripeptides. *Journal of Agricultural and Food Chemistry, 54*, 732-738.

Wulfson, N. S., Puchkov, V. A., Rozinov, B. V., Denisov, Y. V., Bochkarev, V. N., Shemyakin, M. M., et al. (1965). Mass spectrometric determination of the amino (hydroxy) acid sequence in peptides and depsipeptides. *Tetrahedron Letters, 32*, 2805-2812.

Yamagata, Y., Czernik, A. J., & Greengard, P. (1991). Active catalytic fragment of Ca2+/calmodulin-dependent protein kinase II. purification, characterization, and structural analysis. *The Journal of Biological Chemistry, 266*, 15391-15397.

Yamamoto, N. (1997). Antihypertensive peptides derived from food proteins. *Biopolymers, 43*, 129-134.

Yamamoto, N., Akino, A., & Takano, T. (1994). Antihypertensive effect of the peptides derived from casein by an extracellular proteinase from lactobacillus helveticus CP790. *Journal of Dairy Science, 77*, 917-922.

Yamanoto, N. (1997). Antihypertensive peptides derived from food proteins. *Biopoly, 43*, 129-134.

Yan, C., Zhao, A. Z., Bentley, J. K., & Beavo, J. A. (1996). The calmodulin-dependent phosphodiesterase gene PDE1C encodes several functionally different splice variants in a tissue-specific manner. *The Journal of Biological Chemistry, 271*, 25699-25706.

Yan, C., Zhao, A. Z., Bentley, J. K., Loughney, K., Ferguson, K., & Beavo, J. A. (1995). Molecular cloning and characterization of a calmodulin-dependent phosphodiesterase enriched in olfactory sensory neurons. *Proceedings of the National Academy of Sciences of the United States of America, 92*, 9677-9681.

Yang, C. Y., Dantzig, A. H., & Pidgeon, C. (1999). Intestinal peptide transport systems and oral drug availability. *Pharmaceutical Research, 16*, 1331-1343.

Yang, H. Y. T., Erdos, E. G., & Levive, Y. (1970). A dipeptidyl carboxypeptidase that converts angiotensin I and inactivates bradykinin. *Biochimica Et Biophysica Acta, 214*, 374-376.

Yang, S., Kawamura, Y., & Yoshikawa, M. (2003). Effect of rubiscolin, a [delta] opioid peptide derived from rubisco, on memory consolidation. *Peptides, 24*, 325-328.

Yang, S., Yunden, J., Sonoda, S., Doyama, N., Lipkowski, A. W., Kawamura, Y., et al. (2001). Rubiscolin, a [delta] selective opioid peptide derived from plant rubisco. *FEBS Letters, 509*, 213-217.

Yang, Y., Marczak, E. D., Yokoo, M., Usui, H., & Yoshikawa, M. (2003). Isolation and antihypertensive effect of angiotensin I-converting enzyme (ACE) inhibitory peptides from spinach rubisco. *Journal of Agricultural and Food Chemistry, 51*, 4897-4902.

Yokoyama, K., Chiba, H., & Yoshikawa, M. (1992). Peptide inhibitors for angiotensin I-converting enzyme from thermolysin digest of dried bonito. *Bioscience, Biotechnology, and Biochemistry, 56*, 1541-1545.

Yoshiji, H., Kuriyama, S., & Fukui, H. (2002a). Perindopril: Possible use in cancer therapy. *Anti-Cancer Drugs, 13*, 221-228.

Yoshiji, H., Kuriyama, S., & Fukui, H. (2002b). Angiotensin-I-converting enzyme inhibitors may be an alternative anti-angiogenic strategy in the treatment of liver fibrosis and hepatocellular carcinoma. possible role of vascular endothelial growth factor. *Tumour Biology, 23*, 348-356.

Yoshiji, H., Kuriyama, S., Noguchi, R., & Fukui, H. (2004). Angiotensin-I converting enzyme inhibitors as potential anti-angiogenic agents for cancer therapy. *Current Cancer Drug Targets, 4*, 555-567.

Yoshikawa, M., Fujita, H., Matoba, N., Takenaka, Y., Yamamoto, T., Yamauchi, R., et al. (2000). Bioactive peptides derived from food proteins preventing lifestyle-related diseases. *BioFactors (Oxford, England), 12*, 143-146.

Yoshikawa, M., Takahashi, M., & Yang, S. (2003). Delta opioid peptides derived from plant proteins. *Current Pharmaceutical Design, 9*, 1325-1330.

Yousif, M. H., Benter, I. F., & Akhtar, S. (2003). Inhibition of calcium/calmodulin-dependent protein kinase II normalizes diabetes-induced abnormal vascular reactivity in the rat perfused mesenteric vascular bed. *Autonomic & Autacoid Pharmacology, 23*, 27-33.

Yu, J., Wolda, S. L., Frazier, A. L. B., Florio, V. A., Martins, T. J., Snyder, P. B., et al. (1997). Identification and characterisation of a human calmodulin-stimulated phosphodiesterase PDE1B1. *Cellular Signalling, 9*, 519-529.

Zhong, F., Zhang, X., Ma, J., & Shoemaker, C. F. (2007). Fractionation and identification of a novel hypocholesterolemic peptide derived from soy protein alcalase hydrolysates. *Food Research International, 40*, 756-762.