ISOLATION AND FUNCTIONAL CHARACTERIZATION OF ANGIOTENSIN CONVERTING ENZYME INHIBITORY PEPTIDES FROM SIMULATED IN VIVO DIGESTS OF CASEIN AND SOYBEAN PROTEINS

By

VAHID FARZAMIRAD

A thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfilment of the requirements of the degree of

MASTER OF SCIENCE

Department of Human Nutritional Sciences

University of Manitoba

Winnipeg

August 2006

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

*****

COPYRIGHT PERMISSION

ISOLATION AND FUNCTIONAL CHARACTERIZATION OF ANGIOTENSIN CONVERTING ENZYME INHIBITORY PEPTIDES FROM SIMULATED IN VIVO DIGESTS OF CASEIN AND SOYBEAN PROTEINS

by

VAHID FARZAMIRAD

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirement of the degree

Of

MASTER OF SCIENCE

ABSTRACT

Hypertension is a major risk factor for cardiovascular diseases such as heart failure, stroke, coronary heart disease, and myocardial infarction. One approach in the treatment of hypertension decreases the production of angiotensin II (a potent vasoconstrictor) through inhibition of angiotensin-converting enzyme (ACE), using soybean and casein peptides. Because soybean and casein are not able to exhibit their bioactive effects in the form of parent proteins, they were hydrolyzed using pepsin and pancreatin to mimic the digestion process in the gastrointestinal tract, and the hydrolysates were separated into fractions. The ACE-inhibitory activities of the fractions were determined using an *in vitro* method and the IC_{50} (peptide concentration that reduced ACE activity by 50%) was calculated. The ACE-inhibitory activities of all fractions were dose dependent and at low concentrations were non-competitive. The radical scavenging activities of the peptide fractions were evaluated. Fluorescence spectroscopy showed that soy protein and casein peptides can induce structural changes in ACE. This study shows that soy protein and casein derived peptides have ACE-inhibitory activity.

ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Rotimi Aluko for taking me as his student and for the research opportunity I have had. I particularly appreciate his patience, professional knowledge and the friendly atmosphere that helped me during my work.

My gratitude also goes to my research committee members Dr. Suh Miyoung and Dr Trust Beta for their valuable input in my work.

Many thanks to Huan Li, Dennis Labossiere for always being willing to lend a helping hand and Mary Pelton for editing my thesis.

I also appreciate my family, especially my mother, for all their prayers and encouragement when I needed it most.

And finally I thank my sister for the financial support.

TABLE OF CONTENTS

List of Tables

List of Figures

List of Abbreviations

EH: Enzyme Hydrolysis

FPLC: Fast protein liquid chromatography

GPC: Gel permeation chromatography

GF-FPLC: Gel filtration fast protein liquid chromatography

HPLC: High pressure liquid chromatography

IC_{50}: Concentration of peptides which is able to inhibit 50% activity of ACE

IMAC: Immobilized metal affinity chromatography

IPP: Isoleucine-Proline-Proline

Lb.F: Lactobacillus ferementation

LbH: Lactobacillus helveticus

LbD.B: Lactobacillus delbrueckii bulgaricus

M of F: Method of fractionation

SHRs: Spontaneously hypertensive rats

UFM: Ultra filtration membrane

VPP: Valine- Proline- Proline

1.0 INTRODUCTION

In recent years, many researchers have investigated peptides, especially those peptides extracted from partial enzymatic hydrolysates of food protein, because of the possible health benefits resulting from their biological activities. Health benefits such as opiod activity, antihypertensive activity, antibacterial activity, mineral-binding activity and enhanced intestinal activity have been identified in biological peptides derived from food protein hydrolysate (Ariyoshi, 1993). In our study, we are focusing on antihypertensive and antioxidant activities of soy and casein protein hydrolysates.

One of the most comprehensively studied activities is the inhibition of angiotensin converting enzyme (ACE). Antihypertensive peptides can inhibit ACE activity, and decrease blood pressure in vivo (Yamamoto, Maeno, & Takano 1999). Wu and Ding (2001) reported in vivo hypotensive activity from soy-protein-derived ACE inhibitory peptide powder in spontaneously hypertensive rats (SHR). The antihypertensive effect of tryptic casein hydrolysate has been proven in hypertensive human volunteers by Sekiya, Kobayashi, Kita, and Imamura (1992) and compares favourably with the casein ACE inhibitory hydrolysate whose in vivo activity was determined during an animal feeding experiment (Karaki et al., 1990).

Soy protein and casein have been recognized as antioxidizing agents (Laakso, 1984; Pratt & Birac 1979). Although there are various components of soy proteins and foods that could play the role of antioxidant, studies show that whole soy protein isolate may have similar beneficial effects to isoflavones, beta-conglycinin, fibre, and peptides (Chen, Sugiyama, Abe, Kuruto-Niwa, Nozawa, & Hirota, 2005; Takenada, Annaka, Kimura, Aoki, & Igarashi 2003). Moreover, casein hydrolysate fractions as well as other

milk components have been recognized to decrease oxidative stress, helping in the prevention of cardiovascular diseases and cancer (Laakso, 1984; Steijns & Hooijdonk 2000).

Interest has focused on isolating and identifying purified ACE inhibitory peptides from simulated in vivo digests of casein and soybean proteins. These peptides are inactive within the sequence of the parent protein but can be released during enzymatic digestion or food processing (Korhonen, Pihlanto-Leppälä, Rantamäki, & Tupasela, 1998). The in vitro digestion of plant proteins with the enzymes pepsin and pancreatin may result in the production of bioactive peptides that could be used in the formulation of functional foods and nutraceuticals. Because both enzymes are involved in gastrointestinal digestion in the human, the results obtained will also provide information on the generation of bioactive peptides produced during the physiological digestion of plant or similar proteins.

Metabolic studies in animals suggest that the quality of soy protein is less effective in reducing blood pressure compared to casein protein, but confirmatory studies in humans are lacking. The biological value of soy protein may be considered inferior to that of casein in humans (Luiking, Deutz, Jakel, & Soeters, 2005) but previous works have shown that soy proteins have superior bioactive properties to casein. However, information is lacking on the nature and functional properties of the peptides that are responsible for the in vivo bioactive properties of soy proteins. Therefore, the overall aim of this work is to simulate gastrointestinal digestion of casein and soy proteins such that the bioactive peptides that are produced can be isolated and studied in greater detail. After isolation and identification of the bioactive peptides, it will be possible to develop a

large scale manufacturing process that will produce peptides for the functional foods and nutraceuticals industry.

Objective of Study

Soybean and casein proteins will be hydrolysed using the enzymes pepsin and pancreatin to simulate gastrointestinal tract digestion. The obtained hydrolysates will be fractionated into peptides and freeze-dried. ACE-inhibitory and radical scavenging activities of all fractions will be determined in vitro. Moreover, the conformational changes of ACE structure using obtained fractions will be evaluated.

Hypothesis

Soy protein and casein can be hydrolyzed and fractionized to produce ACE inhibitory peptides that will bind to and induce structural changes in ACE and interfere with ACE activity. The inhibitory peptides will also have antioxidant activity.

2.0 LITERATURE REVIEW

2.1 Angiotensin Converting Enzyme (ACE)

ACE is a metallopeptidase located on the vascular endothelial cells in the brain, lungs, liver, intestine, pancreas, spleen, skeletal muscle, adrenal gland, and placenta (Inagami, 1994). ACE has two roles in blood pressure adjustment: First, it converts angiotensin I to angiotensin II by cleavage of a dipeptide (Histidine–Leucine) from a carboxyl terminal of angiotensin I (decapeptide) thereby producing angiotensin II (octapeptide), a strong vasoconstrictor. For this reason ACE is also called dipeptidyl carboxypeptidase. Angiotensin II is responsible for an increase in blood pressure not only by constricting the blood vessels, but also by releasing the hormone aldosterone from the suprarenal glands, reducing water and sodium excretion from the kidney and causing extra cellular fluid retention (Erdos, 1990). The second role of ACE is to inactivate bradykinin, a potent vasodilator (Schmaier, 2002). Because of these two essential roles of angiotensin II, ACE is a main target in the treatment of conditions such as high blood pressure, heart failure, diabetic nephropathy and type 2 diabetes mellitus.

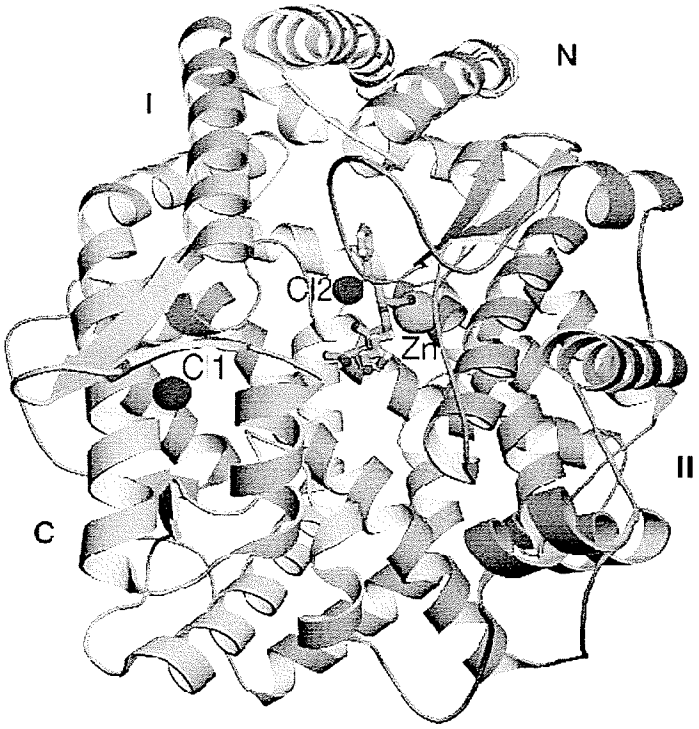
ACE is not only a vasoregulator but also a digestive peptidase in the intestinal tract (Yoshioka, 1987), and is able to hydrolyze other human peptides such as substance P, a potent neuropeptide transmitter, and luliberin, a luteinizing-releasing hormone (Turner & Hooper, 2002). It can act on other substrates such as enkephalins (Melzig & Heder, 1995), neurotensin (Skidgel & Erdos, 2004), and the ß-chain of insulin (Lupi et al., 2006). Because ACE inactivates both bradykinin and substance P, it has a particular role in creating inflammation. Testicular ACE might influence immunity and the reproduction system due to its presence in the reproductive organs (Ehlers & Riordan,

1989). ACE is found in the body in three forms: somatic, testicular (half the size of somatic), and ACE-2. Somatic angiotensin-converting enzyme (ACE) contains two homologous domains (N and C domains) each having a separate active site (Wei, Alhenc-Gelas, Corvol, & Clauser, 1992). Testicular ACE is a single-domain enzyme that is similar to the C-domain of somatic ACE (Corvol, Eyries & Soubrier, 2004). The amino acid sequence of ACE shows a similarity between the two domains, suggesting that the molecule was built from gene duplication (Soubrier et al., 1988). ACE-2 is a recently isolated form of ACE that contains a single active site that is not able to hydrolyze bradykinin. It can only convert angiotensin I to II (Turner et al., 2002).

The three-dimensional structure of ACE is composed of α-helices containing a zinc ion and two chloride atoms. Zinc is localized in the centre of the molecule, and a deep and narrow channel divides the molecule into two parts, shown as parts I and II in Figure 2.1. The role of the chloride atom in ACE has been explained as a stimulator in increasing the activity of both ACE domains with more effect on the C-domain active site compared to the N-domain (Wei, Alhenc-Gelas, Corvol & Calauser, 1991). Therefore, it has been demonstrated that both domains have an independent functional active site that possesses a zinc-dependent dipeptidyl carboxypeptidase activity and finally both domains are sensitive to competitive ACE inhibitors (Wei et al., 1991). In vitro studies show that for complete inhibition of angiotensin I and bradykinin cleavages, inhibitors should block both active sites, however, in vivo experiments indicate that the inhibition of one of the domains (either N or C) of ACE by inhibitors is enough for prevention of angiotensin I to II conversion (Wei et al., 1992).

Figure 2.1

Structure of Angiotensin Converting Enzyme (ACE)



Adapted from Macromolecular Crystallography, Highlights 2003, European Synchrotron Radiation Facility, August 1st, 2006
(http://www.esrf.fr/UsersAndScience/Publications/Highlights/2003/MX/MX10/)

I & II : ACE is composed of two parts
C: C-terminal
N: N-terminal
Zn: Zinc
Cl: Chlorine

2.2 Renin Angiotensin System

ACE plays a crucial role in the regulation of blood pressure (Figure 2.2) and cardiovascular functions (Fleming, 2006) in the renin-angiotensin system (RAS). Within the enzyme cascade of the RAS, ACE converts the inactive decapeptide angiotensin I into the potent vasoconstricting octapeptide called angiotensin II by cleaving a dipeptide from the C-terminus. Angiotensin II, a potent vasoconstritor, is also involved in the release of aldosterone, a sodium-retaining steroid, from the adrenal cortex. Both angiotensin II and aldosterone have a tendency to increase blood pressure.

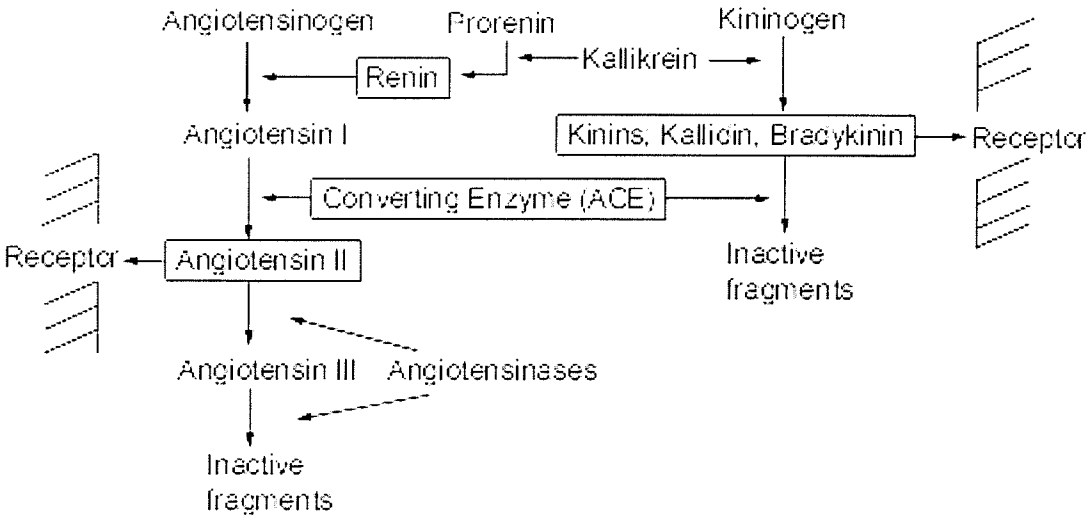
ACE catalyses the degradation of bradykinin, a blood pressure lowering nonapeptide in the kallikrein-kinin system (Johnston and Franz, 1992). In addition, ACE has been shown to degrade neuropeptides including enkephalins, neurotensin, and substance P, which may interact with the cardiovascular system (Wyvratt and Patchett, 1985). Inhibition of ACE is considered to be a useful therapeutic approach in the treatment of hypertension. Therefore, in the development of drugs to control high blood pressure, ACE inhibition has become an important target. A large number of highly potent and specific ACE inhibitors are in the market as orally active drugs that are used in the treatment of hypertension and congestive heart failure (Xie, 1990).

2.3 Angiotensin Converting Enzyme Inhibitory Peptides

Biologically active peptides that can modulate blood pressure have been recognized for a long time. These peptides are obtained from different food sources such as: sake (Yamamoto, 1997); milk (Meisel, 2004); soy protein (Chen, Yang, Suetsana, & Chao, 2004); tuna (Kohama, Matsumoto, Oka, Teramoto, Okabe, & Mimura, 1988);

Figure 2.2

Role of ACE in Blood Pressure Regulation



Adapted from: http://www.scielo.br/img/fbpe/qn/v22n3/1091f1.gif

zein (Miyoshi, et al. 1991); sardine (Seki, Osajima, Matsufuji, Matsui, & Osajima, 1995); salmon (Ono, Hosokawa, Miyashita, & Takahashi, 2003); wheat (Matsui, Li, & Osajima, 1999); porcine muscle (Katayama et al., 2003); and chicken muscle (Fujita, Yokoyama, & Yoshikawa, 2000).

Functional activity of ACE-inhibitory peptides depends on their structural properties. Peptides having aromatic and hydrophobic amino acids such as proline, lysine, and arginine are strong ACE inhibitors (Kitts, & Weiler, 2003). Short-chain peptides, containing two to nine amino acids, show more ACE inhibitory effects and resistance to degradation by gastrointestinal digestive enzymes (Matsufuji, Matsui, Seki,

Osajima, Nakashima, & Osajima, 1994) than long chain peptides. Typical examples include Ile-Pro-Pro (IPP) and Val-Pro-Pro (VPP), which are extracted from sour milk (Hata, Yamamoto, Ohni, Nakajima, Nakamura, & Takano, 1996).

Another key amino acid, which has been demonstrated to inhibit ACE activity, is tryptophan (TRP). Tryptophan, antepenultimate aromatic amino acid, has a key role in enhancing the binding of peptides to ACE, thereby preventing the enzyme from acting on the substrate (Kohama et al., 1988). Hydrolysis is the first step to obtain ACE-inhibitory peptides, and the enzymes usually involved include pepsin, pancreatin, protease, trypsin, chemotrypsin, thermolysin, papain, and alcalase. Compared to the other enzyme digests and parent compounds, a thermolysin hydrolysate of bonito muscle showed the most potent ACE-inhibitory activity (Yokoyama et al., 1992) showing reduction of blood pressure in vivo by about 30 and 50 mm Hg in spontaneously hypertensive rats (SHR) given an intravenous dose of 100 and 30 μg/kg of Leu-Lys-Pro-Asp-Met and Leu-Lys-Pro peptides, respectively. However, oral administration of these two peptides demonstrated an even higher ACE inhibitory activity than the intravenous administration (Fujita, Usui, Kurahashi, & Yoshikawa, 1995a). ACE-inhibitory peptides obtained from α-zein have also shown strong activity. Three peptides including Leu-Arg-Pro, Leu-Ser-Pro, and Leu-Gln-Pro, which contain a common C-terminal proline, showed the lowest IC_{50} in vitro (that is, the concentration of peptide that can inhibit 50% of enzyme activity) and reduced blood pressure in spontaneously hypertensive rats (SHR) 6 hours after oral administration (Matsumura, Fujii, Takeda, Sugita, & Shimizu, 1993).

Recently, two peptides derived from egg white protein have been found to have blood pressure reducing factors. Ovokinin is a vasodilator octapeptide with an amino acid

sequence of Phe-Arg-Ala-Asp-His-Pro-Phe-Leu (FRABHPFL) and acts similarly to bradykinin (Fujita et al., 1995a). Ovokinin showed antihypertensive effects when administered in high doses to SHR and was especially efficient when administered orally in the form of an emulsion in egg yolk. Fujita, Sasaki, and Yoshikawa (1995b) hypothesize that the phospholipids in the egg yolk have a protective effect against intestinal peptidases and increase peptide absorption in the intestine. The second antihypertensive peptide derived from egg white protein, a hexapeptide fraction of the first peptide, called ovokinin "2-7" has the sequence of Arg-Ala-Asp-His-Pro-Phe (RABHPF). Both peptides were isolated from enzymatic hydrolysis of ovalbumin and had vasodilatory activity, but ovokinin "2-7" was 10 times more active than the first one. Further, recent studies show that pepsin hydrolysates of egg white proteins can inhibit ACE and exhibit antihypertensive activity in SHR (Miguel & Aleixandre, 2006).

Chicken breast muscle is another source of ACE-inhibitory peptides (Saiga et al., 2003). SHRs were administered an oral extract prepared from chicken breast muscle, and their blood pressure decreased by 50 mmHg. Breast muscle was boiled in water, pH 4.5, and then hydrolyzed by Aspergillus protease. The peptides possessing hypotensive activity in the chicken extract were examined by measuring the inhibitory activity (IC_{50}) against ACE. Recently, it has been demonstrated that the long-term intake of a hydrolysate of egg white with pepsin, with a potent ACE-inhibitory activity, lowers blood pressure of SHR (Miguel et al., 2006).

Sunflower (*Helianthus annuus*) protein hydrolysates were also studied for ACE-inhibitory peptides (Megias et al., 2005). An ACE-inhibitory peptide with the sequence Phe-Val-Asn-Pro-Gln-Ala-Gly-Ser was extracted by gel filtration chromatography and

high-performance liquid chromatography, following 3 hours incubation with pepsin and 3 hours with pancreatin. These results showed that sunflower seed proteins were a potential source of ACE-inhibitory peptides when hydrolyzed with pepsin and pancreatin.

2.4 Milk

Cow's milk has about 3.5% protein. The milk proteins, casein (casokinins, 80% of milk proteins) and whey (lactokinins), are precursors of many different biologically active peptides that have been shown to have ACE-inhibitory activity (Yalcin, 2006). In fact, peptides extracted from milk proteins are potential modulators of various regulatory processes in the body (Meisel, 2005). They could act as immune system stimulators (Migliore-Samour, Floc`h, & Joll`es, 1989), cytomodulators, having cancer cell growth inhibitory activity (Kayser & Meisel, 1996), and antimicrobial agents (Otani, Kihara, & Park, 2000).

Casein peptides can act as anti-thrombotics by inhibiting fibrinogen binding to the platelet surface and preventing the aggregation of platelets. Maruyama & Suzuki (1982), in the first study on ACE-inhibitory peptides, reported a peptide with IC_{50} of 77 μM by using trypsin hydrolysis. Other researchers have discovered new active peptides in casein and whey with different amino acid sequences (Meisel, Sawatski & Renner, 1993; Takano, 1998; FitzGerald, & Meisel, 1999). Until recently, two fractions from casein and whey proteins with sequence of Val-Ala-Pro and Ala-Leu-Lys-Ala-Trp-Ser-Val-Ala-Arg have been recognized with the lowest IC_{50} values of 2 and 3 mmol/l, respectively (Maruyama, Mitachi, Tanaka, Tomizuka, & Suzuki. 1987; Chiba, & Yoshikawa, 1991). IPP, which is resistant to digestive system enzymes, is another strong and well-known

peptide with IC_{50} of 5 μmol/l of casein (Nakamura, Yamamoto, Sakai, & Takano, 1995b).

Various structural functional studies have discovered that binding peptide inhibitors to ACE are influenced by the C-terminal tripeptide sequence of the substrate (FitzGerald et al., 1999). Moreover, ACE appears to bind effectively with those inhibitory peptides containing hydrophobic (aromatic or branched side-chains) amino acid residues at each of the three C-terminal positions. Cheung, Feng-Lai, Ondetti, Sabo, and Cushman (1980) showed that tryptophan, tyrosine, phenylalanine, and proline can increase ACE binding affinity when they are located at C-terminal. Because some ACE inhibitors have lysine or arginine in C-terminal, Cheung et al. (1980) hypothesized that positively charged side-chain groups of these amino acids are responsible for their ACE-inhibitory effects (Ariyoshi, 1993; Ondetti, Rubin, Cushman, 1977). FitzGerald, and Meisel. (2000) suggested that long-chain side chains of amino acids could also account for the ACE-inhibitory effect of peptides.

2.4.1 Production and Isolation of ACE Inhibitory Peptides from Casein

Casein peptides are produced mainly by enzyme hydrolysis with pepsin, trypsin, and chymotrypsin. Tauzin (2002) separated a tryptic hydrolysate of casein by high-pressure liquid chromatography (HPLC) and obtained 40 fractions with IC_{50} values in the range of 4 to 214 μM. In addition to enzyme hydrolysis, fermentation through incubation with bacterial and plant proteinases is another useful method to obtain these peptides (Abubakar, Tadao, Kitazawa, Kawai, & Itoh, 1998). In fact, during manufacture of fermented dairy products such as cheese and yogurt, many active peptides having ACE-

inhibitory ability are produced (Gobbetti, F,erranti, Smacchi, Goffredi, & Addeo, 2000). Gobbetti (2000) obtained a peptide with IC_{50} value of 173 μM (179 mg/L) after fermenting and separating with *Lactobacillus delbrueckii bulgaricus* and fast protein liquid chromatography (FPLC), respectively. Moreover, *Lactobacillus lactis* and *Lactobacillus helveticus* CP790 are two well-known lactic acid bacteria that produce proteinases with the ability to release potent ACE-inhibitory peptides in vitro (Maeno, Yamamoto, & Takano, 1996). *Lactobacillus helveticus* strains are capable of releasing ACE-inhibitory peptides into fermented milk drinks or yoghurt-type products (Yamamoto et al., 1999). It should be mentioned that the potent casokinins IPP and VPP are found in skim milk fermented with *Lactobacillus helveticus* CP790 and *Saccharomyces cerevisiae* (Yamamoto et al., 1999).

In another study, IPP and VPP with IC_{50} values of 5 and 9 μM were extracted from HPLC after fermentation with *Lactobacillus helveticus* (Nakamura, Yamamoto, Sakai, Okubo, Yamazaki, & Takano. 1995a) .Two further studies, one by Minervini, Algaron, Rizzello, Fox, & Monnet (2003) and another by Robert (2004) introduced ACE inhibitory peptides from casein fermented by *Lactobacillus helveticus*. Minervini et al. identified ACE inhibitory peptides with IC_{50} values of 16 to 100 μM after FPLC separation; Robert separated the fermented product with GPC and HPLC and obtained 14 fractions with IC_{50} values of 15 to 55 μM. In addition, ACE-inhibitory peptides can be obtained during cheese making and from yoghurt-type products (Stepaniak, Jedrychowski, Wroblewska, & Sorhaug, 2001). It seems that cheese ripening is an important method for releasing ACE-inhibitory peptides from milk; however, extension of ripening times can result in the degradation of ACE-inhibitory peptides. Therefore, the

essential strategy for the isolation and production of these peptides is to obtain them from in vitro digests of milk proteins or from fermented dairy products. Synthetic peptides can be used to confirm in vitro tests for primary structure and for the determination of IC_{50} values (FitzGerald et al., 2000).

In vitro studies of ACE-inhibitory effects of lactokinin and casokinin, showed that in vivo administration of these peptides can act as a hypotensive agent. However, in order to get a hypotensive effect in vivo, the peptide must reach the target organ. Therefore, oral administration of milk protein hydrolysates or fermented dairy proteins containing ACE-inhibitory peptides reduces peptide stability against the digestive system. Orally administered peptides should survive during digestion in the intestine where they may be susceptible to brush border and intracellular peptidase activities, and resistant to serum peptidase degradation (FitzGerald & Meisel, 2003). Many in vitro studies have been performed to determine the ability of different ACE-inhibitory peptides to survive gastrointestinal passage and transport through intestinal cells. Two fractions of α_{s1}-casein including f23-27 and f104–109, which had potent ACE inhibitory activity in vitro (Maruyama et al., 1987) were subsequently shown to have no hypotensive effects in vivo. This was presumably due to the degradation into inactive fragments during oral ingestion.

An interesting in vitro experiment was conducted by Vermeirssen, et al. (2002) using the lactokinin, ß-lactoglobulin f142–148. They showed that this peptide could pass through a Caco2-BBe (enterocyte) cell monolayer. However, the concentrations transported were reported to be too low to exert any ACE-inhibitory effect in vitro. Although in vitro model systems that are designed for evaluation of stability and

susceptibility of peptides to intracellular passage can bring much helpful information, only in vivo studies are able to describe the hypotensive effects of a specific peptide.

2.4.2 In Vivo Animal Studies

Numerous rat studies have been done regarding the hypotensive effects of milk proteins. Some focused on casokinin and lactokinin peptides and others worked on fermented sour milk. The strongest hypotensive peptide with 34 mm Hg systolic blood pressure lowering effect is α_{s1}-casein f(23–34) with sequence of Phe-Phe-Val-Ala-Pro-Phe-Pro-Glu-Val-Phe-Gly-Arg (Karaki et al., 1990). Although this fraction did not have the lowest in vitro IC_{50}, 77 μM, it was the most effective peptide in decreasing the blood pressure in rats. In addition, a whey protein derived (lactokinin) tripeptide with sequence of Ile-Pro-Arg decreased systolic blood pressure 31 mm Hg with IC_{50} of 141 μM (Abubakar et al., 1998). Therefore, it seems that there is no relationship between the in vitro ACE-inhibitory activities and in vivo antihypertensive effect of peptides. Although some peptides can be degraded by digestive enzymes, resulting in loss of their effectiveness, other peptides are converted by the digestive enzymes into new peptides with more hypotensive effects (Maeno et al., 1996).

An interesting study showed the presence of ACE-inhibitory peptides Ile-Pro-Pro and Val-Pro-Pro in the aorta of rats after oral administration of sour milk containing these peptides (Masuda, Nakamura, & Takano 1996), after it was previously demonstrated that Ile-Pro-Pro and Val-Pro-Pro showed antihypertensive activity effects in SHR (Nakamura et al., 1995). Nakamura also demonstrated the hypotensive effects of Ile-Pro-Pro and Val-Pro-Pro in SHRs. They orally administered 0.3-0.6 mg/kg Ile-Pro-Pro and Val-Pro-Pro to

rats and showed 20 mmHg reductions in blood pressure after 6-8 hours. As mentioned, some researchers have obtained ACE-inhibitory peptides from fermented milk products by various bacteria proteases and have applied these peptides to rats. These studies generally measure rat blood pressure by tail-cuff method and administered the peptides for a short time. Sipola, Finckenberg, Korpela, Vapaatalo, & Nurminen (2002) conducted a long-term study for 14 weeks, comparing the blood pressure of rats after they were given fermented sour milk containing Ile-Pro-Pro and Val-Pro-Pro or skim milk. The result was a significant decrease in blood pressure by about 21 mm Hg.

2.4.3 In Vivo Human Studies

Although few human studies have been conducted regarding ACE-inhibitory activity of milk peptides, the results demonstrate the antihypertensive ability of casein peptides. One of the first studies, done by Sekiya et al. (1996), indicated that tryptic hydrolysate of casein (20 g/d) can reduce systolic and diastolic blood pressure in human subjects by about 6.6 and 4.6 mm Hg, respectively when administered orally for 4 weeks. A similar experiment showed that smaller amounts of a specific peptide can have the same effect as the tryptic hydrolysate in less than 2 weeks. Nimmagudda (2002) administered orally an extracted peptide of casein (less than 0.2 g/kg) containing 12 amino acids with sequence of Phe-Phe-Val-Ala-Pro-Phe-Glu-Val-Phe-Gly-Lys to human volunteers and obtained 4.5 – 6.5 mm Hg reduction in blood pressure.

Hata et al. (1996) gave 95 ml/d fermented sour milk to hypertensive individuals for 8 weeks in a double blind study and observed systolic blood pressure reductions of 9.4 and 14 mm Hg after 4 and 8 weeks, respectively. Previous studies have been done in

definitely hypertensive individuals (Systolic Blood Pressure: 158 ± 11 mm Hg) but a new study showed that people with borderline blood pressure (Systolic Blood Pressure: 147±9 mm Hg) can benefit from consumption of fermented sour milk (Mizushima et al., 2004). A randomized, single blinded, placebo-controlled study of 8 weeks duration using 131 individuals with high normal to mild hypertension was carried out by Jauhiainen et al. (2005). They administered two tablets containing 0, 1.8, 2.5 or 3.6mg of IPP and VPP at breakfast to the individuals. The group receiving 1.8 mg of VPP and IPP showed a significant decrease in SBP, 5.8 mm Hg, ($P<0.01$) compared to the value at week 0. Significant decreases were also observed in the groups receiving 2.5 mg and 3.6mg after 8 weeks, 6.2 and 9.3 mm Hg, ($P<0.001$ and $P<0.0001$ respectively) compared to the value at the first week.

Table 2.4.1 Casein ACE Inhibitory Activities

In Vitro studies

EH or LbF	M of F	IC_{50} (μM)	Reference
Trypsin	HPLC	4-214	Tauzin et al. (2002)
LbH	GPC & HPLC	15-55	Robert et al. (2004)
LbH	FPLC	16-100	Minervini* et al. (2003)
LbDB	FPLC	173	Gobbetti et al. (2000)
LbH	HPLC	5-9	Nakamura et al. (1995a)

* In this study, Minervini et al. compared the ACE inhibitory activity of peptides obtained from pig, cow, sheep, goat, and human milk. The most potent ACE inhibitory activity of these peptides belonged to a bovine fraction with IC_{50} value of 16 μM.

In Vivo studies

Sample	Product	Dosage	Blood pressure reduction	Reference
SHRs	IPP & VPP	0.6-0.3 mg/kg	20 mmHg 6-8 hrs	Nakamura et al. (1995b)
Humans	Sour milk	95 ml for 8wks	SBP-DBP (14-7 mmHg) after 8 wks	Hata et al. (1996)
Humans	IPP & VPP	22.5-30 mg/d	SBP (5.8 mmHg) for 10 wks	Jauhiainen (2005)

ACE: Angiotensin converting enzyme

AEC: Anion exchange chromatography

EH: Enzyme hydrolysis

FPLC: Fast protein liquid chromatography

GPC: Gel permeation chromatography

GF-FPLC: Gel filtration fast protein liquid chromatography

HPLC: High pressure liquid chromatography

IC_{50}: Concentration of peptides which is able to inhibit 50% activity of ACE

IMAC: Immobilized metal affinity chromatography

IPP: Isoleucine-Proline-Proline

Lb.F: Lactobacillus ferementation

LbH: Lactobacillus helveticus

LbD.B: Lactobacillus delbrueckii bulgaricus

M of F: Method of fractionation

SHRs: Spontaneously hypertensive rats

UFM: Ultra filtration membrane

VPP: Valine- Proline- Proline

SBP: Systolic blood pressure

DBP: Diastolic blood pressure

2.5 Soy Protein

The soybean plant, called Glycine max, originated in China and was imported to America in 1765 (Hymowitz, and Harlan, 1983). Epidemiological studies have indicated that the high intakes of foods containing soy protein in Asian countries (specifically Japan and Taiwan) are significantly correlated with reduced incidence of heart disease, osteoporosis, and some forms of cancer.

In October 1999, the FDA claimed that diets with low saturated fat and cholesterol and containing 25 grams of soy protein may reduce the risk of heart disease. Aside from the health benefits of soy, it should be noted that consumption of large amounts of raw soybean increases the risk of some complications such as bladder cancer (Sun, Yuan, Arakawa, Low, Lee, & Yu, 2002), thyroid disorders (Divi, Chang, & Doerge, 1997), breast cell hyperplasia (McMichael-Phillips et al., 1998), and dementia (White et al., 1996).

There is a broad spectrum of bioactive components in soybean that can be divided into two main groups; proteins and phytochemicals. Each group has been recognized for particular health benefits such as antihypertensive and antioxidant effects. Most of the protein of soybean is found in the seed and root of the plant. Nitrogen fixation by root nodule bacteria in soybean plants is the ultimate source of soy proteins (Burris & Roberts, 1993). The concentration of protein in the soybean seed is 5-6 times greater in the hypocotyls (outer layer) than in the cotyledon (inner part).

Previous studies have shown that some soy protein-derived peptides are able to inhibit ACE in vitro and decrease blood pressure during rat studies (Wu & Ding, 2001). Isolation and extraction of soy peptides is from two sources; one is fermented soy foods

like natto (Okamoto, Hanagata, Kawamura, & Yanagida, 1995), and soy paste (Shin et al., 1995); and second is soy protein isolate (SPI) hydrolysis (Ahn, Kim, Yu, Noh, & Suh, 2000) with one or two enzymes like pepsin, pancreatin, trypsin, alcalase, papain, or *Bacillus subtilis* protease. Then various kinds of chromatography, for example, gel chromatography, high-pressure liquid chromatography (HPLC), or fast protein liquid chromatography (FPLC), are used to separate the peptides obtained from enzyme hydrolysis.

One of the latest studies on soy protein has determined an IC_{50} value of 0.28 mg/ml for soy protein hydrolysate after digestion with pepsin and pancreatin (Lo et al., 2005). However, Wu et al. (2002) obtained an alkaline hydrolysate of SPI with IC_{50} value of 0.065 mg/mL. They collected two purified peptide fractions after chromatography with amino acid sequences Asp-Leu-Pro and Asp-Gly that exhibited IC_{50} values of 4.8 and 12.3 μM, respectively. As seen in the Wu et al. (2002) study and one by Chen et al. (2003), which obtained four peptide fractions with sequences of Ile-Ala (IC_{50}, 153 μM), Tyr-Leu-Ala-Gly-Asn-Gln (IC_{50}, 14 μM), Phe-Phe-Leu (IC_{50}, 37 μM), and Ile-Tyr-Leu-Leu (IC_{50}, 42 μM), leucine exists in most of the fractions, sometimes at the C-terminal position. IC_{50} values are different in the Lo et al. (2005) and Wu et al. (2002) studies, however, they used different enzymes for hydrolysis such as alcalase, pepsin, and pancreatin. Fermented soy foods have also been shown to contain ACE-inhibitory peptides. Natto, a traditional Japanese fermented food made from boiled soybeans, has been discovered to have ACE-inhibitory peptides that is more potent than the bean extract (Okamoto et al., 1995). Shin et al. (2001) isolated a tripeptide HHL from soy paste with IC_{50} value of 2.2 μM that was injected into rats to evaluate hypotensive

effects. Triple injections of soy protein HHL at 5 mg/kg of body weight/injection resulted in a significant decrease of SBP by 61 mmHg ($p < 0.01$) after the third injection.

2.5.1 In Vivo Rat Studies

Wu et al. (2001) orally (by gavage) administered soy peptides to SHR in 100, 500, and 1000 mg/kg of body weight/day for one month and observed a significant blood pressure reduction up to 38 mm Hg in the SHR, while normotensive rats did not show any blood pressure changes. A study by Chen et al. (2004) compared the hypotensive effects of soy protein isolate and casein hydrolysate with IC_{50} values of 0.73 and 0.82 mg/ml, respectively among SHRs. The systolic blood pressure of the soybean protein hydrolysate-supplemented group was significantly lower than that of the casein hydrolysate-supplemented group during the study period. Another study by Martin, Williams, Breitkopf &, Eyster (2002) showed that the mean arterial pressure (MAP) of soy-fed SHRs (144 mm Hg) was significantly less when compared with the value obtained in casein-fed SHRs (165 mm Hg). This finding indicates that the ability of soy protein in reducing the blood pressure is more than that of casein.

2.5.2 In Vivo Human Studies

Until recently, no specific human studies have been carried out to evaluate the effects of soy protein isolate in hypertensive individuals. However, some research shows that soy foods lower blood pressure in humans. In a double-blind study using 40 men and women, Rivas, Garay, Escanero, Cia, and Alda (2002) compared the effects of soymilk with cow's milk in reducing blood pressure. The primary blood pressure for soymilk and cow`s milk was 155 ± 12.5 and 151.7 ± 13.8 mm Hg for systolic BP; and 100.3 ± 8.8 and 99.2 ± 5.4 mm Hg for diastolic BP, respectively. The results indicated that 3 months'

consumption of soy milk (500 ml twice per day) decreased systolic blood pressure to 18.4 ± 10.7 mm Hg when compared to 1.4 ± 7.2 mm Hg in the cow's milk group; and diastolic blood pressure to 15.9 ± 9.8 mm Hg vs. 3.7 ± 5.0 mm Hg in the cow's milk group.

A long-term study (Yang et al., 2005), designed to show the relation between soy intake and blood pressure among 45,694 normotensive women aged 40–70 years having no history of diabetes or cardiovascular disease at recruitment, indicates that soy protein intake (more than 25 g/d) was inversely associated with systolic and diastolic blood pressure. This inverse association was stronger among the elderly (older than 60 years).

Table 2.5.1 Soy ACE Inhibitory Activities

In Vitro studies

EH	M of F	Product	IC_{50}	Reference
Alcalase	HPLC	Ase-Leu-Pro Ase-Gly	4.8 μM 12.3 μM	Wu et al. (2001)
Fermented soy	HPLC	His-His-Leu	2.2 μg/ml	Shin et al. (2001)
Alcalase* Flavourzyme Trypsin Chymotrypsin Pepsin	UFM**	———	0.668-0.078 mg/ml	Chiang (2005) et al.
Protease	UFM 10 kDa	———	0.048 mg/ml	Cha et al. (2005)
Pepsin Pancreatin	AEC HPLC GF-FPLC IMAC	———	13-93 mg/ml 1.1-8.3 μM	Lo et al. (2005)
Tofuyo	GF & HPLC	Ile-Phe-Leu Trp-Leu	44.8 μM 29.9 μM	Kuba et al. (2003)

In Vivo studies

Sample	Product	Dosage	Blood Pressure reduction	Reference
SHRs	Alcalase hydrolysate of soy	100 mg/kg/day for one month	38 mmHg	Wu et al. (2001)
SHRs	peptic *** hydrolysate	5% of diet	soy >casein	Chen et al. (2003)
SHRs	HHL (natto)	5mg/kg 3 Injections/day	61 mmHg	Shin et al. (2001)

* Alcalase hydrolysate showed the most potent ACE inhibitory activity (Lowest IC_{50})

** Membranes with molecular weight cut-offs (MWCFs) of 1000-30,000 Da were used to filter the hydrolysate

*** Peptic hydrolysate of soy and casein after GF and Ion Chromatography with IC_{50} values of 0.73 and 0.83 mg/ml, respectively.

ACE: Angiotensin converting enzyme

AEC: Anion exchange chromatography

EH: Enzyme hydrolysis

FPLC: Fast protein liquid chromatography

GPC: Gel permeation chromatography

GF-FPLC: Gel filtration fast protein liquid chromatography

HHL: Hip-His-Leu

HPLC: High pressure liquid chromatography

IC_{50}: Concentration of peptides which is able to inhibit 50% activity of ACE

IMAC: Immobilized Metal Affinity Chromatography

Lb.F: *Lactobacillus* ferementation

LbH: *Lactobacillus helveticus*

LbD.B: *Lactobacillus delbrueckii bulgaricus*

M of F: Method of Fractionation

SHRs: Spontaneously hypertensive rats

UFM: Ultra filtration Membrane

2.6 Free Radicals and Prevention of Chronic Diseases

Free radicals appear to play an important role in the mechanism of chronic diseases such as atherosclerosis, cancers, degenerative diseases, and diabetes. During past decades, many studies have focused on the role of antioxidants, specifically food derived peptides, in hindering oxidation.

2.6.1 Production of Free Radicals

A free radical is defined as any independent species with one or more unpaired electrons. The presence of unpaired electrons makes free radicals highly reactive because they require another electron to fill the orbital and become stable. Free radicals, including reactive oxygen species (ROS), are produced in the body by natural metabolism and are controlled by the endogenous antioxidant system. Food oxygenation takes place in cell mitochondria to produce adenosine triphosphate (ATP). During food oxygenation, electrons are lost from food materials and added to electron carriers such as nicotinamide adenine dinucleotide (NAD+), flavin mononucleotide (FMN), and flavin adenine dinucleotide (FAD). These compounds are oxidized again in the mitochondria to produce ATP; cytochrome oxidase gives four electrons to oxygen as an end stage of this process (Halliwell, 1994).

When a single electron is added to O_2, a superoxide radical is produced ($O_2^{\cdot}$). The other organic molecules such as flavins, adrenaline, L-dopa, dopamine, and cysteine may be oxidized in the presence of O_2 to produce superoxide radicals. Superoxide radicals are produced whenever the integrity of the mitochondrial electron transport chain is compromised. Therefore, any damage to proteins and lipids in DNA resulting in

mutations in mitochondrial DNA produces oxygen radicals, which cause a broad range of chronic diseases (Shigenaga, Hagen, & Ames, 1994). Superoxide radicals are also produced when any electron leaks through the C—C bond formation into fatty acids.

Superoxide radicals decrease the effectiveness of antioxidant defence enzymes such as catalase, glutathione peroxidase, and several other molecules in the energy metabolism scheme such as NADH dehydrogenase. Superoxide radicals also damage ribonucleotide reductase required for DNA synthesis, as well as calcineurin and aprotein that are necessary for signal transduction. Superoxides can produce hydrogen peroxide (H_2O_2) by adding one electron to H_2O. H_2O_2 is not a free radical but can attack some enzymes such as glyceraldehyde-3-phosphate dehydrogenase, an enzyme in the glycolytic pathway; oxidize certain keto-acids such as pyruvate; induce depletion of ATP; reduce glutathione, and NADPH; increase intracellular Ca^{2+} and activate a polymerase resulting in cell death.

Free radicals can be released by enzymes NADPH oxidase and myeloperoxidase of macrophages and neutrophils during the immune system activation to kill bacteria (Punchard & Kelly, 1997), and are produced during tissue damage and ischemia. In ischemia, xanthine oxidase converts to xanthine dehydrogenase, and is responsible for free radical production. During reperfusion of ischemic tissue with blood and oxygen, xanthine oxidase converts hypoxanthine and xanthine to uric acid. Uric acid is responsible for increased levels of superoxide and peroxide. This is the major process in the tissue damage seen in ischemia/reperfusion injury. Ultraviolet exposure, smoking, and air pollution are other causes of free radical production (Chow, 1993).

2.6.2 Antioxidants and Chronic Diseases

Halliwell and Gutteridge (1999) defined an antioxidant as any substance that, when present at low concentrations, compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate. Peroxidases and metal chelating proteins are first line antioxidants because they can prevent the generation of free radicals. Radical-scavenging antioxidants are the second line of defense against free radicals. This group includes vitamin E and C, as well as food-derived peptides that prohibit the initiation or development of chain oxidation. The line of defense against oxidants involves those antioxidants that can repair the damages of cell components or membranes such as lipases, proteases, DNA repair enzymes, and transferases (Niki, 1997). Catalases, superoxide dismutase (SOD), glutathione peroxidase (GPX) are some examples of endogenous antioxidants. They are present inside the cell to remove most superoxides and peroxides before they react with metal ions to form more reactive free radicals (Mates, Pereez-Gomez, & Nunez, 1999). Moreover, diet plays an important role in providing the body with necessary exogenous antioxidants such as Vitamin C & E, β-carotene, flavanoids. For example, polyunsaturated fatty acids need to have enough exogenous antioxidant to prevent double bond oxidation by the attack of free radicals.

Any oxidative imbalance between the free radicals inside a cell and the antioxidant defense system is an initiating factor for cell injury. Oxidative stress can damage all biomolecules including DNA, lipids, and proteins that are associated with many chronic diseases. Oxidative stress can be the primary process of a chronic disease or a consequence of chronic disease.

2.6.2.1 Antioxidants and cardiovascular diseases

Atherosclerosis, plaque formation, is the most common cardiovascular disease. Formation of foam cells, fatty streaks, and fibrous plaques are three stages of atherosclerosis resulting in coronary artery occlusion. Mechanism of atherosclerosis includes 4 stages. First step is the migration of monocytes to intima and conversion to macrophages (Frostegard, Hagerstrand, Gidlund, & Nilsson, 1991). Free radicals are responsible for initial damage of endothelium and release of cytokines. It is supposed that cytokines stimulate the migration of monocytes to intimae. In addition, free radicals accelerate lipid peroxidation in intimae and provide oxidized lipids required for the second step of plaque formation. The role of antioxidants is important because they inhibit free radicals and reduce the rate of endothelium damage and lipid peroxidation. Therefore, the process of plaque formation and atherosclerosis decreases in presence of antioxidants. As mentioned, next step of plaque formation and atherosclerosis is the production of oxidized LDL by free radicals increasing the uptake of LDL by macrophages (Henriksen, Mahoney, & Steinberg, 1981). Third, formation of foam cells by macrophages increases (Pratico, Iuliano, & Mauriello, 1997) and finally the aggregation of foam cells to produce fatty streaks. Some studies have shown that oxidized LDL antibodies are present in the blood and play a role in atherosclerosis formation (Palinski et al., 1989; Salonen, Yla-Herttulal, & Yamamoto, 1992).

2.6.2.2 Antioxidants and Cancer

Epidemiological studies show that there is an inverse relationship between cancer and ingestion of micronutrients such as β-carotene, vitamin E & C, and selenium. In 21 lung cancer studies, 15 studies were found to have an inverse correlation between

β–carotene intake and disease pathology (McLarty, 1997). Metastatic cells produce a huge amount of hydrogen peroxide that function as signal molecules necessary for cancer cell survival. Antioxidants can inhibit hydrogen peroxide production, resulting in prevention of cancer cell proliferation (Loo, 2003).

It has been mentioned that the role of free radicals in cancer induction occurs through DNA damage (Loft et al., 1996). Mutation to the P gene, a suppressing factor for cell division, is considered a mechanism in cancer cell proliferation. Free radical attack to this gene inactivates it, resulting in DNA damage and cancer cell production. Almost 75% of colorectal cancers and 90% of squamous cell skin cancers have P gene mutation (Heart Protection Study Collaborative Group, 2002). During oxidative stress, some changes in DNA structure happen through the conversion of double-ringed purines like adenine (A) and guanine (G) as well as the conversion of single-ringed pyrimidines like cytosine (C) and thymine (T) which were observed by Cerutti (1994) in human cancers. Moreover, 8-hydroxy-2'-deoxyguanosine (8-OHdG) is a reliable marker for oxidative stress during cancer cell proliferation. Apart from direct damage of free radicals to DNA, free radicals are able to destroy the proteins, which are involved in DNA repair enzymes.

2.6.3 Antioxidant Effects of Soy

Since an antioxidant compound in soy protein was discovered in 1979, scientists have demonstrated how the antioxidant activities of soy compounds can decrease the risk of cardiovascular diseases and cancers. Recently, a new compound in dou-chi, a soybean fermented food (Chen et al., 2005) having DPPH radical-scavenging effect, has been found. A meta-analysis by Anderson, Johnstone, and Cook-Newell (1995) concluded that soy protein lowers total and LDL concentration by 9% and 13%, respectively. However,

other studies, using an average dose of 47 g/d of soy, showed smaller effects (2-7%) of soy protein, on total and LDL cholesterol (Teixeira, Potter, & Weigel, 2000). Teixeira et al. (2000) observed hypocholesterolemic effects of soy occurred after 4 weeks in men, but after 24 weeks in women. Although some studies indicate that soy protein isoflavones are responsible for hypocholesterolemia, a multiple regression analysis shows that after 12 weeks of treatment, plasma total antioxidant status is positively affected by soy protein but not by soy isoflavones (Swain, Alekel, Dent, Peterson, & Reddy, 2002).

Soy protein isolate is able to prevent oxidative stress in rats that have been treated with chemical oxidants. Takenada et al. (2003) showed that an intake of either dietary SPI or a soy peptide (PEP), but not an amino acid mixture resembling soy protein, had the effect of reducing paraquat (PQ)-induced oxidative stress in rats. A previous study showed that there is an increasing risk of oxidative stress during and after exercise causing fatigue, and increasing risk of muscle injury and cancer in some types of athletes (Karlsson, 1997). Box et al. (2005) demonstrated that soy intake (40 mg/d) decreased lipid peroxide production at 3 and 24 hours after exercise in 18 recreationally trained, young adult women who did a moderate intensity, weight resistance exercise session for a 4 week period. During a meta-analysis by Badger et al. (2005), the preventive role of soy protein isolate was indicated in breast, colon, and prostate cancers. Badger et al. (2005) induced cancers in rats by injecting them with azoxymethane (AOM) and dimethyl-benz anthrocene (DMBA). Possible mechanisms of action for SPI were considered such as: 1) increasing the mammary gland differentiation, 2) decreasing the activation of pro-carcinogens to carcinogens and 3) regulation of genes in signal transduction pathways underlying tumor initiation, promotion and/or progression.

2.6.4 Antioxidant Effect of Casein

Dairy products have beneficial effects in the oxidative defense system of the body. Milk products have an important role in the prevention of lipid peroxidation and maintenance of milk quality (Steijns et al., 2000). Several milk components such as whey, casein, lactoferrin have been found to have antioxidant activities. Laakso (1984) reported that milk casein inhibited lipoxygenase-catalyzed lipid autoxidation and proposed that quenching of free radicals by oxidation of amino acid residues in casein explained the results. However, many studies have demonstrated other possible mechanisms for casein and milk product antioxidant activities. For example, Kitts et al. (2005) have proposed that a bioactive phosphopeptide derived from casein (CPP) has both primary and secondary antioxidant activity through ferrous ion sequestering and direct free radical quenching effects. Various methods have been applied to measure the potential antioxidant activity of dairy products. The hydroxyl radical ($OH^{\cdot}$) is a very potent oxidant that is an important factor in initiating lipid peroxidation reactions and can be measured indirectly using the deoxyribose assay (Halliwell et al., 2004). Casein has been shown to scavenge superoxide, hydroxyl radicals and stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Suetsuna, Ukeda & Ochi, 2000) and to sequester iron through intermolecular iron bridging (Gaucheron, Famelart & Le Graet, 1996). As shown in the preceding sections, casein and soy protein isolate show antioxidant activities in vitro and in vivo. This is consistent with several epidemiological studies that have shown that consumption of these foods has resulted in decreased incidence of cardiovascular diseases and cancers.

3.0 MATERIALS AND METHODS

Soy protein and casein do not show their biological effects in form of parent protein. They should be hydrolyzed and isolated by chromatography to produce the bioactive peptides. In our study, we hydrolyzed soybean and casein by pepsin and pancreatin to mimic digestion processes in the gastrointestinal system. After hydrolysis, the hydrolysates were separated by chromatography and freeze-dried to evaluate the ACE-inhibitory and radical scavenging activities. Also conformational changes of ACE affected by isolated peptides were assessed.

3.1 Materials

Soy protein isolate; PRO-FAM 974, having a protein content of 90%, was a gift from Archer Daniels Midland Company (ADM, Decatur, IL, USA). Casein sodium salt from bovine milk, ACE, and Hippuryl-histidyl-lecine (HHL) were purchased from Sigma Chemicals (St Louis, MO, USA), while other analytical reagents (including DPPH) were obtained from Fisher Scientific (Oakville, ON).

3.2 Hydrolysis

In vitro digestion was carried out in triplicate according to the method of Garrett, Failla & Sarama. (1999). Soy protein isolate (SPI) or casein solution (4.5% w/v, in distilled water) was adjusted to pH 2.0 with 2 M HCl. The solution was heated to 37°C and pepsin (4% w/w, protein basis) added. After 2 hr of peptic hydrolysis, the reaction mixture was adjusted to pH 7.5 with 2 M NaOH, and pancreatin (4% w/w, protein basis) was added. The solution was incubated at 37°C for 4 hr and the pH was adjusted to 5.0 with 2 M HCl to stop enzyme action. The reaction mixture was heated to 95°C for 15

minutes to ensure complete destruction of enzyme protein. The SPI peptide digest was centrifuged at 8,500xg for 15 min, and the supernatant containing soy peptides was collected, freeze-dried and stored at -20°C. Protein content (68% for soy and 78% for casein) was determined using the method of Markwell, Maas, Biebar, and Tolbert. (1978).

3.3 Fast Protein Liquid Chromatography

The freeze-dried peptide digests were fractionated on a cation-exchange chromatography column using a Fast Protein Liquid Chromatography (FPLC) system (AKTA FPLC, Amersham Biosciences, Montreal, PQ) according to the method of Li and Aluko et al. (2005). A solution of the protein hydrolysate (concentration 140 mg/ml) was prepared using 0.1 M ammonium acetate (pH 7.5) buffer and filtered through a 0.2 micron membrane. An aliquot (1 ml) of the sample solution was injected onto the column. Prior to sample loading, the column was equilibrated with 60 ml of 0.1 M ammonium acetate buffer (pH 7.5). After sample loading, the column was washed with 60 ml of ammonium acetate buffer to remove unbound peptides. Adsorbed peptides were then eluted using a linear gradient between 0 and 0.5 M ammonium carbonate (pH 8.8) in 0.1 M ammonium acetate (pH 7.5) buffer; eluted peptides were monitored through absorbance values at 214 nm. Fractions within each peak were pooled and freeze-dried.

3.4 Protein Content Determination

Protein content of peptides was determined by the method of Markwell et al. 1978. The following reagents were used:

- Reagent A: 2% Na_2CO_3 + 0.4% NaOH + 0.16% sodium tartrate + 1% sodium dodecyl sulphate (all dissolved in distilled water)

- Reagent B: 4% $CuSO_4.5H_2O$ dissolved in distilled water

- Reagent C: 100 parts of reagent A + 1 part of reagent B

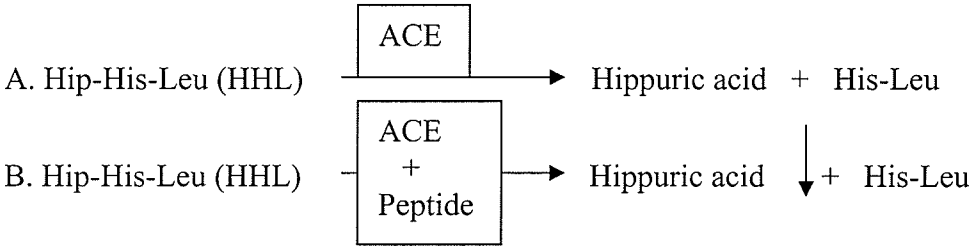
- Reagent D: 1 part of Folin-Ciocalteu phenol reagent + 1 part of distilled water.

A 1 mL aliquot of peptide sample containing 20-100 μg/ml protein was prepared and mixed with 3 mL of reagent C followed by incubation at room temperature for 1 hr. Reagent D (0.3 mL) was added to the samples and mixed vigorously using a vortex. The mixture was kept at room temperature for 45 min and the absorbance at 660 nm was measured using a 1 cm pathlength cuvette in a spectrophotometer (Milton Roy, Spectronic 3000 array, Ivyland, PA, USA). Standard protein solutions that contained 20-100 μg/mL bovine serum albumins (BSA) were prepared and absorbance was measured following the same steps. Triplicate determinations were used to calculate protein concentrations.

3.5 Enzyme Inhibition Kinetics

ACE-inhibitory activity was determined according to the method described by Aluko and Monu (2003). The substrate (hippuryl-histidyl-leucine, HHL) with different concentrations (5, 2.5, 1.25, and 0.625 μM), protein sample (1.25 & 2.5 mg/ml) and ACE solutions (5 μM) were all prepared using 50 mM sodium borate buffer, pH 8.3 containing 0.5 M NaCl. An aliquot (50 μL) of the peptide fraction (sample), or 50 μL of borate buffer (control) was mixed with 50 μL of ACE solution (25 mU) and incubated at 37°C for 5 min. An aliquot (150 μL) of the substrate solution was then added and incubation continued for 30 minutes. The reaction was terminated by adding of 250 μL of 1 M HCl

solution followed by addition of 1.5 mL of ethyl acetate to extract the hippuric acid. After mixing for 1 min, the mixture was centrifuged at 1000xg for 5 min and 1 mL of the upper layer transferred into a glass test tube. The ethyl acetate was evaporated to dryness using a heating block and the residue dissolved in 3 mL of distilled water; concentration of hippuric acid was determined by measuring absorbance of the aqueous solution at 228 nm. The concentration of peptide fraction that inhibited 50% of ACE activity was defined as the IC_{50} value. Type of inhibition was determined from the Lineweaver-Burk plots of 1/velocity versus 1/peptide concentration for each peptide fraction. The corresponding parameters (Vmax and Km) were estimated by non-linear squares fit of the data.



3.6 Radical Scavenging Activity (RSA)

Scavenging activity of the isolated peptide fractions against 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was determined according to the method described by Aluko and Monu (2003). Protein solutions were prepared in 0.1 M sodium phosphate buffer, pH 7.0, containing 1% (w/v) Triton X-100, while DPPH (100 μM) was prepared in methanol. An aliquot (1.5 mL) of the protein solution (sample) or 1.5 mL of buffer (control) was mixed with 1.5 mL of DPPH solution, and left in the dark at room temperature for 30 min. Absorbance of the solution was measured at 517 nm. An RSA (absorbance of control/absorbance of sample) value above 1 indicates a scavenging activity.

3.7 Determination of Peptides-Induced Modulation of Angiotensin Coverting Structure by Spectrofluorimetry

Fluorescence spectroscopy was applied to determine possible changes in the structure of ACE when bound to peptides. This method uses light absorption by protein components, amino acids, and radiation emission in the UV range of the spectrum. After light absorption by the molecules, the electron movements will increase and the molecules will be promoted to excited state from ground state. Then the fluorescence emission will be manifested when excited electrons revert from excited state back to the ground state (Schmid, 1989). Any changes in fluorescence intensity indicate the conformational changes such as folding or unfolding of the enzyme. Three aromatic amino acids including phenylalanine, tyrosine, and tryptophan have the principal roles in fluorescence emission of proteins. The optimum excitation wavelengths of phenylalanine, tyrosine and tryptophan are 257, 274 and 295 nm respectively. Because the absorbance at excitation wavelength and the quantum yield of emission radiation of tryptophan are higher than those of tyrosine and phenylalanine, the excitation wavelength applied for this experiment was 295 nm and emission spectrum was 300-450 nm. Stock solutions of ACE and peptides (with two concentrations: 1.25 and 2.5 mg/ml) were prepared with 50 mM sodium borate buffer, pH 8.3 containing 0.5 M sodium chloride using the same concentration as in the enzyme kinetics, but diluted 25 times to keep the total protein concentration within the suitable range for fluorescence measurement.

The fluorescence measurements were recorded using a JASCO FP-6300 spectrofluorimeter (JASCO Inc, Tokyo, Japan). Excitation wavelength was fixed at 295

nm for tryptophan and the fluorescence emission spectra were recorded between 300-450 nm. All measurements were taken using a 0.5 cm pathlength 100 μl quartz cuvette.

- Concentration of ACE diluted to 25 times: 5 μM / 25 = 0.2 μM

- Low concentration of peptide (LCP): 1.25 (mg/ml) / 25 = 0.05 (mg/ml)

- High concentration of peptide (HCP): 2.5 (mg/ml) / 25 = 0.1 (mg/ml)

The following fluorescence intensity of samples was measured:

A. Fluorescence intensity of ACE

B. Fluorescence intensity of ACE+ peptide (LCP)

C. Fluorescence intensity of ACE+ peptide (HCP)

The following calculations were made to obtain the structure of ACE:

1. (ACE+ peptide, LCP) - ACE, (B-A)

2. (ACE+ peptide, HCP) - ACE, (C-A)

3.8 Statistical Analysis

Each analysis was done in duplicate and two-way analysis of variance (Proc ANOVA) was performed using each measured parameter as the dependent variable. The mean values were compared by Duncan's multiple range tests using the Statistical Analysis Systems software, Version 9.1 (Statistical Analysis System, Cary, NC). Significant differences were taken at $p<0.05$.

4.0 RESULTS AND DISCUSSION

4.1 Hydrolysis

In the present study, enzymatic hydrolysis by pepsin and pancreatin was used to mimic the GI system. Digestion in the GIT plays an important role in increasing the bioavailability of ACE-inhibitory peptides. We hydrolyzed casein with pepsin for 2 hours at pH 2 and pancreatin for 4 hours at pH 7.5. After freeze-drying and storing at $-20^{\circ C}$, we obtained a white, soft powder having a protein content of 76%. After isolating the sample by FPLC, 7 fractions were obtained with IC_{50} values of 0.55, 0.44, 0.26, 0.35, 0.16, 0.13, 0.48 mg/ml for fractions 1, 2, 3, 4, 5, 6, and 7, respectively, which were higher than those of previous studies (Minervini et al., 2003; Quiros, Hernandez, Amigo, & Recio, 2005). For example Minervini et al. (2003) indicated IC_{50} values of 0.016-0.1 mg/ml in a study that used milk fermented by *Lactobacillus helveticus*. The differences may be due to differences between the protease activities produced by *Lactobacillus helveticus* compared to the proteases present in pancreatin (Hernandez et al., 2004).

In the case of soy protein, hydrolysis by pepsin and pancreatin was applied using similar conditions as for casein. A white and soft powder was obtained after freeze-drying. IC_{50} values of obtained fractions after FPLC separation were 0.16 mg/ml, 0.31 mg/ml, and 0.76 mg/ml for fractions 4, 3, and 2, respectively, similar to previous studies. Lo et al. (2005) obtained IC_{50} values in the range of 0.13 to 0.93 mg/ml for soy protein after pepsin and pancreatin hydrolyzation and chromatographic separation, which are close to our study. They also showed that the IC_{50} of pepsin hydrolysate was increased after subsequent digestion with pancreatin, indicating a higher potential to generate ACE-inhibitory peptides during digestion in the lower sections of the GIT. Previous studies by

Cha and Park (2005) and Wu et al. (2001) evaluated the stability of soybean derived ACE-inhibitory peptides after protein hydrolysis by SS103 protease was obtained from *alkalophilic bacillus* and alcalase, respectively. In the Wu et al. (2001) study, soybean protein and casein sodium salt from bovine milk were hydrolyzed by pepsin and pancreatin for four and two hours, respectively, to mimic stomach and small intestine digestion in the body. They found that soy protein peptides were resistant to enzymatic digestion of the gastro-intestinal system indicating that the obtained peptides can pass through the gastrointestinal tract without losing their effects. Lo et al. (2005) used pepsin and pancreatin, for one and two hours respectively, for soybean digestion, but this method was not designed to mimic the GI system digestion because of the short time of hydrolysis.

4.2 Peptides Separation and Isolation by Chromatography

The freeze-dried casein and soy protein powders obtained from the previous enzymatic hydrolysis step were loaded (5 ml) onto the cation-exchange chromatographic column using the FPLC system with the concentration of 140 mg/ml. Cation-exchange chromatography was chosen for peptide separation since previous studies (Chen, Okada, Muramoto, Suetsuna & Yang, 2003; Lo et al., 2005), using anion-exchange chromatography, did not detect much difference between the ACE-inhibitory activity of the protein digest and the peptide fractions. Therefore, the use of a cation exchange column in this work provides a unique approach towards identifying ACE-inhibitory peptides present in casein and soybean protein digests.

Five peaks from soy protein and seven peaks from casein were collected with a flow rate of 3 ml/min and 5 ml/min respectively (Figures. 4.2.1 & 4.2.2). The first 3 casein fractions eluted produced a very sticky paste with low protein concentrations (40%-50%) after freeze-drying. In contrast, fractions 4, 5, 6, and 7 produced completely white powders with high protein concentrations (> 65%) after freeze-drying. Separation of peptides is on the basis of their positive charges. Peptides having more positive charge bind more tightly to the negatively charged column and are released later during elution compared to peptides with fewer positive charges. As shown in Figure 4.2.1, the first three fractions of casein peptides were eluted in large amounts during the first 100 minutes, indicating that they had less net positive charge than the last four fractions. In comparison with our study, Vincenzetti et al. (2005) obtained six peaks from HPLC cation exchange separation followed by gel filtration and anion exchange HPLC. In our study, protein concentrations of fractions were measured by the Markwell et al. method (1978). For casein fractions, protein assay data ranged from 32% for fraction three (the lowest) to 76% for fraction five (the highest), as shown in Table 4.3.1. In contrast to casein fractions, most of the soy peptides were eluted in the middle of the chromatography spectrum (Figure 4.2.2).

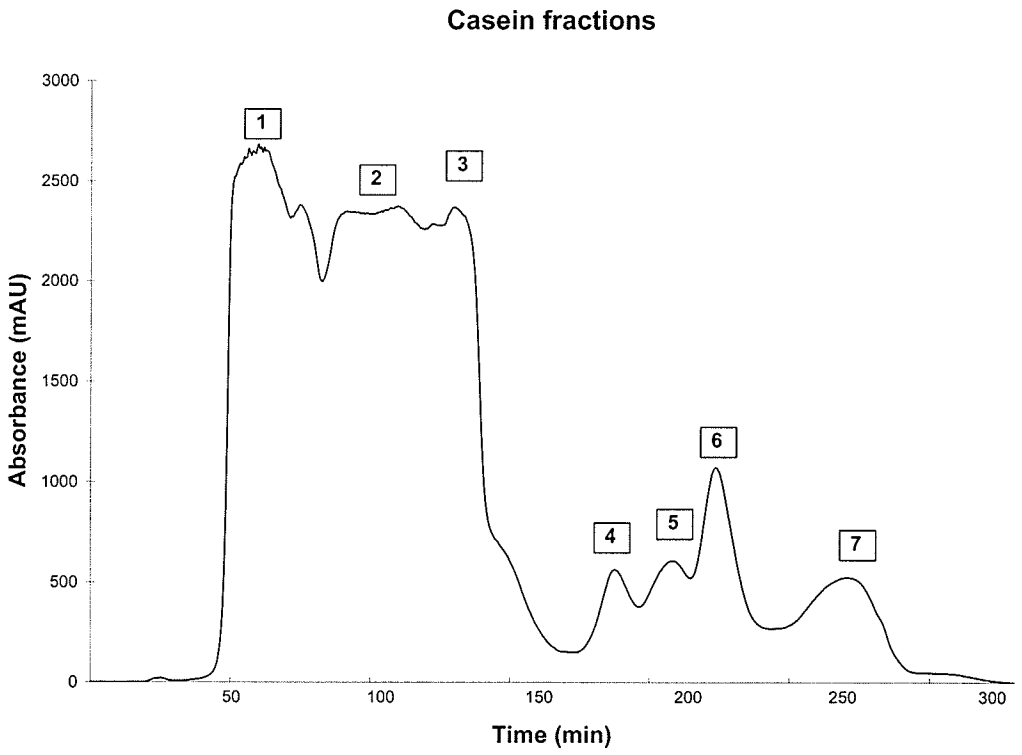
All soy fractions were a yellowish, soft powder. The other soy components such as isoflavones, fibers may be responsible for yellowish color of our sample. Large amounts of fractions 2, 3, and 4 were collected and considered for further experiments. Fraction one did not have enough protein concentration (10%) and fraction 5 was not collected in sufficient quantities for the next steps. Therefore, we deleted these two fractions for future experiments and focused only on fractions 2, 3, and 4 with 60%, 75%,

and 50% protein assay, respectively, using the Markwell et al. (1978) method. In comparison, a study by Gibbs, Zougman, Masse, and Mulligan (2004) obtained two fractions from plasma proteases digest of soy protein after HPLC separation.

FPLC in our work produced five peptide fractions of pepsin and pancreatin digest of soy protein. Shin et al. (1994 & 2001) isolated peptides by HPLC from fermented soy paste in two separate studies. In the first study, Shin et al., 1994 designed a step gradient system of water and acetonitrile as the first mobile phase, and then an ion-exchange column was applied with a mobile phase of isocratic system of sodium succinate buffer/acetonitrile. The fraction obtained from this experiment was used in another two-step study using the same HPLC protocol of previous work (Shin et al., 2001). In the first step, a fraction was eluted isocratically with trifluoroacetic acid/acetonitrile, and in the second step, an ion-exchange column was applied which finally yielded seven fractions.

Figure 4.2.1

FPLC chromatogram showing separation of the casein hydrolysate on a SP-Sepharose cation exchange column using buffers A (0.1 M ammonium acetate, pH 7.5) and B (0.5 M ammonium carbonate). After sample was loaded, the column (58 mL capacity) was washed with 180 mL of 0.1 M ammonium acetate buffer (pH 7.5) to remove unbound peptides. Adsorbed peptides were eluted with a gradient of 0-100 % of buffer B in 300 minutes at a flow rate of 3 ml/min; 2 mL fractions were collected and pooled peak fractions were freeze-dried.

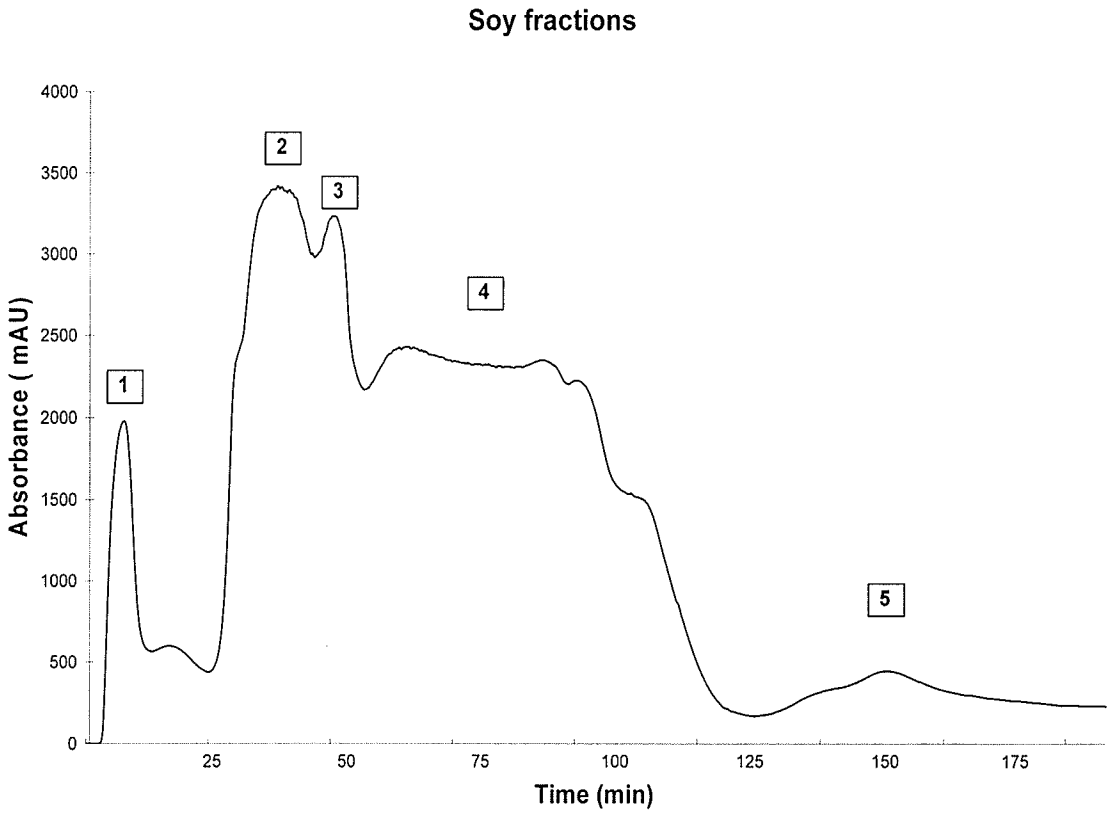


Fractions 1, 2, and 3 were yellow, sticky paste after freeze-drying

Fractions 4, 5, 6, and 7 were white, soft powder

Figure 4.2.2

FPLC chromatogram showing separation of the soy hydrolysate on a SP-Sepharose cation exchange column using buffers A (0.1 M ammonium acetate, pH 7.5) and B (0.5 M ammonium carbonate). After sample was loaded, the column (58 mL capacity) was washed with 180 mL of 0.1 M ammonium acetate buffer (pH 7.5) to remove unbound peptides. Adsorbed peptides were eluted with a gradient of 0-100 % of buffer B in 180 minutes at a flow rate of 3 ml/min; 2 mL fractions were collected and pooled peak fractions were freeze-dried.



Fraction 1 had small amount of protein (10%)

Fraction 5 had insufficient quantities to be useful

4.3 ACE Inhibitory Activity of Isolated Fractions

Each fraction obtained from FPLC chromatography was evaluated for its angiotensin converting inhibitory activity.

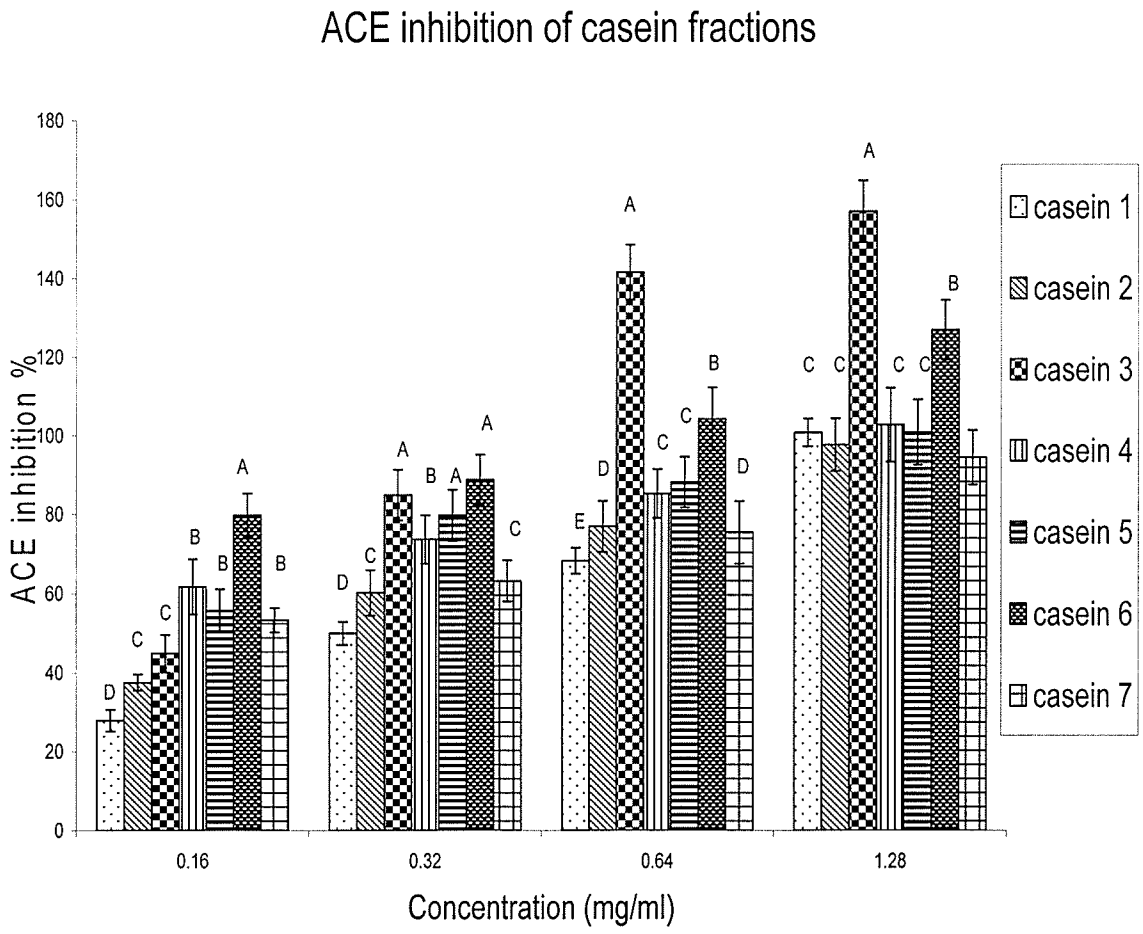
Casein fractions

Protein concentration of casein fractions collected from FPLC were determined as 58%, 62%, 32%, 60%, 76%, 60%, 62% for fractions 1, 2, 3, 4, 5, 6, and 7 respectively. To evaluate ACE inhibitory activity of peptides, we measured IC_{50}, which is the concentration of peptide inhibiting 50% of ACE activity: a lower IC_{50} value represents a higher ACE-inhibitory activity. IC_{50} for peaks 5 and 6 of casein was 0.16 mg/ml and 0.13 mg/ml respectively after considering their protein concentration (Table 4.3.1). A significant decrease ($p<0.05$) in ACE activity was observed for fractions 6 and 2 of casein in low concentration (0.16mg/ml), while little change occurred for fractions 1, 3, 4, 5, and 7. With concentrations of 0.32 mg/ml, 0.64 mg/ml and 1.28 mg/ml, fractions 5, and 6 significantly decreased ACE activity while there was a little change for fractions 1, 2, 3, 4, and 7. We observed that those fractions of casein eluted after 150^{th} minute from FPLC had stronger ACE-inhibitory activity than the early-eluted fractions. Fractions 5 and 6 that were collected from 130 to 200 minute had the most ACE-inhibitory activity. Therefore, casein fractions with more positive charge have more ACE-inhibitory activity than less positively charged fractions, except for the last fraction.

The data in Figure 4.3.1 demonstrates the ACE-inhibitory potential of FPLC-casein fractions indicating dose-dependent properties of casein peptides. This figure shows that after consideration the protein content of casein fractions, ACE inhibitory activity of fraction 3 increased significantly ($p<0.05$) with high concentrations 0.64 and

Figure 4.3.1

Higher ACE Inhibitory Activity of Casein Fractions Associated with Peptide Concentration Increasing (Dose-Dependent)



1.28 mg/ml. IC_{50} values obtained in this study for casein fractions ranged between 0.13-0.55 mg/ml, compared to peptide isolated by Gobbetti et al. (2000) with IC_{50} value of 179 mg/L (0.179 mg/ml), using *Lactobacillus helveticas* for fermentation of casein followed by FPLC separation. On the other hand, Nakamura et al. (1995a) got two specific tripeptides with amino acid sequence of IPP and VPP having IC_{50} values of 5 and 9 μM, respectively, after fermentation with *Lactobacillus helveticas* and HPLC separation. The other study by Tauzin et al. (2002), which hydrolyzed casein with trypsin and separated the hydrolysate by HPLC, extracted 40 fractions with IC_{50} values ranging from 4 to 214 μM/L.

Soy fractions

The first four soy protein fractions produced by FPLC had peptide concentrations of 10%, 60%, 75%, and 50% for peaks 1, 2, 3, and 4, respectively. Fraction 1 of soy protein had a low protein concentration and did not show ACE-inhibitory activity. Fraction 5 was in insufficient quantity to be used for the experiments. After identifying IC_{50} values of soy fractions (Table 4.3.2), fractions 3 and 4 significantly ($p<0.05$) inhibited ACE activity with peptide concentrations of 0.125 mg/ml, 0.25 mg/ml, and 1 mg/ml; with peptide concentration of 0.5 mg/ml, all soy fractions significantly ($p<0.05$) inhibited ACE activity. In comparing soy and casein ACE inhibitory peptides, casein fractions showed more ACE-inhibitory activity than those of soy protein. Their retention times in FPLC were between 70 to 95 minutes for fraction 3 and 95 to 140 minutes for fraction 4. Fraction 2, collected from 37 to 70 minutes, did not show strong ACE inhibitory activity compared with fractions 3 and 4, indicating that those soy protein

peptide fractions, which had the most positive charge, inhibit ACE more strongly than fractions with less net positive charge.

The data in Figure 4.3.2 demonstrates the ACE-inhibitory potential of FPLC-soy fractions indicating dose-dependent properties of soy peptides. Figure 4.3.2 shows that fractions 3 and 4 had the same ACE inhibitory activity at 1 mg/ml which is about 63%-65%. Also, we did not find a significant difference between fractions 3 and 4 except in peptide concentration of 0.5 mg/ml. The results of our study, with IC_{50} from 0.16-0.76 mg/ml, are in the range of those obtained by Lo, Eunice, and Li (2005). They applied anion exchange chromatography (AEC), HPLC, GF-FPLC, and IMAC to extract the soy fractions. In their research, IC_{50} values found for soy protein-derived peptides ranged from 0.13 to 0.93 mg/ml. Chiang et al. (2003) compared soy protein hydrolysates of 5 enzymes including alcalase, flavourzyme, trypsin, chymotrypsin, and pepsin. They found that alcalase hydrolysate had the most potent ACE inhibitory activity among the other enzyme hydrolysates after ultra filtration. The IC_{50} values of their study ranged between 0.668 and 0.078 mg/ml.

Figure 4.3.2

Higher ACE Inhibitory Activity of Soy Protein Fractions Associated with Peptide Concentration Increasing (Dose-Dependent)

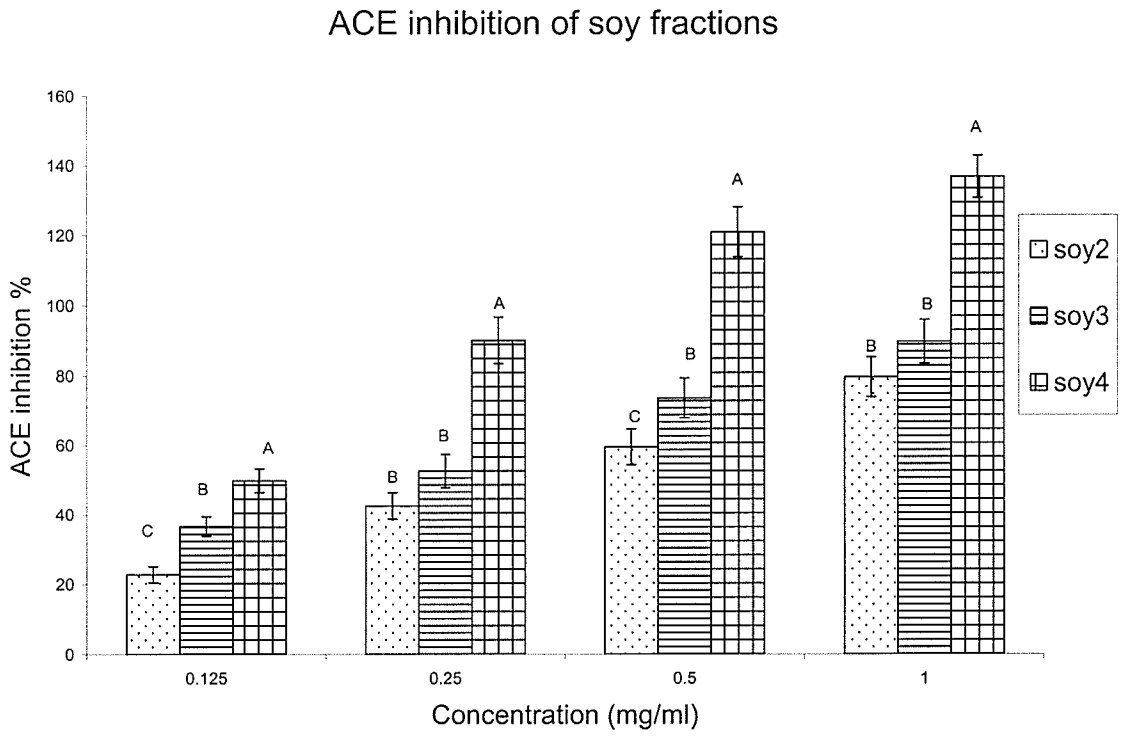


Table 4.3.1 IC_{50} (mg/ml) of Peptides Fractions of Casein

	Casein fractions						
	1	2	3	4	5	6	7
IC_{50} (mg/ml) *	$0.55\pm^{a}$ 0.062	$0.44\pm^{ab}$ 0.021	$0.26\pm^{cd}$ 0.063	$0.35\pm^{cb}$ 0.016	$0.16\pm^{ed}$ 0.022	$0.13\pm^{e}$ 0.021	$0.48\pm^{a}$ 0.013
Protein concentration	58%	62%	32%	60%	76%	60%	62%

* IC_{50} is the peptide concentration which inhibits the enzyme activity by 50%.

Table 4.3.2 IC_{50} (mg/ml) of Peptides Fractions of Soy

	Soy fractions		
	2	3	4
IC_{50} (mg/ml)	$0.76\pm^{a}$ 0.032	$0.31\pm^{b}$ 0.017	$0.16\pm^{c}$ 0.011
Protein concentration	60%	75%	50%

Note:

1. According to protein content, fraction one did not show ACE inhibitory activity.

2. Fraction five was too small a quantity.

3. Values are means of two independent determinations. Means with different letters are significantly different at $p< 0.05$

** IC_{50} data shown in Table 4.3.1 and 4.3.2 are corrected IC_{50} values, which have been calculated after IC_{50} values were multiplied at protein concentration. For example IC_{50} value of fraction 5 of casein was calculated directly in the experiment as 0.21 mg/ml which was multiplied at 76% (protein concentration) and obtained 0.16 mg/ml.

$A \times B = C$

A: IC50 value obtained directly from experiment

B: Protein concentration of fraction

C: Corrected IC_{50}

4.4 Enzyme Kinetics

Because chemical reactions in the body occur at very slow rates, enzymes are required to activate the reactions and increase their velocity. It is necessary, however, to clarify inhibition mechanisms of each enzyme, depending upon the method of binding to the peptide. The inhibition methods of enzyme by peptides are categorized as: competitive, non-competitive, and mixed. Non-competitive inhibition means that the inhibitor (peptide) binds to the enzyme at a site which is different from the substrate binding site (active site of the enzyme). Another explanation is that peptide binding may cause some changes in the ACE structure, reducing its ability to effectively catalyze conversion of the substrate to products. Table 4.4.1 and 4.4.2 show the Km and Vmax values of all casein and soy peptides for ACE inhibition. Km is the concentration of substrate required to reach half of maximal velocity (Vmax/2), while Vmax is the maximal velocity for enzyme reaction. Non-competitive enzyme inhibition occurs when Km does not change. If Km decreases or increases, it indicates that inhibition is of the mixed-type.

One method of analyzing enzyme kinetic data, the Lineweaver-Burk plot, was used to determine the ACE inhibition pattern of casein and soy protein peptides. Figures (4.4.1) to (4.4.9) show enzyme reaction velocity for ACE at different concentrations of casein and soy peptides, indicating a non-competitive and mixed inhibition for various fractions. For example, fraction 1 of casein at a low concentration shows a non-competitive inhibition, because the line of the low peptide concentration (1.25 mg) and uninhibited reaction (control) intersect the x-axis almost at the same point (see Figure 4.4.1) and Km values for control and low concentration are similar (Table 4.4.1).

Table 4.4.1 Km Values (μM) for the Inhibition of ACE by Casein and Soy Protein Peptides

		Casein*	
	Control	Low concentration (1.25 mg/ml)	High concentration (2.5 mg/ml)
Fraction 1	2.597±0.24	2.791±0.32	3.115±0.42
Fraction 2	2.950 ±0.37	3.721±0.3	3.324±0.26
Fraction 3	3.062±0.18	3.624±0.46	4.259±0.42
Fraction 4	3.536±0.52	2.974±0.21	2.829±0.35
Fraction 5	2.04±0.22	2.038±0.18	4.401±0.52
Fraction 6	2.443±0.19	2.29±0.16	2.532±0.42

		Soy	
	Control	Low concentration (1.25 mg/ml)	High concentration (2.5 mg/ml)
Fraction 2	5.249±0.7	5.364±0.5	3.748±0.4
Fraction 3	14.854±1.7	21.728±3.5	28.834±4.3
Fraction 4	2.807± 0.2	2.862±0.3	4.694±0.6

Note:

Km is the substrate (HHL) concentration required to reach half of maximal velocity (Vmax/2)

* Because of the lack of adequate quantities of fraction 7, we were not able to evaluate it for this experiment.

Table 4.4.2 Vmax Values for the Inhibition of ACE by Casein and Soy Protein Peptides

	Casein*		
	Control	Low concentration (1.25 mg/ml)	High concentration (2.5 mg/ml)
Fraction 1	0.1599±0.027	0.2213±0.034	0.297±0.021
Fraction 2	0.1182±0.041	0.176±0.015	0.2691±0.019
Fraction 3	0.1696±0.031	0.2313±0.026	0.2723±0.028
Fraction 4	0.1621±0.025	0.2673±0.031	0.3278±0.017
Fraction 5	0.1637±0.014	0.5443±0.032	0.6459±0.038
Fraction 6	0.2203±0.025	0.4327±0.017	0.5172±0.037

	Soy		
	Control	Low concentration (1.25 mg/ml)	High concentration (2.5 mg/ml)
Fraction 2	0.1095±	0.1691±0.015	0.2944±0.031
Fraction 3	0.0411±	0.0912±0.01	0.1075±0.01
Fraction 4	0.1501±	0.2561±0.018	0.2415±0.019

Note:

Vmax is maximum velocity

* Because of the lack of adequate quantities of fraction 7, we were not able to evaluate it for this experiment.

But there is a mixed-type inhibition at a higher concentration shown by the different points of intersection of x-axis with the line for 2.5 mg/ml compared to the control line (Figure 4.4.1); moreover, the Km values are also different (see Table 4.4.1).

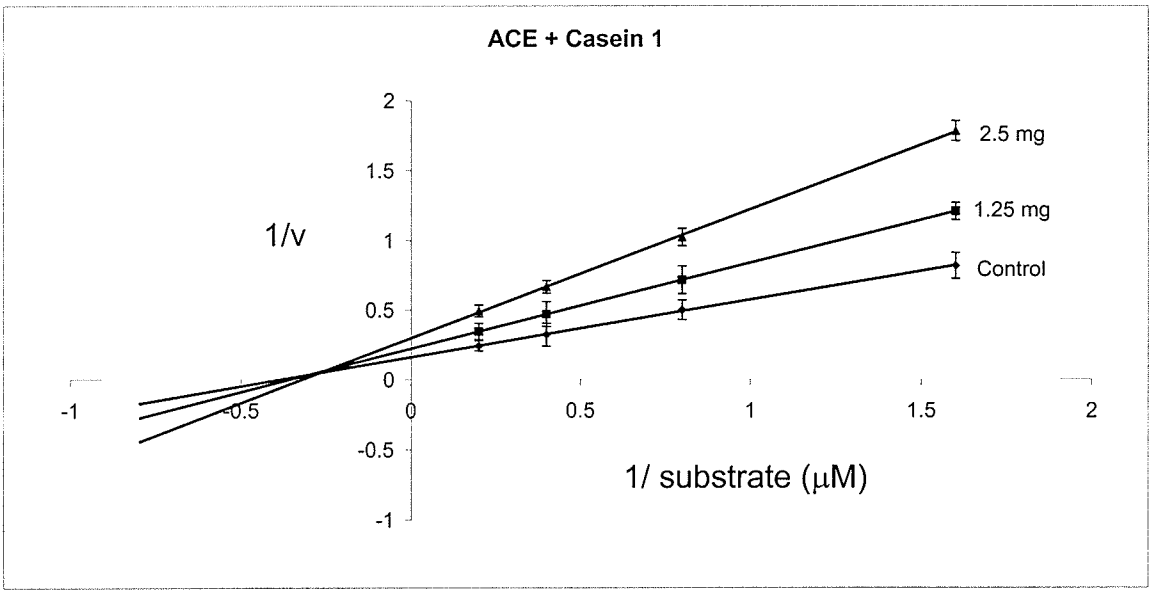
When inhibition shifts from non-competitive to mixed, it demonstrates that the peptide can compete with the substrate to bind to the active site of the enzyme and also bind to the enzyme at sites different from the active site. Fractions 2, 3, 4, and 7 inhibited ACE in a mixed-type manner because the x-axis and slope lines do not intersect at the same point (Figures 4.4.2, 4.4.3, 4.4.4, and 4.4.6). Moreover, Km values of the control and the fractions are not the same for fractions 2, 3, 4, and 6 of casein and fraction 3 of soy (Table 4.4.1). On the other hand, fraction 5 at a low concentration inhibited ACE non-competitively with regard to Km values at low concentration which shows Km (2.04 μM) for control and Km (2.038 μM) for fraction 5.

In addition to fraction 1 and 5 of casein, fraction 6 of casein showed the same results. According to Km values of the control and low concentration of fraction 6 which are close to each other (Table 4.4.1) and Figure 4.4.6 which shows the same intersection point of control line and fraction 6 with low concentration (1.25 mg/ml), we observe that fraction 6 also had the non-competitive behaviour with low concentration but at an increased concentration, started to compete with the buffer in binding to ACE. In fact, fractions 6, 5, and 1 are good examples indicating that with increasing the concentration of peptides, the difference between Km values of substrate (control) and sample (fraction 1, 5, and 6) increase. This describes our hypothesis that peptides compete with the substrate to bind and inhibit enzyme (ACE) resulting in decreased ACE activity and blood pressure.

In regard to soy peptides, all fractions exhibited mixed-type inhibition activities except fractions 2 and 4 at low concentrations, which had similar Km values to the control. Km values of substrate and fraction 2 of soy in low concentration were 5.249, 5.364 (μM) respectively. Km values of substrate and fraction 4 of soy in low concentration were 2.807, 2.862 (μM) respectively (Table 4.4.1). Comparing these data, indicates the same logical result as discussed for fraction 1 and 5 of casein. Therefore, it was concluded that the higher the inhibitor concentration, the higher the slope which indicates reduction in the ability of ACE to convert HHL (substrate) to products. Table 4.4.2 shows the velocity of reaction between enzyme and peptides. Fraction 5 of casein had the fastest reaction with enzyme compared with the others. It decreased the reaction time by a factor of 3 and changed the velocity from 0.1637 (substrate with enzyme) to 0.5443 (peptide with enzyme). Similarly, fraction 3 of soy decreased the reaction time between peptide and enzyme more rapidly than fractions 2 and 4 of soy. Velocity changed from 0.0411 (substrate with enzyme) to 0.0912 (peptide with enzyme) for fraction 3 of soy protein.

Figure 4.4.1

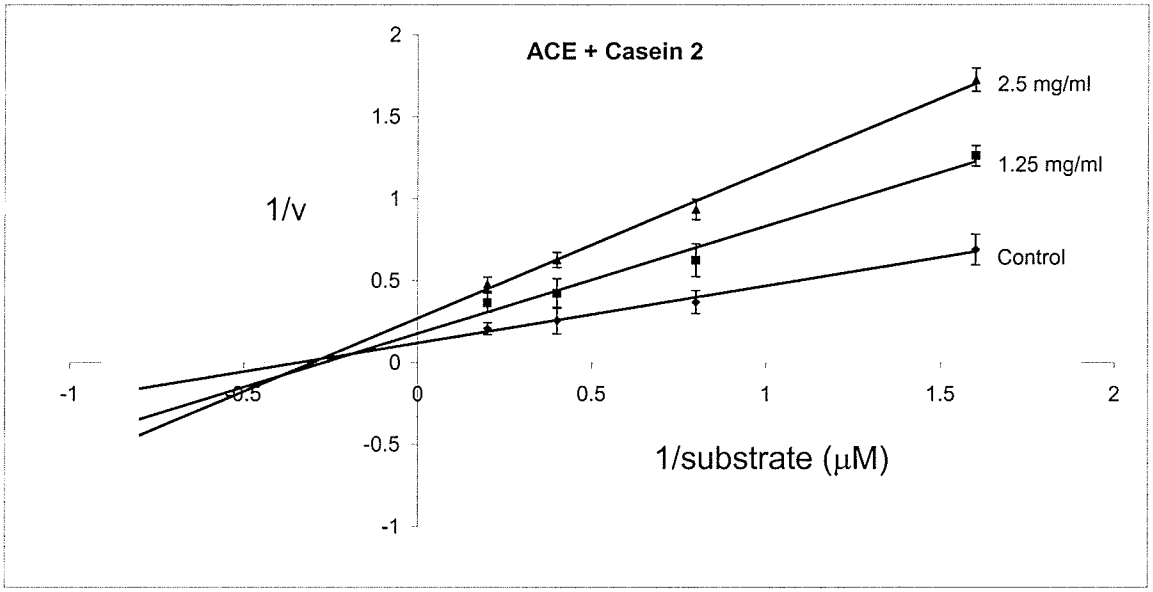
Lineweaver-Burk Plots of ACE Inhibition with Peptide Fraction 1 of Casein



Slope lines of substrate and peptide join each other close to x-axis. But Km value of peptide (Table 4.4.1) shows that only Km of peptide in low concentration is close to Km value of substrate indicating a non-competitive inhibition for low concentration of fraction 1 and mixed-typed inhibition for high peptide concentration.

Figure 4.4.2

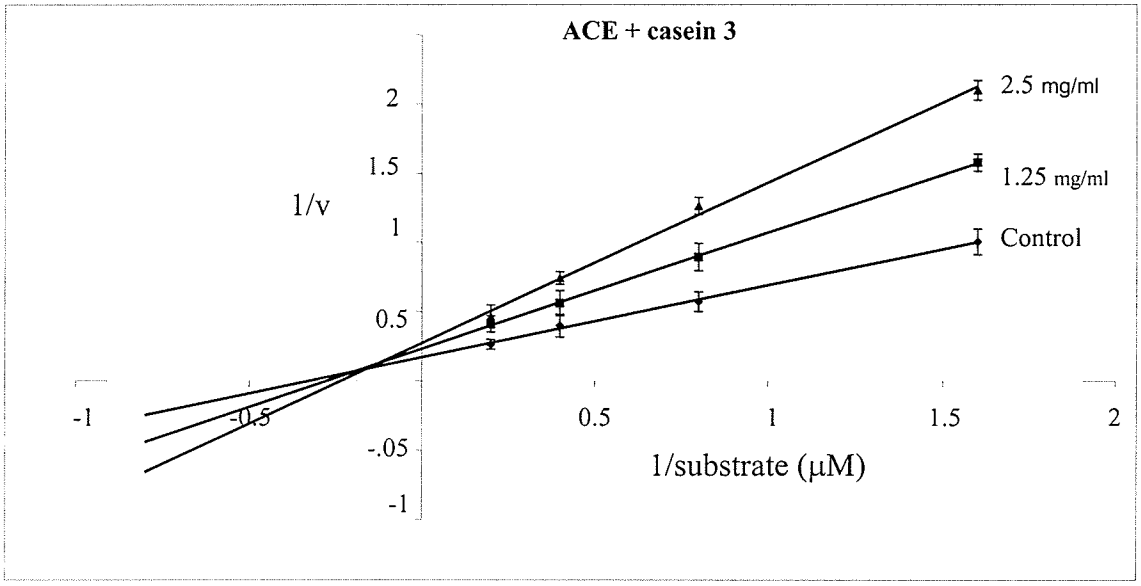
Lineweaver-Burk Plots of ACE Inhibition with Peptide Fraction 2 of Casein



Slope lines of sample and substrate do not cross each other at the same point at x-axis indicating a mixed-typed inhibition in both low and high peptide concentration.

Figure 4.4.3

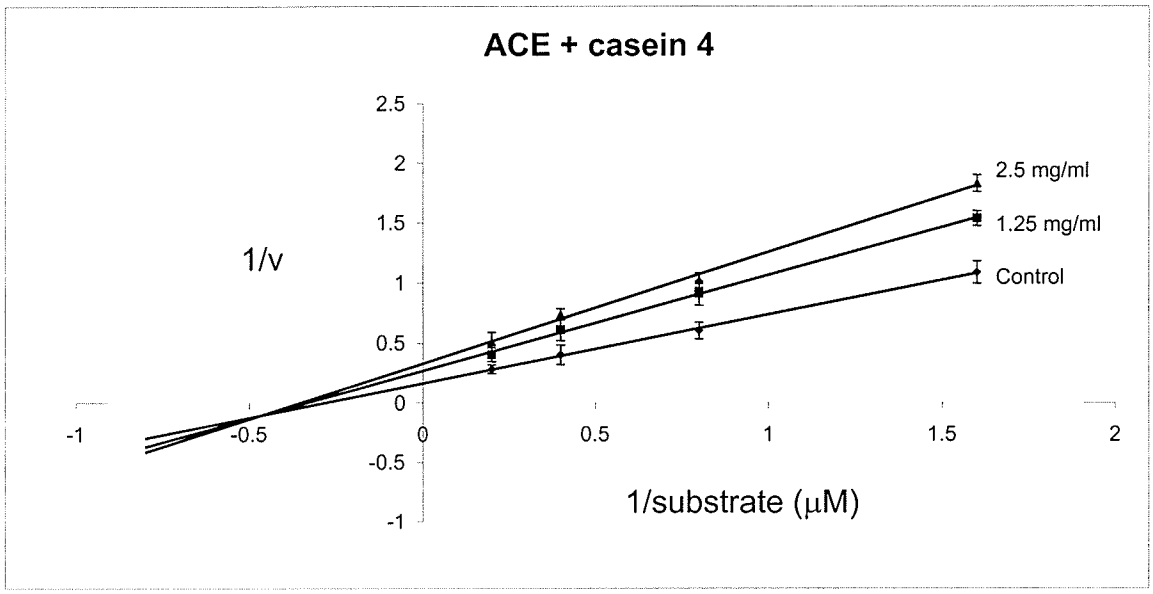
Lineweaver-Burk Plots of ACE Inhibition with Peptide Fraction 3 of Casein



Slope lines of sample and substrate do not cross each other at the same point at x-axis indicating a mixed-typed inhibition in both low and high peptide concentration.

Figure 4.4.4

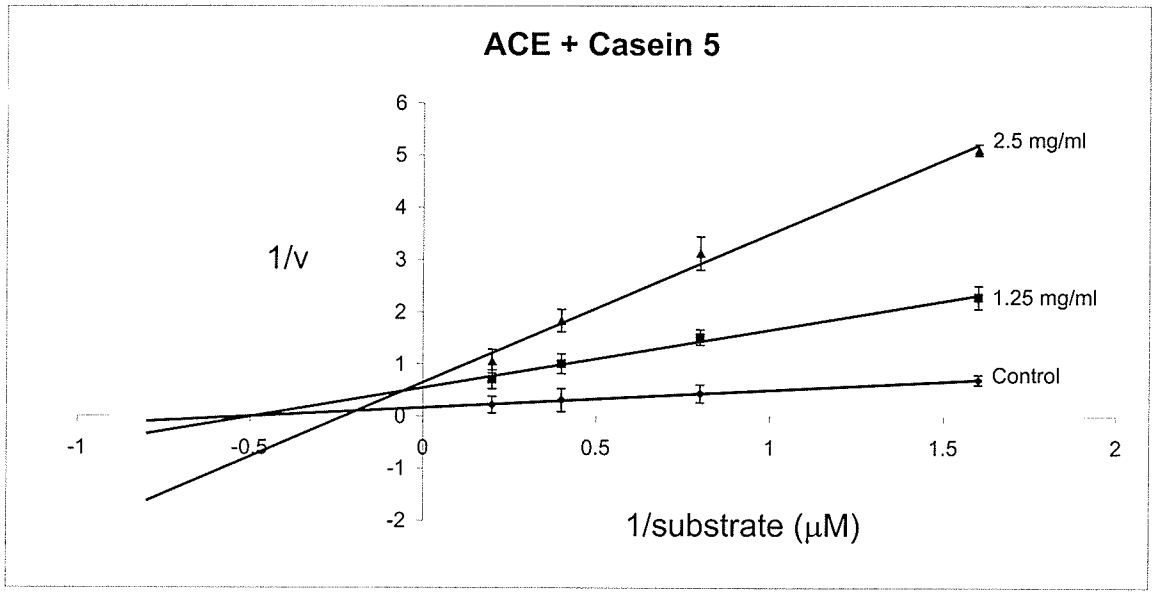
Lineweaver-Burk Plots of ACE Inhibition with Peptide Fraction 4 of Casein



Slope lines of sample and substrate do not cross each other at the same point at x-axis indicating a mixed-typed inhibition in both low and high peptide concentration.

Figure 4.4.5

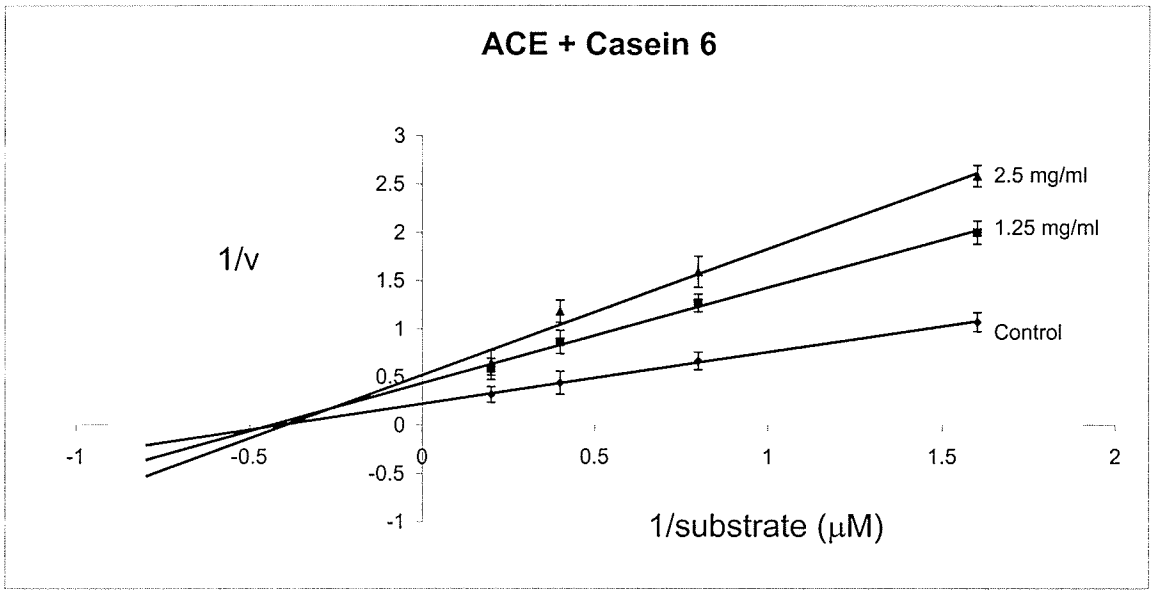
Lineweaver-Burk Plots of ACE Inhibition with Peptide Fraction 5 of Casein



Slope lines of substrate and peptide in low concentration join each other close to x-axis. Also Km value of peptide (Table 4.4.1) shows that Km of peptide in low concentration is close to Km value of substrate which indicates a non-competitive inhibition for low concentration of fraction 5.

Figure 4.4.6

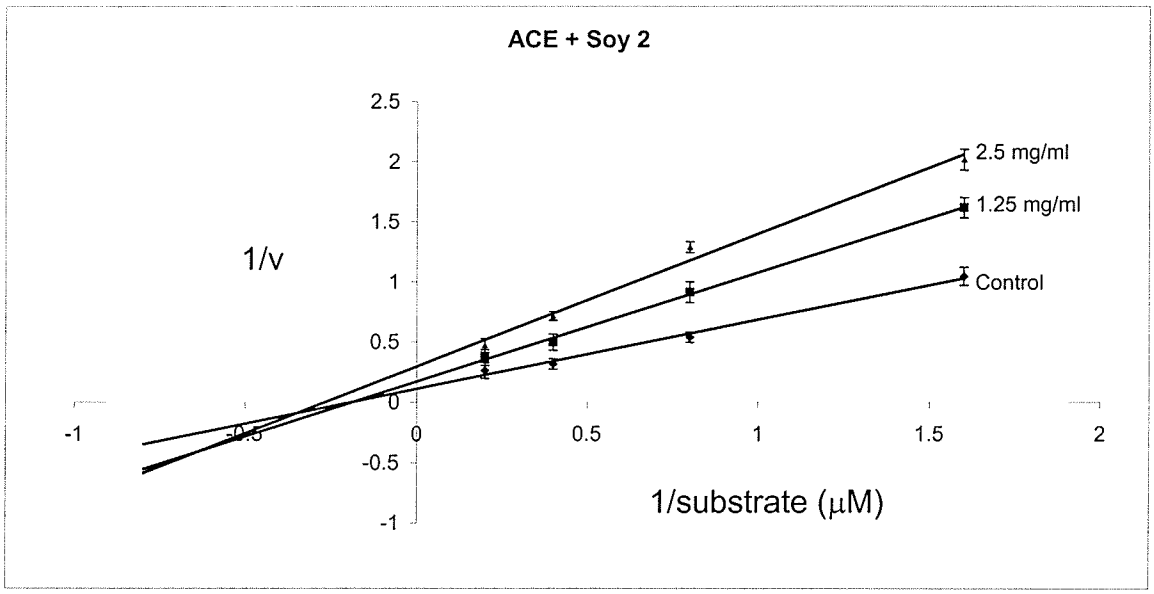
Lineweaver-Burk Plots of ACE Inhibition with Peptide Fraction 6 of Casein



Slope lines of sample and substrate do not cross each other at the same point at x-axis indicating a mixed-typed inhibition in both low and high peptide concentration.

Figure 4.4.7

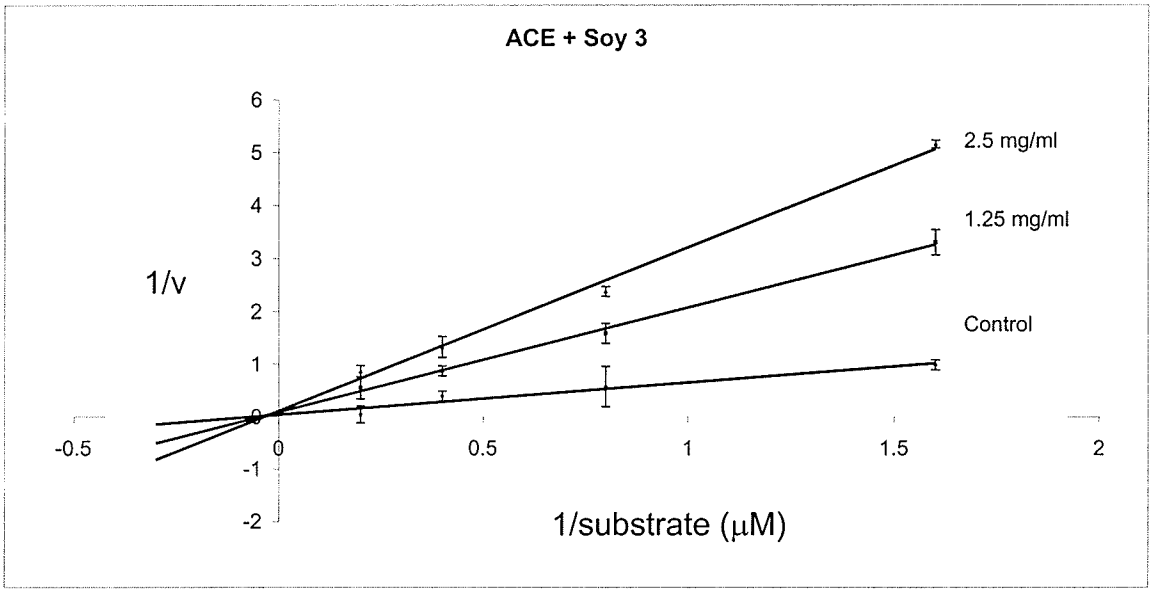
Lineweaver-Burk Plots of ACE Inhibition with Peptide Fraction 2 of Soy



Slope lines of substrate and peptide in low concentration join each other close to x-axis. Also Km value of peptide (Table 4.4.1) shows that Km of peptide in low concentration is close to Km value of substrate indicating a non-competitive inhibition for low concentration of fraction 2 of soy. Km value of peptide with high concentration is different from that of substrate indicating a mixed-typed inhibition.

Figure 4.4.8

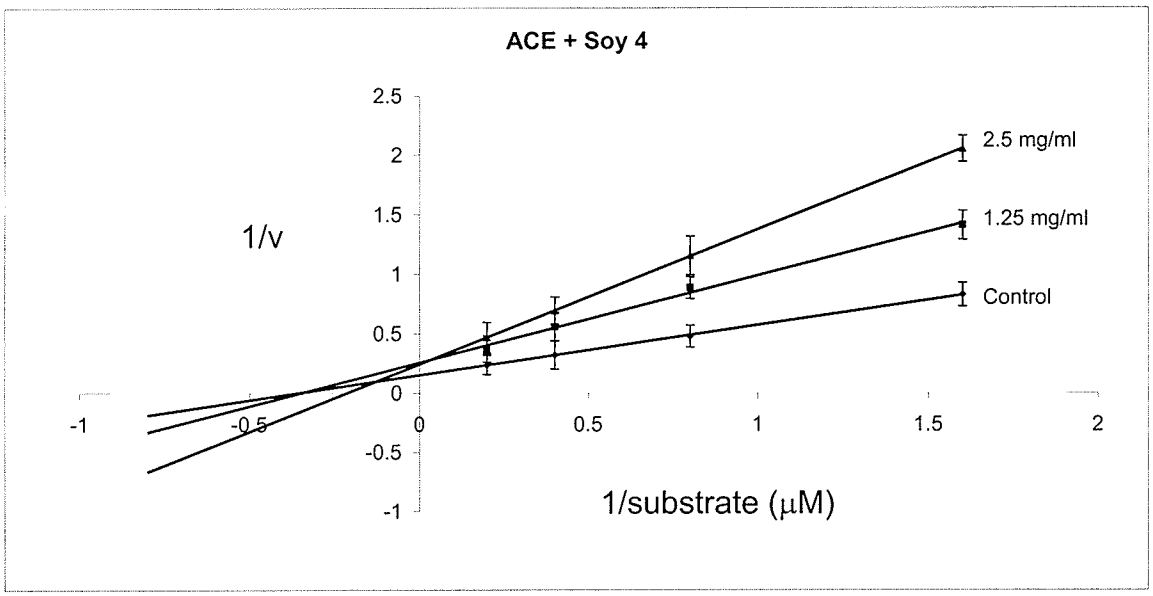
Lineweaver-Burk Plots of ACE Inhibition with Peptide Fraction 3 of Soy



Slope lines of sample and substrate seem to cross each other at the same point at x-axis in this figure. Considering Km values in Table 4.4.1, it appears that Km values of peptide concentrations and substrate are very different from each other indicating a non-competitive inhibition in both low and high peptide concentration.

Figure 4.4.9

Lineweaver-Burk Plots of ACE Inhibition with Peptide Fraction 4 of Soy



Slope of substrate and peptide in low concentration join each other close to x-axis. Km value of peptide (Table 4.4.1) shows that Km of peptide in low concentration is close to Km value of substrate which indicates a non-competitive inhibition for low concentration of fraction 4. Meanwhile, Km value of peptide with high concentration is different from that of substrate indicating a mixed-typed inhibition.

4.5. Determination of ACE Structure when Bound to Peptides

Fluorescence studies of ACE in the absence and presence of peptides with high and low concentrations determined the structural consequences to ACE structure after interaction with peptides. Table 4.5.1 shows the ratios of fluorescence intensity (FI) of ACE + peptide (FPEP) to FI of ACE (FA) as well as maximum emission wavelength (λmax) values. Fluorescence intensity of all fractions was measured with high sensitivity, except fraction 6 of casein and fraction 3 of soy, which were measured with medium sensitivity because of their high protein concentration. Addition of peptides to ACE increased the FI of peptide (FPEP) of all fractions. But the interesting finding was that FPEP significantly ($p<0.05$) decreased when the concentration of peptide increased from 0.05 to 0.1 mg/ml for all fractions with both low and high concentrations, except fractions 1 and 6 of casein and fraction 3 of soy. After subtraction the FI of ACE (FA) from ACE + Peptide (FPEP), ratio of FPEP/FA increased significantly ($p<0.05$) for fraction 1 of casein (Table 4.5.1 & Figure 4.5.1). As shown in Figure 4.5.1 the FPEP of fraction 1 of casein increased from 25 to 55 with the increasing of the concentration of peptide from 0.05 mg/ml to 0.1 mg/ml. Also FPEP and FPEP/FA ratio of casein 6 and soy 3 increased with medium sensitivity. We can explain that the increasing of the peptide concentration of fractions 1, and 6 of casein and fraction 3 of soy can unfold the ACE structure and expose more aromatic amino acids such as tryptophan.

The rest of casein and soy fractions, including fractions 2, 3, 4, and 5 of casein and fractions 2 and 4 of soy, showed different results. According to figures (4.5.2, 4.5.3, 4.5.4, 4.5.5) and table (4.5.1), FPEP/FA ratios of fractions 2, 3, 4, and 5 of casein decreased, significantly ($p<0.05$) for fraction 2 and 5, when the concentration of peptide

Table 4.5.1

Changes of Fluorescence Intensity of ACE in the Presence of ACE Inhibitor Peptides of Casein with low concentration (0.05 mg/ml)

Sample	λmax(nm)*	FPEP *	FPEP/FA
ACE	331±1.2[c]	94.22±1.8[d]	
C1 (B-A)	347±2.1[a]	25.79±1.9[a]	0.265
C2 (B-A)	347±1.6[a]	52.91±2.1[b]	0.553
C3 (B-A)	341±1.5[b]	49.19±1.4[b]	*0.521*
C4 (B-A)	344±0.7[ab]	33.22±1.6[c]	*0.351*
C5 (B-A)	346±1.4[ab]	38.85±0.8[c]	*0.404*
C6 (B-A)	341±0.9	20.92±0.5	0.212

* Values are Mean±SD

Italic: Increasing peptide concentration concludes enzyme unfolding

Black: Increasing peptide concentration conclude enzyme folding

Excitation wavelength: 295 nm

FPEP: Maximum FI of ACE+Peptide

FA: FI of ACE

Table 4.5.2

Changes of Fluorescence Intensity of ACE in the Presence of ACE Inhibitor Peptides of Casein with high concentration (1 mg/ml)

Sample	λmax(nm)*	FPEP *	FPEP/FA
ACE	331±1.2[c]	94.22±1.8[d]	
C1 (C-A)	343±1.1[a]	55.53±2.3[a]	0.585
C2 (C-A)	326±0.8[b]	9.03±0.73[b]	0.095
C3 (C-A)	325±1.2[b]	26.22±2.9[c]	*0.276*
C4 (C-A)	343±0.6[a]	28.88±1.8[c]	*0.297*
C5 (C-A)	345±0.5[a]	15.38±1.5[b]	*0.159*
C6 (C-A)	340±0.4	30.12±2.2	0.319

* Values are Mean±SD

Italic: Increasing peptide concentration concludes enzyme unfolding

Black: Increasing peptide concentration conclude enzyme folding

Excitation wavelength: 295 nm

FPEP: Maximum FI of ACE+Peptide

FA: FI of ACE

Table 4.5.3

Changes of Fluorescence Intensity of ACE in the Presence of ACE Inhibitor Peptides of Soy

Sample	λmax(nm)*	FPEP *	FPEP/FA
ACE	331±1.2	94.22±1.8	
S2 (B-A)	353±1.6	78.06±2.7	*0.828*
S2 (C-A)	356±0.6	49.06±3.2	*0.520*
S3 (B-A)	348±0.8	3.28±0.2	0.168
S3 (C-A)	354±1.5	5.01 ±0.5	0.258
S4 (B-A)	353±0.9	35.51±1.1	*0.376*
S4 (C-A)	361±1.4	13.42±0.3	*0.142*

* Values are Mean±SD

Italic: Increasing peptide concentration concludes enzyme unfolding

Black: Increasing peptide concentration conclude enzyme folding

Excitation wavelength: 295 nm

FPEP: Maximum FI of ACE+Peptide

FA: FI of ACE

increased from 0.05 to 0.1 mg/ml. For example, FPEP and FPEP/FA ratio of fraction 2 of casein decreased from 52 to 9 and 0.55 to 0.09, respectively. Also FPEP and FPEP/FA of fraction 5 of casein decreased significantly ($p<0.05$) from 38 to 15 and 0.40 to 0.15, respectively (Figure 4.5.5). In addition, FPEP and FPEP/FA of fractions 3 and 4 of casein decreased significantly ($p<0.05$). Although, FI of fraction 6 of casein was measured by medium sensitivity, FPEP and FPEP/FA of fraction 6 increased from 20 to 30 and 0.21 to 0.31, respectively (Figure 4.5.6). Also FPEP and FPEP/FA of fractions 2 and 4 of soy, decreased from 78 to 49, 0.82 to 0.52 for fraction 2 and from 35 to 13, 0.37 to 0.14 for fraction 4, respectively (Figures 4.5.7 & 4.5.9).

We conclude that ACE structure was folded after increasing the peptide concentration of fractions 2, 3, 4, and 5 of casein and fractions 2 and 4 of soy, indicating less exposure of hydrophobic groups, especially tryptophan. In addition, it could be concluded that any conformation changes in ACE structure prevents light absorption, resulting in less light emission and reducing fluorescence intensity.

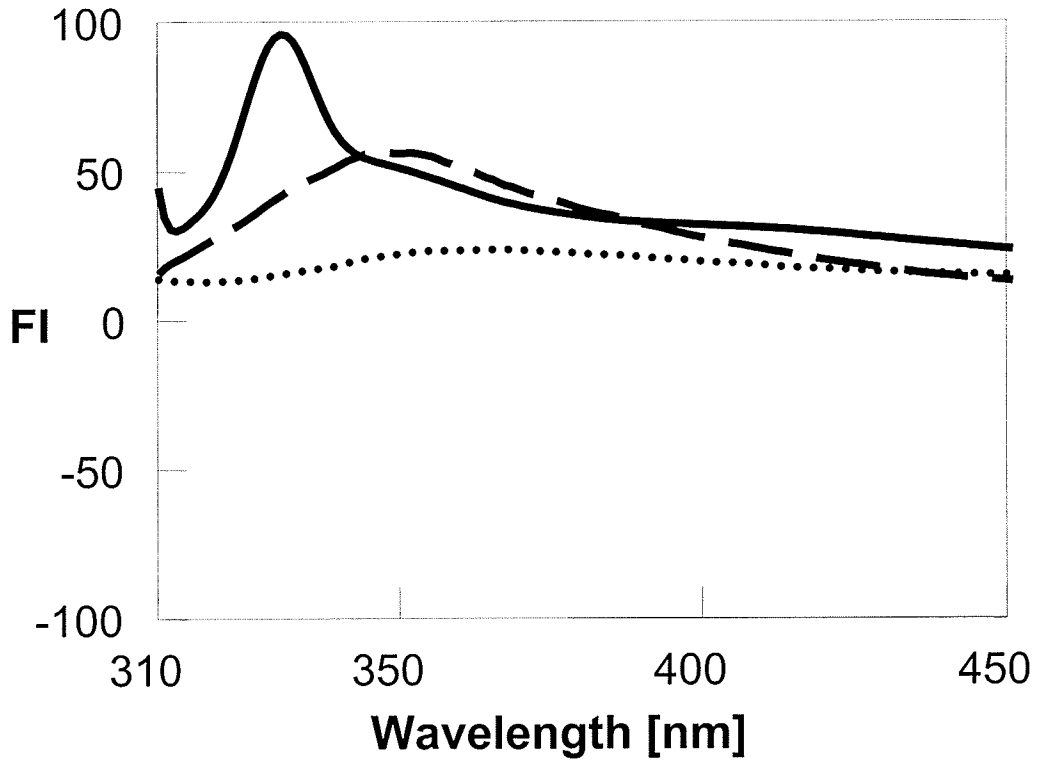
Another hypothesis is that peptide molecules may adhere to the enzyme in those sites adjacent to the tryptophan amino acids. In this case, tryptophan amino acids are not able to absorb and emit the light properly. Also, a significant change ($p<0.05$) was observed in maximum wavelength when peptides were added to ACE compared with maximum wavelength of ACE in both low and high concentration for all fractions of casein. As shown in Table 4.5.1, the maximum emission wavelength of casein fractions (λmax) had a blue shift with the increase of peptide concentration from 347 to 343, 347 to 326, 341 to 325, 344 to 343, 346 to 345, and 341 to 340 for fractions 1 to 6

respectively indicating that tryptophan components were exposed to a more hydrophobic environment.

Conversely, the data obtained from soy fraction 3 (Table 4.5.1) indicate that the maximum emission wavelength of soy fraction 3 had a red shift from 348 to 354 indicating tryptophan residues exposed in a hydrophilic environment. The maximum emission wavelength of fractions 2 and 4 of soy increased from 353 to 356 and 353 to 361, respectively (red shift) while we expected a decrease (blue shift) relating to the decreasing ratio of FPEP/FA (Table 4.5.1). Therefore, the FPEP/FA ratios of fractions 2, 3, 4, and 5 of casein and fractions 2 and 3 of soy decreased when we increased the peptide concentration which described the decreased binding ability of peptide to ACE, and hence less tryptophan exposure. However, the FPEP/FA ratios of fractions 1 and 6 of casein and fraction 3 of soy had opposite data (Table 4.5.1). The FPEP/FA ratio of fraction 1 and 6 of casein increased from 0.265 to 0.585 (casein 1) and 0.212 to 0.319 (casein 6). In the case of soy, the FPEP/FA ratio of fraction 3 increased from 0.168 to 0.258.

Figure 4.5.1 (Casein 1)

Effect of Peptide Fraction 1 of Casein on ACE Structure



This figure shows that the increasing of the concentration of peptide (casein 1) increases the FI of ACE. This demonstrates the unfolding effect of fraction 1 of casein on ACE.

A. FI of ACE

B. FI of *ACE+ peptide* with low concentration of peptide (LCP): 0.05 (mg/ml)

C. FI of *ACE+ peptide* with High concentration of peptide (HCP): 0.1 (mg/ml)

B-A = (ACE+ peptide, LCP) - ACE

C-A = (ACE+ peptide, HCP) - ACE

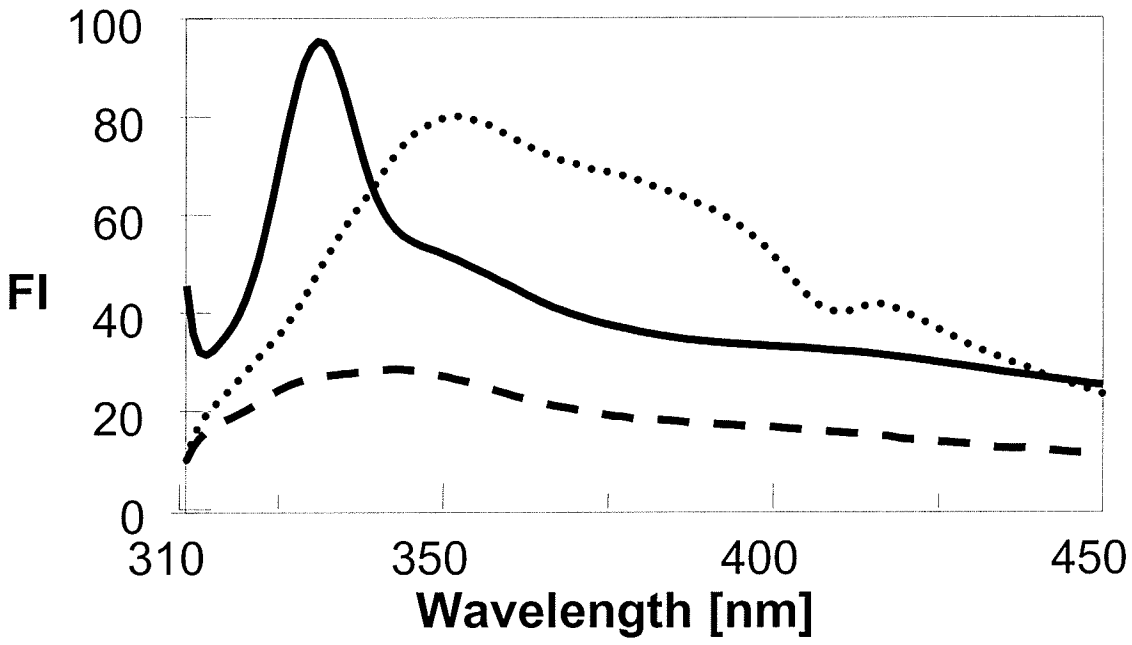
——— ACE

••••••••• C1 (B-A) Low concentration

– – – C1 (C-A) High concentration

Figure 4.5.2 (Casein 2)

Effect of Peptide Fraction 2 of Casein on ACE Structure



This figure shows that the increasing of the concentration of peptide (casein 2) decreases the FI of ACE. This demonstrates the folding effect of fraction 2 of casein on ACE.

A. FI of ACE

B. FI of *ACE+ peptide* with low concentration of peptide (LCP): 0.05 (mg/ml)

C. FI of *ACE+ peptide* with High concentration of peptide (HCP): 0.1 (mg/ml)

B-A = (ACE+ peptide, LCP) - ACE

C-A = (ACE+ peptide, HCP) - ACE

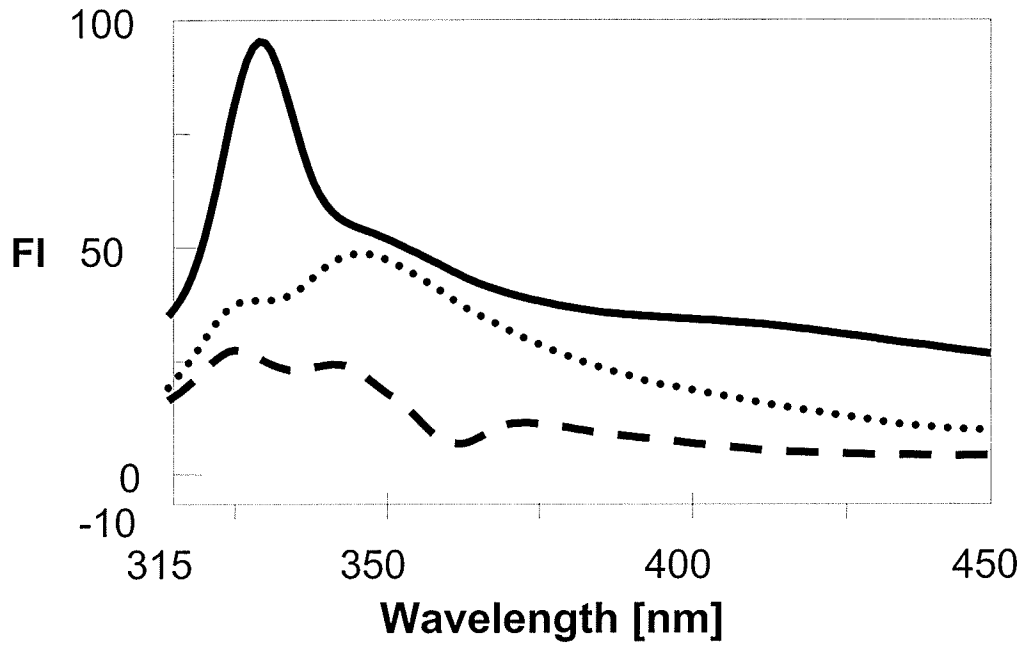
——— ACE

••••••••• C2 (B-A) Low concentration

– – – C2 (C-A) High concentration

Figure: 4.5.3 (Casein 3)

Effect of Peptide Fraction 3 of Casein on ACE Structure



This figure shows that the increasing of the concentration of peptide (casein 3) decreases the FI of ACE. This demonstrates the folding effect of fraction 3 of casein on ACE.

A. FI of ACE

B. FI of *ACE+ peptide* with low concentration of peptide (LCP): 0.05 (mg/ml)

C. FI of *ACE+ peptide* with High concentration of peptide (HCP): 0.1 (mg/ml)

B-A = (ACE+ peptide, LCP) - ACE

C-A = (ACE+ peptide, HCP) - ACE

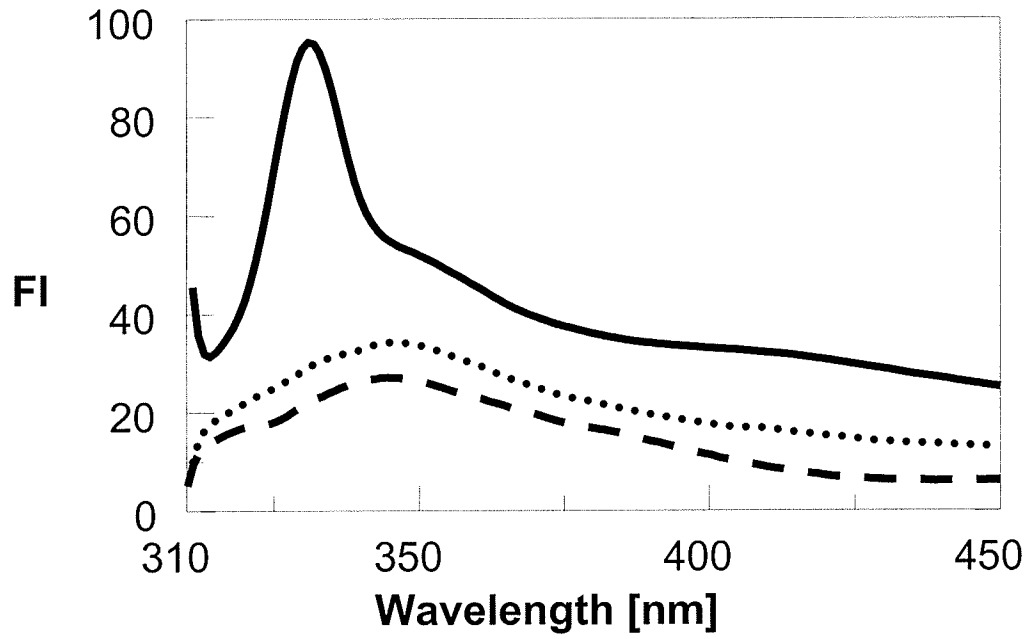
——— ACE

••••••••• C3 (B-A) Low concentration

– – – C3 (C-A) High concentration

Figure 4.5.4 (Casein 4)

Effect of Peptide Fraction 4 of Casein on ACE Structure



This figure shows that the increasing of the concentration of peptide (casein 4) decreases the FI of ACE. This demonstrates the folding effect of fraction 4 of casein on ACE.

A. FI of ACE

B. FI of *ACE+ peptide* with low concentration of peptide (LCP): 0.05 (mg/ml)

C. FI of *ACE+ peptide* with High concentration of peptide (HCP): 0.1 (mg/ml)

B-A = (ACE+ peptide, LCP) - ACE

C-A = (ACE+ peptide, HCP) - ACE

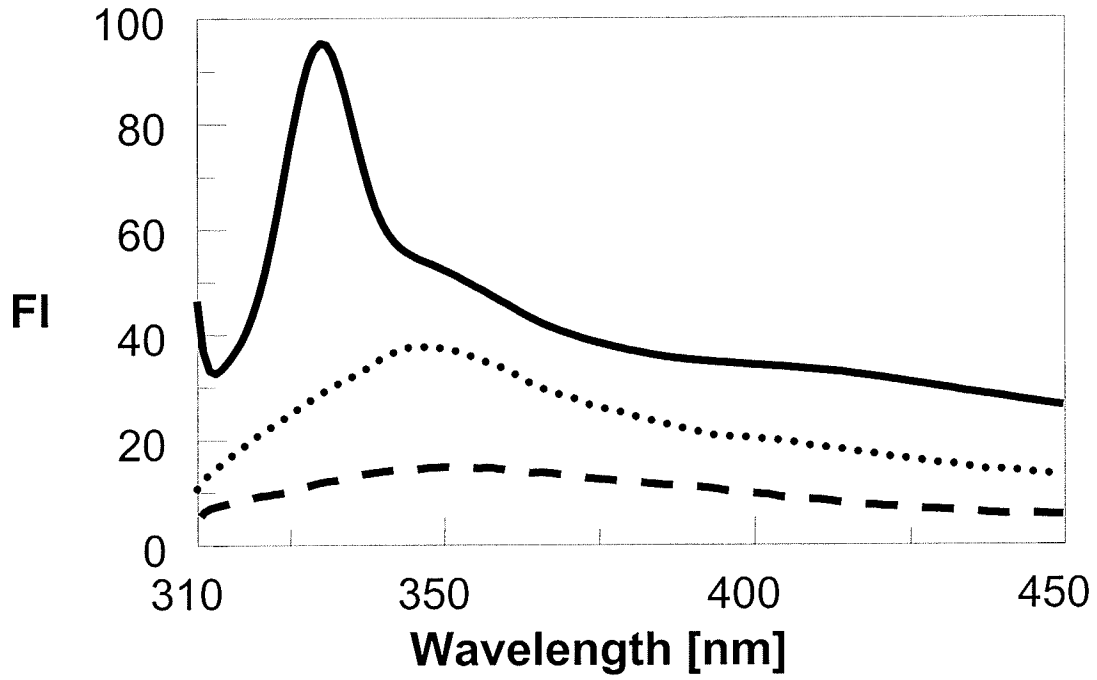
——— ACE

..... C4 (B-A) Low concentration

– – – C4 (C-A) High concentration

Figure: 4.5.5 (Casein 5)

Effect of Peptide Fraction 5 of Casein on ACE Structure



This figure shows that the increasing of the concentration of peptide (casein 5) decreases the FI of ACE. This demonstrates the folding effect of fraction 5 of casein on ACE.

A. FI of ACE

B. FI of *ACE+ peptide* with low concentration of peptide (LCP): 0.05 (mg/ml)

C. FI of *ACE+ peptide* with High concentration of peptide (HCP): 0.1 (mg/ml)

B-A = (ACE+ peptide, LCP) - ACE

C-A = (ACE+ peptide, HCP) - ACE

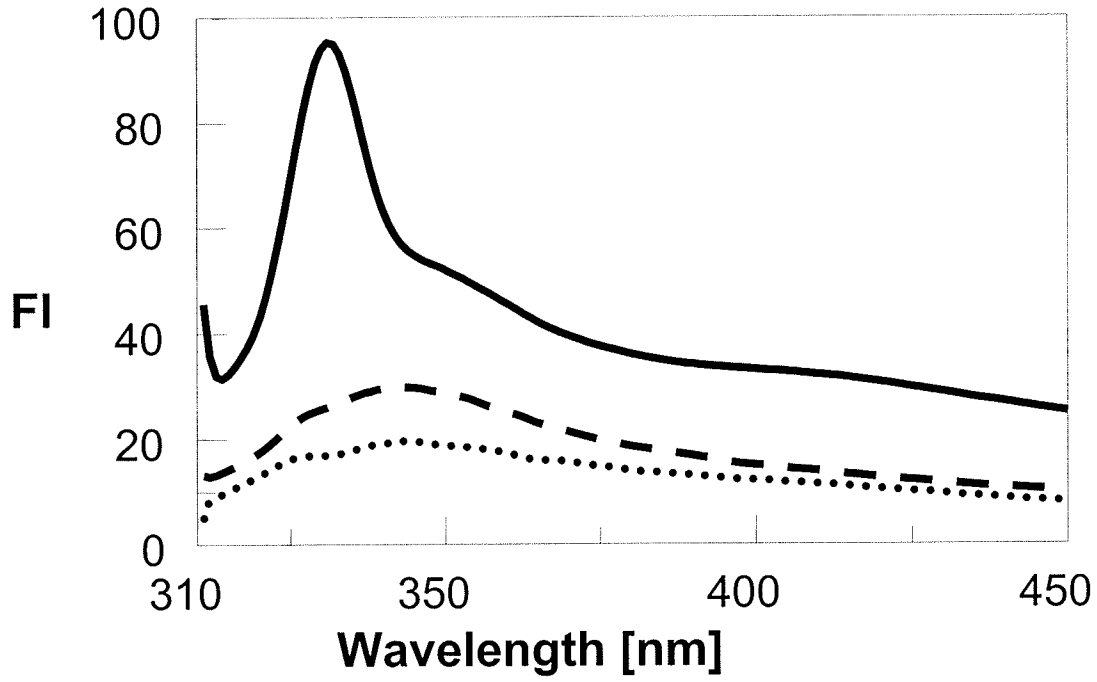
——— ACE

..... C5 (B-A) Low concentration

– – – C5 (C-A) High concentration

Figure: 4.5.6 (Casein 6)

Effect of Peptide Fraction 6 of Casein on ACE Structure



This figure shows that the increasing of the concentration of peptide (casein 6) increases the FI of ACE. This demonstrates the unfolding effect of fraction 6 of casein on ACE.

A. FI of ACE

B. FI of *ACE+ peptide* with low concentration of peptide (LCP): 0.05 (mg/ml)

C. FI of *ACE+ peptide* with High concentration of peptide (HCP): 0.1 (mg/ml)

B-A = (ACE+ peptide, LCP) - ACE

C-A = (ACE+ peptide, HCP) - ACE

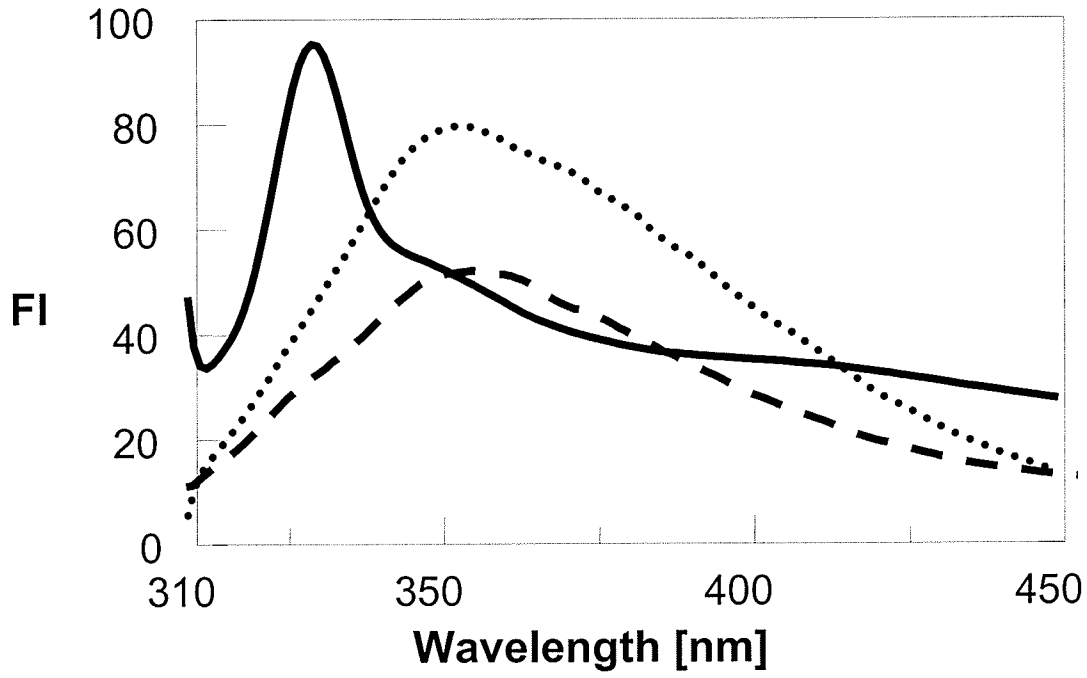
——— ACE

..... C6 (B-A) Low concentration

– – – C6 (C-A) High concentration

Figure: 4.5.7 (Soy 2)

Effect of Peptide Fraction 2 of Soy on ACE Structure



This figure shows that the increasing of the concentration of peptide (soy 2) decreases the FI of ACE. This demonstrates the folding effect of fraction 2 of soy on ACE.

A. FI of ACE

B. FI of *ACE+ peptide* with low concentration of peptide (LCP): 0.05 (mg/ml)

C. FI of *ACE+ peptide* with High concentration of peptide (HCP): 0.1 (mg/ml)

B-A = (ACE+ peptide, LCP) - ACE

C-A = (ACE+ peptide, HCP) - ACE

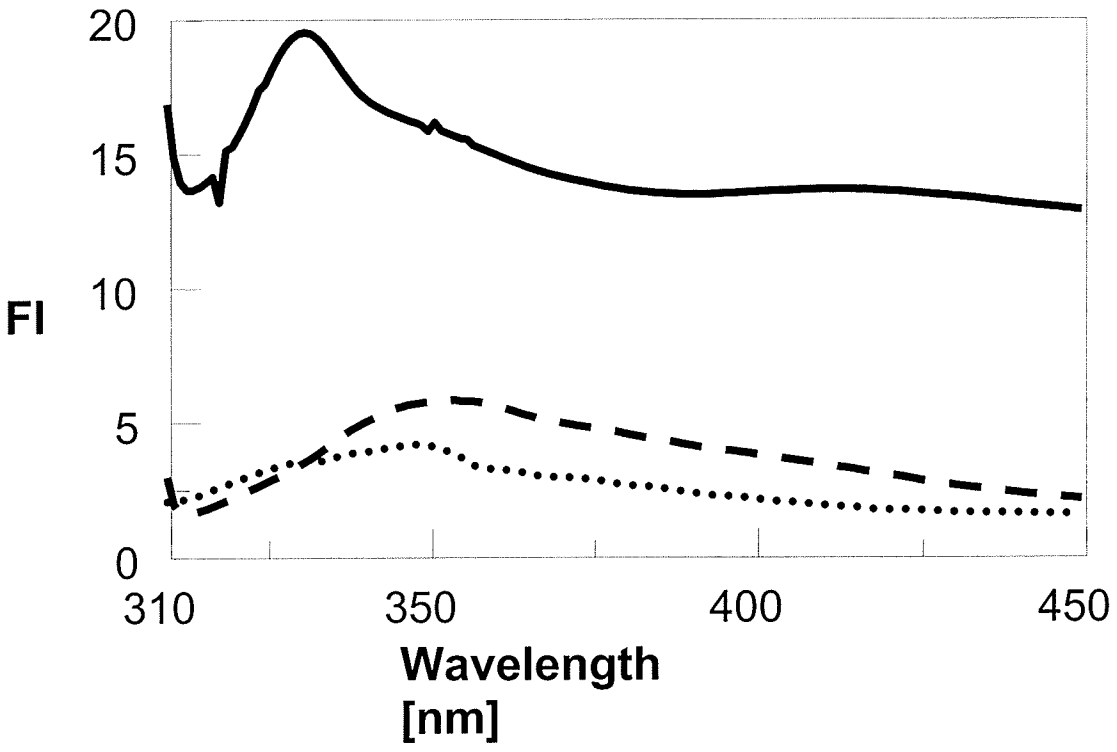
——— ACE

••••••••• S2 (B-A) Low concentration

– – – S2 (C-A) High concentration

Figure: 4.5.8 (Soy 3)

Effect of Peptide Fraction 3 of Soy on ACE Structure



This figure shows that the increasing of the concentration of peptide (soy 3) increases the FI of ACE. This demonstrates the unfolding effect of fraction 3 of soy on ACE.

A. FI of ACE

B. FI of *ACE+ peptide* with low concentration of peptide (LCP): 0.05 (mg/ml)

C. FI of *ACE+ peptide* with High concentration of peptide (HCP): 0.1 (mg/ml)

B-A = (ACE+ peptide, LCP) - ACE

C-A = (ACE+ peptide, HCP) - ACE

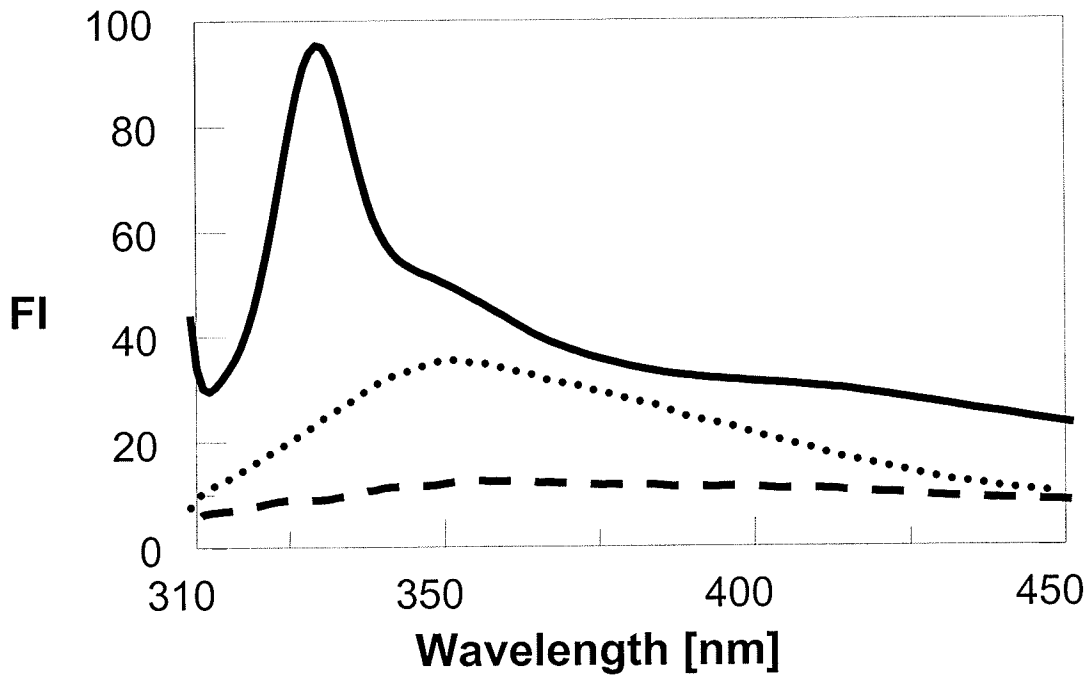
——— ACE

..... S3 (B-A) Low concentration

– – – S3 (C-A) High concentration

Figure: 4.5.9 (Soy 4)

Effect of Peptide Fraction 4 of Soy on ACE Structure



This figure shows that the increasing of the concentration of peptide (soy 4) decreases the FI of ACE. This demonstrates the folding effect of fraction 4 of soy on ACE.

A. FI of ACE

B. FI of *ACE+ peptide* with low concentration of peptide (LCP): 0.05 (mg/ml)

C. FI of *ACE+ peptide* with High concentration of peptide (HCP): 0.1 (mg/ml)

B-A = (ACE+ peptide, LCP) - ACE

C-A = (ACE+ peptide, HCP) - ACE

——— ACE

..... S4 (B-A) Low concentration

– – – S4 (C-A) High concentration

4.6 Radical Scavenging Activity (RSA)

Previous studies regarding antioxidant activity of soy protein mainly focused on radical scavenging effects of isoflavones (Lee, 2006) or soy protein isolate (Greaves et al., 1999) and it was not certain if isoflavones were responsible for soy antioxidant activity or the other components of soy such as polyunsaturated fats, fiber, vitamins, and minerals found in soy protein. Antioxidant activities of peptides have been found mostly among His-containing peptides. For example, the antioxidative properties of carnosine, a dipeptide from animal skeletal muscle, have been extensively reviewed (Chan & Decker, 1994; Quinn et al., 1992). In our study, we evaluated the radical scavenging ability of soy and casein fractions using DPPH, a free stable radical. We measured SC_{50}, the concentration of peptide required to scavenge one-half of free radical (SC_{50}), of casein and soy fractions to evaluate their radical scavenging activities. Fraction 5 of casein was identified as the strongest scavenging fraction among the other casein peptides, when SC_{50} value was calculated as 5.58 mg/ml with corrected value equal to 5.58 × 76.77% (Protein concentration) = 4.28 mg/ml. A significant radical scavenging activity ($p<0.05$) was observed for fraction 5 with all three concentrations (1.56 mg/ml, 3.125 mg/ml, and 6.25 mg/ml) and fraction 1 with lowest concentration (1.56 mg/ml), while there was little change for the other fractions with different concentrations (Figure 4.5.1).

Compared to other studies, Suetsuna et al. (2000) separated a strong radical scavenging activity peptide from casein protein hydrolysate by ion-exchange chromatography and gel filtration. They further separated the obtained fractions by octadecylsilano-high performance liquid chromatography and extracted a peptide with amino acid sequence of Tyr-Phe-Tyr-Pro-Glu-Leu. The SC_{50} values of this peptide for

superoxide anion scavenging activity (SOSA) and DPPH were 79.2 μM and 98 μM, respectively. In the case of soy, SC_{50} of fraction 3 was calculated as 1.43 mg/ml; however, with regard to 75% protein content of fraction 3, the corrected SC_{50} of fraction 3 is $1.43 \times 0.75 = 1.07$ mg/ml. Moreover, all three fractions of soy with peptide concentrations of 1.25 (mg/ml) and 2.5 (mg/ml) significantly ($p<0.05$) scavenged DPPH. As shown in Figures 4.6.1 and 4.6.2 radical scavenging ability of soy fractions was more than that of casein fractions at the same concentration. Further, the scavenging ability of both soy and casein is dose-dependent.

Compared with other studies, few specific works have reported the role of soy peptides in radical scavenging activity, while soy protein isolates or isoflavone studies have generally demonstrated antioxidant activities. Chen et al. (1998) investigated the radical scavenging effects of 22 synthetic His-containing peptides, designed on the basis of an antioxidative peptide derived from a proteolytic digest of soybean protein. They applied various methods to evaluate antioxidant effects of the peptides and found that the histidine-containing peptides had a quenching activity on singlet oxygen; however, they did not show antioxidant activity in an (2, 4-dimethylvaleronitrile)-induced oxidation system or scavenging effects on 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH) and superoxide. Radical scavenging ability of soy and casein can explain the antihypertensive (Yang et al., 2005), anti-atherosclerosis (Anderson et al. 1995), and some cancer preventive characteristics of these proteins (Badger et al. 2005; Kayser et al., 1996), in regard to the epidemiological studies in Asian countries, where people consume more soy foods and suffer less from cardiovascular diseases (Sacks et al., 2006). However, there are some controversial theories about the role of soy in cancers specifically breast.

Figure 4.6.1

A Comparison of Radical Scavenging Activity of Casein Fractions in Different Concentrations of Peptides

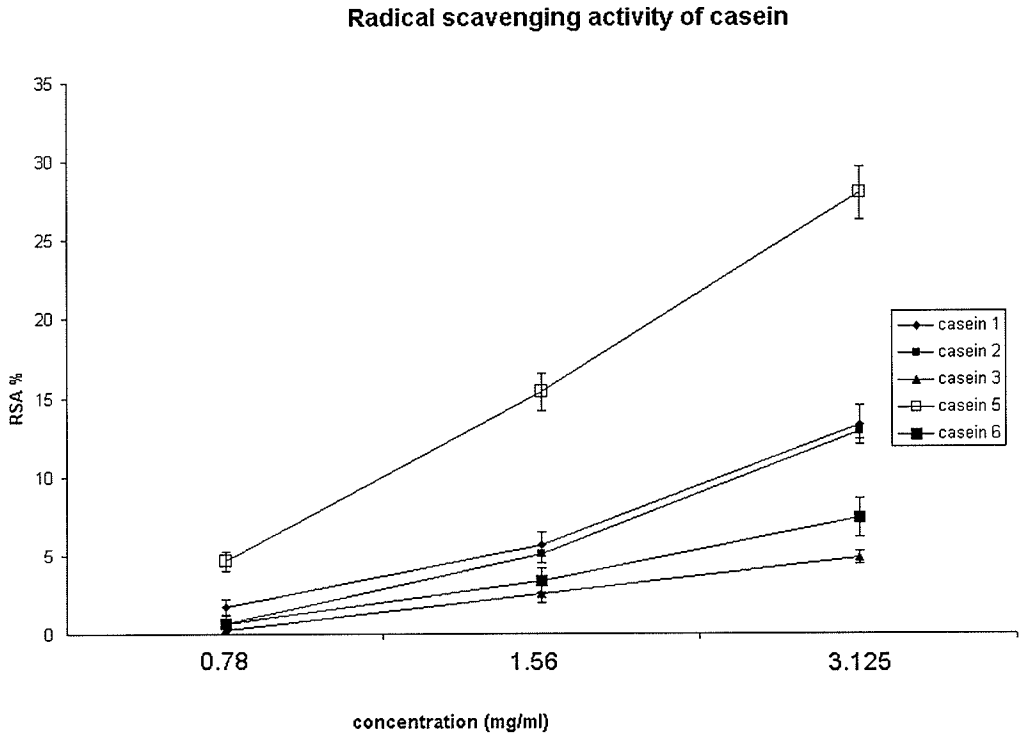
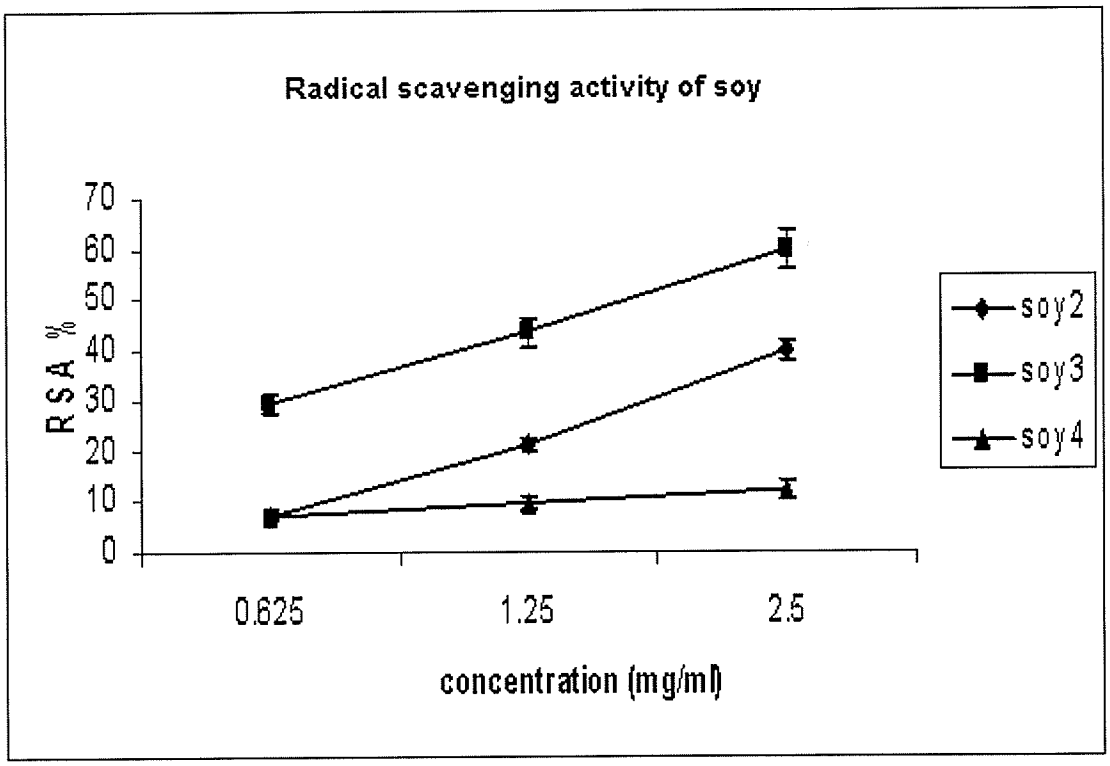


Figure 4.6.2

A Comparison of Radical Scavenging Activity of Soy Fractions in Different Concentration of Peptides



CONCLUSION AND FUTURE STUDIES

Soy protein and casein were successfully hydrolyzed by pepsin and pancreatin; seven fractions were obtained from casein and 5 fractions were obtained from soy protein after fast protein liquid chromatography (FPLC). The first 3 eluted fractions of casein were in the form of a sticky paste, with low protein content, compared with the last four fractions, in the form of a very soft and white powder. All five fractions of soy were obtained in the form of a yellow powder. The first fraction of soy did not have enough protein content to show the biological activities. Fractions 2 and 6 of casein and 3 and 4 of soy significantly ($p<0.05$) inhibited ACE, while fraction 5 of casein, and all fractions of soy scavenged DPPH significantly ($p<0.05$). Soy fractions showed more scavenging activity than casein fractions. Conversely, casein fractions inhibited ACE more potently than soy fractions. Potent ACE inhibitor fractions showed stronger scavenging activity in both soy and casein. Soy and casein peptides inhibited ACE non-competitively in low concentration, which indicates that isolated soy or casein peptides bind at a site other than the active site of ACE. On the other hand, binding of peptides led to conformational changes in ACE enzyme (folding of structure) that decreased catalytic activity, as shown with spectrofluorometric studies.

For future studies, obtained soy protein and casein peptides can be further separated by HPLC and GPC to extract smaller peptides. The amino acid sequence of eluted peptides should be identified. Also, in vivo studies to determine the potential health benefits of the peptide fractions are essential. Florescence studies for other aromatic amino acids like tyrosine and phenylalanine should be assessed.

REFERENCES

Abubakar, A., Tadao, S., Kitazawa, H., Kawai, Y. & Itoh, T. (1998). Structural analysis of new antihypertensive peptides derived from cheese whey protein by proteinase K digestion. *J. Dairy Sci. 81*: 3131–3138.

Ahn, S. W., Kim, K. M., Yu, K. W., Noh, D. O., Suh, H. J. (2000). Isolation of angiotensin I converting enzyme inhibitory peptide from soybean hydrolysate. *Food Sci. Biotechnol. 9*, 378-381

Aluko, R.E. and Monu, E. (2003). Functional and bioactive properties of quinoa protein hydrolysates. *J. Food Sci. 68*: 1254-1258.

Aluko, R.E., Yada, R.Y., Lencki, R.W. and Marangoni, A.G. (1997). Structural and functional properties of a partially purified cowpea globulin modified with protein kinase and glycopeptidase. *J. Agric. Food Chem. 45*: 2907-2913.

Angiotensin converting enzyme system, accessed August 1st, 2006 from http://www.scielo.br/img/fbpe/qn/v22n3/1091f1.gif

Anderson, J.W., Johnstone, B.M., Cook-Newell, M.E. (1995). A meta-analysis of the effects of soy protein intake on serum lipids. *N Engl J Med. 333*: 276–82.

Ariyoshi, Y. (1993). Angiotensin-converting enzyme inhibitors derived from food proteins. *Trends Food Sci. Technol. 4*: 139–144.

Burris, R. H., Roberts, G. P. (1993). Biological nitrogen fixation. *Ann. Rev. Nutr. 13*, 317-335.

Carpenter, K. J. (1994). *Protein and Energy*. A Study of Changing Ideas in Nutrition. Cambridge University Press. Cambridge, U.K., p 170.

Cerutti, P. (1994). Oxy-radicals and cancer. *Lancet. 344*: 862–865.

Cha M, Park JR. (2005). Production and characterization of a soy protein-derived angiotensin I-converting enzyme inhibitory hydrolysate. *J Med Food. 8*(3):305-310.

Chan, K.M., Decker, E.A. (1994). Endogenous skeletal muscle antioxidants. *Crit Rev Food Sci Nutr. 34* (4): 403-26.

Chen, J. R., Okada, T., Muramoto, K., Suetsuna, K., Yang, S. C. (2003). Identification of angiotensin I-converting enzyme inhibitory peptides derived from the peptic digest of soybean protein. *J. Food Biochem. 26*, 543-554.

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. *J Agric Food Chem.*, *46* (1): 49-53

Chen, Y.C., Sugiyama, Y., Abe, N., Kuruto-Niwa, R., Nozawa, R., Hirota, A. (2005). DPPH radical-scavenging compounds from dou-chi, a soybean fermented food. *Biosci Biotechnol Biochem.*, *69* (5): 999-1006

Cheung, H.S., Feng-Lai, W., Ondetti, M.A., Sabo, E.F. and Cushman, D.,W. (1980) Binding of peptide substrates and inhibitors of angiotensin-converting-enyzme. *J Biol Chem. 255*, 401-407

Chiang, W. D., Tsou, M. J., Tsai, Z. Y., Tsai, T. C. (2003). AngiotensinI-converting enzyme inhibitor derived from soy protein hydrolysate and produced by using membrane reactor. *Food Technology.* Accessed August 1st from. http://www.aseanfood.info/Articles/11016188.pdf

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

Corvol, P., Eyries, M. & Soubrier, F. (2004). Peptidyl-dipeptidase A/ angiotensin I-converting enzyme. *Handbook of Proteolytic Enzymes* (Barrett, AJ, Rawlings, ND & Woessner, JF, eds), pp. 332–346. Elsevier/Academic Press, Amsterdam.

Divi, R. L., Chang, H. C., Doerge, D. R. (1997). Anti-thyroid isoflavones from the soybean: Isolation, characterization, and mechanisms of action. *Biochem. Pharmacol.*, *54*, 1087-1096.

Ehlers, R. W. & Riordan, J. F. (1989). Angiotensin-converting enzyme: New concepts concerning its biological role. *Biochemistry 28*: 5311-5318.

Elitsur, Y., Luk, G.D. (1991). β-Casomorphin (BCM) and human colonic lamina propria lymphocyte proliferation, *Clin Exp Immunol. 85*, 493–497.

Erdos, E.G. (1990). Angiotensin I converting enzyme and the changes in our concepts through the years: Lewis K. Dahl memorial lecture. *Hypertension. 16*, 363–370.

FitzGerald, R.J. and Meisel, H. (1999). Lactokinins: Whey protein-derived ACE inhibitory peptides. *Nahrung. 43*, 165-167.

FitzGerald, R. J. & Meisel, H. (2000). Milk protein-derived peptide inhibitors of angiotensin-I-converting enzyme. *Br. J. Nutr. 84*, 33–37.

FitzGerald, R. J. & Meisel, H. (2003). Milk protein hydrolysates and bioactive peptides. Fox, P. F. McSweeney, P.L.H. eds. 3rd Edition, *Part B Advanced Dairy Chemistry 1*:675-698. Kluwer Academic/Plenum Publishers New York

Fleming, I. (2006). Signaling by the angiotensin-converting enzyme. *Circ Res.*; *98 (7)*: 887-96.

Frostegard, J., Hagerstrand, A., Gidlund, M., and Nilsson, J. (1991). Biologically modified low density lipoprotein increases the adhesive properties of endothelial cells. *Atherosclerosis 90*: 119–126.

Fujita, H., Usui, H., Kurahashi, K., Yoshikawa, M. (1995a). Isolation and characterization of Ovokinin, a bradykinin B1 agonist peptide derived from ovalbumin. *Peptides. 16*: 785–90.

Fujita, H., Sasaki, R., Yoshikawa, M. (1995b). Potentiation of the antihypertensive activity of orally administered Ovokinin, a vasorelaxing peptide derived from ovalbumin, by emulsification in egg phosphatidyl-choline. *Biosci Biotechnol Biochem. 59*: 2344–5

Fujita, H., Yokoyama, K., Yasumoto, R., Yoshikawa, M. (1995). Antihypertensive effect of thermolysin digest of dried bonito in spontaneously hypertensive rat. *Clin Exp Pharmacol Physiol Suppl., 22* (1): S304-5.

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

Garrett, D. A., Failla, M. L., Sarama, R. J. (1999). Development of an in vitro digestion method to assess carotenoid bioavailability from meals. *J. Agric. Food Chem. 47*, 4301-4309.

Gaucheron, F., Famelart, M. H., & Le Graet, Y. (1996). Iron- supplemented caseins: Preparation, physicochemical charac- terization and stability. *J. Dairy. Res. 63*, 233–243.

Gibbs, B. F., Zougman, A., Masse, R., Mulligan, C. (2004). Production and characterization of bioactive peptides from soy hydrolysate and soy-fermented food. *F. Res. Intl. 37*, 123-131

Gobbetti, M., Ferranti, P., Smacchi, E., Goffredi, F. & Addeo, F. (2000). Production of angiotensin-I-converting enzyme inhibitory peptides in fermented milks started by Lactobacillus delbruekii subsp. bulgaricus SS1 and Lactococcuslactis subsp cremoris FT4. *Appl. Envir. Microbiol. 66*: 3898–3904

Greaves, K. A., Parks, J. S., Williams, J. K., Wagner, J. D. (1999). Intact dietary soy protein, but not adding an isoflavone-rich soy extract to casein, improves plasma lipids in ovariectomized cynomolgus monkeys. *J Nutr, 129* (8): 1585-92.

Halliwell, B., and Gutteridge, J. M.C. (1999a). Ch. 1 Antioxidant Defences. In *Free Radicals in Biology and Medicine 3rd Ed.*, Oxford University Press-New York

Halliwell, B. (1994). Free radicals and antioxidants: A personal view. *Nutr Rev, 52*, 253–265.

Halliwell, B., & Whiteman, M. (2004). Measuring reactive species and oxidative damage in vivo and in cell culture: How should you do it and what do the results mean? *Br J Pharmacol, 142*, 231–255.

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. *Am J Clin Nutr*; *64* (5): 767-71.

Heart Protection Study Collaborative Group, (2002). MRC/BHF Heart Protection Study of antioxidant vitamin supplementation in 20,536 high-risk individuals: A randomized placebo-controlled trial. *Lancet 360*, 23–33.

Henriksen, T., Mahoney, E. M., and Steinberg, D. (1981). Enhanced macrophage degradation of low density lipoprotein previously incubated with cultured endothelial cells: Recognition of receptors for acetylated LDL. *Proc. Natl. Acad. Sci. USA 78*: 6499–6503.

Human Angiotensin I Converting Enzyme Structure Revealed, Macromolecular Crystallography. Accessed August 1,2006. From http://www.esrf.fr/UsersAndScience/Publications/Highlights/2003/MX/MX10/

Hymowitz, T., Harlan, J. R. (1983). Introduction of soybeans to North America by Samuel Bowen in 1765. *Econ. Bot. 37*, 371-379.

Inagami T. (1994). The renin-angiotensin system. *Essays Biochem. 28*: 147-64.

Johnston, J.I., Franz, V.L. (1992), Renin-angiotensin system: a dual tissue and hormonal system for cardiovascular control. *J Hypertens.10,* pp. s13–26

Karaki, H., Doi, K., Sugano, S., Uchiya, H., Sugai, R., Murakami, U. & Takemoto, S. (1990). Antihypertensive effect of tryptic hydrolysate of milk casein in spontaneously hypertensive rats. *Comp. Biochem. Physiol. 96C*: 367-371

Karlsson, J., & Karisson, J. (1997). *Antioxidants and exercise*, P. O. Box 5076, Champaign, IL, Human Kinetics.

Katayama, K., Fuchu, H., Sakata, A., Kawahara, S., Yamauchi, K., Kawamura, Y., Muguruma, M. (2003). Angiotensin I-converting enzyme inhibitory activities of porcine skeletal muscle proteins following enzyme digestion. *Asian-Aust. J. Anim. Sci. 16*, 417-424.

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

Kitts, D. D., Weiler, K. (2003). Bioactive proteins and peptides from food sources. Applications of bioprocesses used in isolation and recovery. *Curr Pharm. 9*(16): 1309-1323.

Kohama, Y., Matsumoto, S., Oka, H., Teramoto, T., Okabe, M., Mimura, Y. (1988). Isolation of angiotensin-converting enzyme-inhibitor from tuna muscle. *Biochem. Biophys. Res. Commun. 155*, 332-337

Korhonen, H., Pihlanto-Leppälä, A., Rantamäki, P. and Tupasela, T. (1998). Impact of processing on bioactive proteins and peptides. *Trends Food Sci Technol, 9* 307–319.

Laakso, S. (1984). Inhibition of lipid peroxidation by casein: Evidence of molecular encapsulation of 1,4-pentadiene fatty acids. *Biochim. Biophys. Acta . 792* , 11–15.

Lee, J. S. (2006). Effects of soy protein and genistein on blood glucose, antioxidant enzyme activities, and lipid profile in streptozotocin-induced diabetic rats. *Life Sci.*; [Epub ahead of print]

Lee, N. Y., Cheng, J. T., Enomoto, T., Nakamura, I. (2006). The antihypertensive activity of angiotensin-converting enzyme inhibitory peptide containing in bovine lactoferrin. *Chin J Physiol.*; *49* (2): 67-73.

Li, H. & Aluko, RE. (2005). Kinetics of the inhibition of calcium/calmodulin-dependent protein kinase II by pea protein-derived peptides. *J Nut Biochem. 16*, 656-662

Lo, W. M., Li-Chan, E. C. (2005). Angiotensin I converting enzyme inhibitory peptides from in vitro pepsin-pancreatin digestion of soy protein. *J Agric Food Chem., 53* (9): 3369-3376.

Loft, S., and Poulsen, H. (1996). Cancer risk and oxidative DNA damage in men. *J. Mol. Med. 74*: 297.

Loo, G. (2003). Redox-sensitive mechanisms of phytochemical-mediated inhibition of cancer cell proliferation. *J. Nutr. Biochem. 14*: 64–73.

Luiking, Y. C., Deutz, N. E., Jakel, M., Soeters, P. B. (2005). Casein and soy protein meals differentially affect whole-body and splanchnic protein metabolism in healthy humans.J. Nutr.; *135*(5): 1080-1087

Lupi, R., Guerra, S. D., Bugliani, M., Boggi, U., Mosca, F., Torri, S., Del Prato, S. and Marchetti, P. (2006). The direct effects of the angiotensin-converting enzyme inhibitors, zofenoprilat and enalaprilat, on isolated human pancreatic islets. *Eur. J. Endocrinol.*; *154*: 355 – 361

Manso, M. A., Lopez-Fandino, R. (2003). Angiotensin I converting enzyme-inhibitory activity of bovine, ovine, and caprine kappa-casein macropeptides and their tryptic hydrolysates. *J food prot ,66:* 9, 1686 –1692.

Maeno, M., Yamamoto, N. & Takano, T. (1996) Identification of antihypertensive peptides from casein hydrolysate produced by a proteinase from Lactobacillus helveticus CP790. *J. Dairy Sci. 73*:1316-1321.

Markwell, M. A. C., Maas, S. M., Biebar, L. L. and Tolbert, N. E.: (1978). A modification of the Lowry procedure to simplify protein determination in membrane and in protein samples. *Anal. Biochem. 87*: 206-211.

Martin, D. S., Williams, J. L., Breitkopf, N. P., Eyster, K. M. (2002). Pressor responsiveness to angiotensin in soy-fed spontaneously hypertensive rats. *Can J Physiol Pharmacol.*; *80* (12): 1180-1186.

Maruyama, S., Suzuki, H. (1982). A peptide inhibitor of angiotensin I converting enzyme in the tryptic hydrolysate of casein. *Agric. Biol. Chem. 46*:1393–1394

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. *Agric. Biol. Chem. 51*:1581-1586.

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. *J Nutr. 126* (12): 3063-3068.

Mates, J. M., Perez-Gomez, C., and Nunez De Castro, I. (1999). Antioxidant enzymes and human diseases. *Clin Biochem 32*: 595–603.

Matsufuji, H., Matsui, T., Seki, E., Osajima, K., Nakashima, M., Osajima, Y. (1994) Angiotensin I-converting enzyme inhibitory peptides in an alkaline protease hydrolyzate derived from sardine muscle. *Biosci Biotechnol Biochem*; *58* (12): 2244-2245.

Matsui, T., Li, C. H., Osajima, Y. (1999). Preparation and characterization of novel bioactive peptides responsible for angiotensin I-converting enzyme inhibition from wheat germ. *J. Pept. Sci. 5*, 289-297.

Matsumura, N., Fujii, M., Takeda, Y., Sugita, K., Shimizu, T. (1993). Angiotensin I-converting enzyme inhibitory peptides derived from bonito bowels autolysate. *Biosci Biotechnol Biochem*; *57* (5): 695-697

McLarty, J. W. (1997). Antioxidants and Cancer: The Epidemiologic Evidence. (Ch. 5). Garewal, H. S (Ed). *Antioxidants and Disease Prevention*, Boca Raton, New York., CRC Press.

McMichael-Phillips, D. F., Harding, C., Morton, M., Roberts, S. A., Howell, A., Potten, C. S., Bundred, N. J. (1998). Effects of soy-protein supplementation on epithelial proliferation in the histologically normal human breast. *Am. J. Clin. Nutr. 68*, 1431S-1435S.

Megias, C., Del Mar Yust, M., Pedroche, J., Lquari, H., Giron-Calle, J., Alaiz, M., Millan, F., Vioque, J. (2004). Purification of an ACE inhibitory peptide after hydrolysis of sunflower (Helianthus annuus L.) protein isolates. *J Agric Food Chem*; *52* (7): 1928-1932.

Meisel, H., Sawatski, G. and Renner, B. (1993). Casokinins as inhibitors of angiotensin-I converting enzyme. *New Perspect Inft Nutr.* 153-159

Meisel, H. (2004). Multifunctional peptides encrypted in milk proteins. *Biofactors. 21*, 55-61

Meisel, H. (2005). Biochemical properties of peptides encrypted in bovine milk proteins. *Curr Med Chem. 12* (16): 1905-1919.

Melzig, M. F., Heder, G. (1995). Dexamethasone induced enhanced enkephalin degradation by angiotensin-converting enzyme (ACE) of endothelial cells. *Pharmazie*; *50* (2): 139-141.

Migliore-Samour, D., Floc'h, F., Joll´es, P. (1989). Biologically active casein peptides implicated in immunomodulation, *J Dairy Res. 56*, 357–362.

Miguel, M., Aleixandre, A. (2006). Antihypertensive peptides derived from egg proteins. *J Nutr*; *136 (6):* 1457-1460.

Minervini, F., Algaron, F., Rizzello, C. G., Fox, P. F., Monnet, V., Gobbetti, M. (2003). Angiotensin I-converting-enzyme-inhibitory and antibacterial peptides from Lactobacillus helveticus PR4 proteinase-hydrolyzed caseins of milk from six species. *Appl Environ Microbiol. 69*(9): 5297-5305.

Miguel, M., López-Fandiño, R., Ramos, M., Aleixandre, A. (2006). Long-term intake of egg white hydrolysate attenuates the development of hypertension in spontaneously hypertensive rats. *Life Sci*, 2960-2966.

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. *Agric. Biol. Chem. 55*, 1313-1318.

Mizushima, S., Ohshige, K., Watanabe, J., Kimura, M., Kadowaki, T., Nakamura, Y., Tochikubo, O., Ueshima, H. (2004). Randomized controlled trial of sour milk on blood pressure in borderline hypertensive men. *Am J Hypertens*; *17* (8):701-706.

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

Nakamura, Y., Yamamoto, N., Sakai, K., Takano, T. (1995b). Antihypertensive effect of sour milk and peptides isolated from it that are inhibitors to angiotensin I-converting enzyme. *J Dairy Sci. 78* (6): 1253-1257.

Niki, E., Hiramatsu, M. (1997). *Food and Free radicals*, New York, Springer

Nimmagudda, R. (2002) New bioactive peptides for the nutritional industry. Oral presentation at: SupplySide West Trade Show and Conference 2002 Las Vegas, NV December 4th to 6th

Okamoto, A., Hanagata, H., Kawamura, Y., Yanagida, F. (1995). Antihypertensive substances in fermented soybean, natto. *Plant Foods Hum. Nutr. 47*, 39-47.

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. 196*: 441–444.

Ono, S., Hosokawa, M., Miyashita, K., Takahashi, K. (2003). Isolation of peptides with angiotensin I-converting enzyme inhibitory effect derived from hydrolysate of upstream chum salmon muscle. *J. Food Sci. 68*, 1611-1614.

Otani, H., Kihara, Y., Park, M. (2000). The immunoenhancing property of a dietary casin phosphopeptide preparation in mice, *Food Agric Immunol. 12*, 165–173.

Palinski, W., Rosenfeld, M. E., Yla-Herttuala, S., Gurtner, G. C., Socher, S. S., Butler, S. W., Parthasarathy, S., Carew, T. E., Steinberg, D., and Witztum, J. L. (1989). Low-density lipoprotein undergoes oxidative modification in vivo. *Proc. Natl. Acad. Sci. 86*: 1372–1376.

Parrot, S., Degraeve, P., Curia, C., Martial-Gros, A. (2003). In vitro study on digestion of peptides in Emmental cheese: Analytical evaluation and influence on angiotensin I converting enzyme inhibitory peptides. *Nahrung/Food*. 87 – 94.

Pratico, D., Iuliano, L., Mauriello, A., et al. (1997). Localization of distinct F2-isoprostanes in human atherosclerotic lesions. *J. Clin. Invest. 100*: 2028–2034.

Pratt, D. E., and Birac, P. M., (1979). Source of antioxidant activity of soybeans and soy products. *J. Food Sci. 44*, 1720–1722

Punchard, N. A., and F. Kelly (1997). *Free Radicals-A Practical Approach*. Oxford University Press, New York.

Quiros, A., Hernandez, L. B., Ramos, M., Amigo, L., Recio, I. (2005). Angiotensin-converting enzyme inhibitory activity of peptides derived from caprine kefir. *J Dairy Sci.*; *88 (10):* 3480-3487.

Quinn, P. J., Boldyrev, A. A., Formazuyk, V. E. (1992). Carnosine: its properties, functions and potential therapeutic applications. *Mol Aspects Med. 13 (5):* 379-444.

Reynolds, E. C. (1997), Premineralization of enamel surface lesions by casein phosphopeptide-stabilized calium phosphate solutions, *J. Dent. Res. 76*, 1587–1595.

Rivas, M., Garay, R. P., Escanero, J. F., Cia, P. J., Alda, J. O. (2002). Soy milk lowers blood pressure in men and women with mild to moderate essential hypertension. *J Nutr*; *132* (7): 1900-1902.

Sacks, F. M., Lichtenstein, A., Van Horn, L., Harris, W., Kris-Etherton, P. Winston, M. (2006). Soy protein, isoflavones, and cardiovascular health: a summary of a statement for professionals from the american heart association nutrition committee. *Arterioscler Thromb Vasc Biol.*; *26* (8): 1689-1692

Saiga, A., Okumura, T., Makihara, T., Katsuta, S., Shimizu, T., Yamada, R., Nishimura, T. (2003). Angiotensin I-converting enzyme inhibitory peptides in a hydrolyzed chicken breast muscle extract. *J. Agric. Food Chem., 51*, 1741-1745

Salonen, T., Yla-Herttulal, S., and Yamamoto, R. (1992). Autoantibodies against oxidized LDL and progression of carotid atherosclerosis. *Lancet. 339*: 883–887.

Schmid, F.X. Spectral methods of characterizing protein conformation and conformational changes, In *Protein Structure: A Practical Approach*, T.E. Creghton, ed. pp 251-285, Oxford., Oxford University Press.

Schmaier, A. H., (2002). The plasma kallikrein-kinin system counterbalances the renin-angiotensin system. *J Clin Invest. 109*: 1007–1009

Seki, E., Osajima, K., Matsufuji, H., Matsui, T., Osajima, Y. (1995). Val-Tyr, an angiotensin-I converting enzyme-inhibitor from sardines that have resistance to gastrointestinal proteases. *J. Jpn. Soc. Biosci. Biotechol. Agrochem. 69*, 1013-1020.

Sekiya, S., Kobayashi, Y., Kita, E., Imamura, Y. & Toyama, S. (1992). Antihypertensive effects of tryptic hydrolysate of casein on normotensive and hypertensive volunteers. *J. Jap. Soc. Nutr. Food Sci. 45*:513-517.

Shigenaga, M., Hagen, T., and Ames, B. (1994). Oxidative damage and mitochondrial decay in ageing. *Proc. Natl. Acad. Sci. USA 91*: 10771–10778.

Shin, Z. I., Ahn, C. W., Nam, H. S., Lee, H. J., Lee, H. J., Moon, T. H. (1995). Fractionation of angiotensin converting enzyme (ACE) inhibitory peptides from soybean paste. *Korean J. Food Sci. Technol. 27*, 230-234.

Shin, Z. I., Yu, R., Park, S. A., Chung, D. K., Ahn, C. W., Nam, H. S., Kim, K. S., Lee, H. J. (2001). His-His-Leu, an angiotensin I converting enzyme inhibitory peptide derived from Korean soybean paste, exerts antihypertensive activity in vivo. *J Agric Food Chem.*; *49*(6): 3004-3009.

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. *J Dairy Res.*; *69* (1):103-11

Skidgel, R. A., Erdos, E. G. (2004). Angiotensin converting enzyme (ACE) and neprilysin hydrolyze neuropeptides: a brief history, the beginning and follow-ups to early studies. *Peptides*; *25* (3): 521-5.

Soubrier, F., Alhenc-Gelas, F., Hubert, C., Allegrini, J., John, M., Tregear, G., Corvol, P. (1988). Two putative active centers in human angiotensin I-converting enzyme revealed by molecular cloning. *Proc Natl Acad Sci. 85*: 9386–9390.

Steijns, J. M., van Hooijdonk, A. C. (2000). Occurrence, structure, biochemical properties and technological characteristics of lactoferrin. *Br J Nutr*; *84*, 1:S11-7.

Stepaniak, L., Jedrychowski, L., Wroblewska, B. & Sørhaug, T. (2001) Immunoreactivity and inhibition of angiotensin I converting enzyme and lactococcal oligopeptidase by peptides from cheese. Ital. *J. Food Sci. 13*:373-381.

Suetsuna, K., Ukeda, H. & Ochi, H. (2000). Isolation and characterization of free radical scavenging activity peptides derived from casein. *J Nutr Biochem*, *11*, 128–131.

Sun, C. L., Yuan, J. M., Arakawa, K., Low, S. H., Lee, H. P., Yu, M. C. (2002). Dietary soy and increased risk of bladder cancer: The Singapore Chinese Health Study. *Cancer Epidemiol. Biomarkers Prev. 11*, 1674-1677.

Swain, J. H., Alekel, D. L., Dent, S. B., Peterson, C. T., Reddy, M. B. (2002). Iron indexes and total antioxidant status in response to soy protein intake in perimenopausal women. *Am J Clin Nutr*; *76*: 165-171.

Takano, T. (1998) Milk derived peptides and hypertension reduction. *Int Dairy J. 8*, 375-381.

Takenaka, A., Annaka, H., Kimura, Y., Aoki, H., Igarashi, K. (2003). Reduction of paraquat-induced oxidative stress in rats by dietary soy peptide. *Biosci Biotechnol Biochem, 67*: 278-283.

Teixeira, S. R., Potter, S. M., Weigel, R. (2000). Effects of feeding 4 levels of soy protein for 3 and 6 wk on blood lipids and apolipoproteins in moderately hypercholesterolemic men. *Am J Clin Nutr*; *71*:1077–1084.

Trock, B. J., Hilakivi-Clarke, L. Clarke, R. (2006). Meta-analysis of soy intake and breast cancer risk. *J Natl Cancer Inst. 98* (7): 459-471.

Turner, A. J. & Hooper, N. M. (2002). The angiotensin-converting enzyme gene family. *Genomics and pharmacology. Trends Pharmcol. Sci. 23*:177-183.

Vermeirssen, V., Deplanke, B., Tappenden, K. A., Van Camp, J., Gaskins, H. R. & Verstraete, W. (2002) Intestinal transport of the lactokinin Ala-Leu-Pro-Met-His-Ile-Arg through a Caco-2 Bbe monolayer. *J. Pep. Sci. 8*:95-100.

Vincenzetti, S., Polidori, P., Salimei, E. Mariani, P. L., Allegri, S., Cammertoni, N., Quadrini, B. and Vita, A. (2005). Purification and Identification of α_{s1}- and β-Caseins from Asses Milk. *Vet Res Commun.*;*2*:211-213

Wei, L., Alhenc-Gelas, F., Corvol, P., and Clauser, E. (1991). Expression and characterization of recombinant human angiotensin I-converting enzyme. *J. Biol. Chem. 266*, 9002-9008

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. *J Biol Chem.*; *267*: 13398–133405

White, L., Petrovitch, H., Ross, G. W., Masaki, K. H., Abbott, R. D., Teng, E. L., Rodriguez, B. L., Blanchette, P. L., Havlik, R. J., Wergowske, G., Chiu, D., Foley, D. J., Murdaugh, C., Curb, J. D. (1996). Prevalence of dementia in older Japaneses-American men in Hawaii: The Honolulu-Asia Aging Study. *J. Am. Med. Assoc. 276*, 955-960.

Wu, J., Ding, X. (2001). Hypotensive and physiological effect of angiotensin converting enzyme inhibitory peptides derived from soy protein on spontaneously hypertensive rats. *J. Agric. Food Chem. 49*, 501-506.

Wu, J., Ding, X. (2002). Characterization of inhibition and stability of soy-protein-derived angiotensin I-converting enzyme inhibitory peptides. *Food Res. Int. 35*, 367-375.

Wyvratt, M. J., Patchett, A. A. (1985). Recent developments in the design of angiotensin converting enzyme inhibitors. *Med Res Rev. 5*. 483–531

Xie, M. H., (1990). Progress of research on angiotensin converting enzyme inhibitors. *Chin J Pharm. 21*. 277–285

Yalcin, A. S. (2006). Emerging therapeutic potential of whey proteins and peptides. *Curr Pharm, 12* (13):1637-1643.

Yamamoto, N. (1997). Antihypertensive peptides derived from food proteins. *Biopolymers*, *43* (2):129-134.

Yamamoto, N., Maeno, M. & Takano, T. (1999). Purification and characterisation of an antihypertensive peptide from yoghurt-like product fermented by Lactobacillus helveticus CPN4. *J. Dairy Sci. 82*:1388-1393.

Yang, G., Shu, X. O., Jin, F., Zhang, X., Li, H. L., Gao, Y. T. and Zheng, W. (2005). Longitudinal study of soy food intake and blood pressure among middle-aged and elderly Chinese women. *Am. J. Clinical Nutrition*, *81*(5): 1012 - 1017.

Yoshioka, M. (1987). Role of rat intestinal brush border membrane angiotensin converting enzyme in dietary protein digestion. *Am. J. Physiol. 253*: G781-G786.

Yamamoto, N., Maeno, M. & Takano, T. (1999). Purification and characterization of an antihypertensive peptide from yoghurt-like product fermented by Lactobacilluse helveticus CPN4. *J. Dairy Sci. 82*: 1388-1393.