University of Alberta

Angiotensin converting enzyme inhibitory peptides released from the hydrolysate of casein and the milk fermented by *Lactobacillus casei* ADA03

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

Food Science and Technology

Department of Agricultural, Food and Nutritional Science

Edmonton, Alberta

Fall 2006

Your file Votre référence
ISBN: 978-0-494-23057-2
Our file Notre référence
ISBN: 978-0-494-23057-2

ABSTRACT

Milk proteins are considered not only a valuable nutrient but also a good source of many bioactive peptides including angiotensin converting enzyme (ACE) inhibitory peptides. ACE, which is a multifunctional enzyme present in the rennin-angiotensin system, has a prominent role in the regulations of blood pressure as well as water and electrolyte balance. ACE elevates blood pressure by generating vasoconstrictor, angiotensin II, in conjunction with inactivating vasodilator, bradykinin. Inhibition of ACE results in antihypertensive effect. The present study was focused on 1) the optimization of the production of ACE inhibitory peptides by modes of hydrolysis of casein with pancreatin and fermentation of milk by *Lactobacillus casei* ADA03 and 2) on the isolation of such peptides using membrane and liquid chromatographic techniques. Furthermore, characterization of ACE inhibitory peptides was carried out by LC/MS/MS technique. The results indicated that hydrolysate of casein at 37 °C for 8 h and milk fermented with *Lactobacillus casei* ADA03 at 37 °C for 24 h exhibited the highest ACE inhibitory activity when compared within the same mode of production. Ultrafiltration with molecular weight cut-off of 2000 Da membrane and Bio-Gel P2 gel filtration chromatography were the effective methods for the purification of ACE inhibitory peptides. Amino acid sequence analysis of ACE inhibitory peptides by LC/MS/MS revealed 1) the formation of novel ACE inhibitory peptides in either casein hydrolysate or fermented milk and 2) the significance of hydrophobic amino acid at the C-terminal end of such peptides. The ACE inhibitory peptides isolated from this study can be incorporated into food, promoting the opportunity of a food product containing

antihypertensive property in the functional food market. In addition, milk fermented with *Lactobacillus casei* ADA03 can be regarded as a probiotic product with multifunctional properties.

Acknowledgement

There have been a lot of supports for the present study, of which the majority has come from professional, knowledgeable and experienced individuals from the Department of Food and Nutritional Science and from other departments. Many people I came to know through professional contact have become true friends, which I cannot express my words enough for their valuable friendship.

Firstly, I would like to gratefully acknowledge my supervisor, Prof. Dr. Ozimek. His leadership, friendship and consistent support has guided me through difficulties both academic and personal. He has taught me how to be a good student, a dear friend and a wonderful supervisor.

I would like to thank Dr. Takuo Nakano from our research group. His professional experience and valuable insight has played a significant role in the present study. Also his friendship and patience has taught me more than I can express my words.

Sue and Dr. David Schroder whom I regard as my family have been very encouraging throughout my stay in Canada. They make me feel that Edmonton is my second home.

I am thankful to Nakeeran Ponnampalam and Dr. Arvind Prasad. Their love, friendship and support cannot be grateful enough.

Drs. Sylvia Chan-Remillard, Eryck Silva-Hernandes, and Min Cheng, Judy Yeung, and Vivian Gee, who are both colleagues and dear friends, have been very supportive with my research and personal experience.

I have received supports from many Thai students in Edmonton. Some of them have gone back to Thailand and some are studying at the time I am writing this section. Their friendships are always cherished.

A few steps in the present study involved in different techniques. Without the assistance from experienced scientists, this work would have been difficult. Drs. Gary Sedgwick and Kelvin Lien from Chromatography Laboratory, Department of Food and Nutritional Science have been helpful with the chromatographic technique and instrumental handling. Dr. Randy Whittal and Jing Zheng from Mass Spectrometry

Laboratory, Department of Chemistry, have assisted the present study with their mass spectrometric technique knowledge.

I am truly grateful to Sayan Paichayonritta. Without his understanding, love and support, my stay and study in Canada would have been unpleasant.

I am extremely thankful to my family including my mother, my father, and my sisters. The success of my study would have been impossible without their unconditional love and support.

Last but not least, I am deeply indebted to the Greatest Buddha, His teaching and His venerable disciples. Without them, I would not have come this far.

Sirinda Kusump
Edmonton, Alberta
August 7, 2006

To My Parents

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CHAPTER 1*

INTRODUCTION

1.1 FUNCTIONAL FOODS

The concept of functional foods has been well-known in Asian countries more than centuries ago albeit not well-documented. It was also mentioned by Hippocrates who suggested,

"Let food be thy medicine and medicine be thy food".

The concept of functional food was introduced in Japan in the late 1980's as foods for specified health use (FOSHU). The Japanese Ministry of Health and Welfare defined FOSHU as 'foods that are expected to exhibit a health or physiological effect when consumed as part of an ordinary diet'. However, up till now, there is no absolute definition of functional foods. According to the European Functional Food Science Programme, funded by the European Union and led by the International Life Sciences Institute (ILSI), 'A food can be regarded as 'functional' if it is satisfactorily demonstrated to affect beneficially one or more target functions in the body, beyond adequate nutritional effects in a way that is relevant to either an improved state of health and well-being and/or reduction of risk of disease' [1]. The Institute of Medicine of the US National Academy of Sciences defines functional foods as 'those foods in which the concentration of one or more ingredients have been manipulated or modified to enhance their contribution to a healthful diet' [2]. The definition given by Health Canada is 'Functional foods are food components that provide demonstrated physiological benefits or reduce the risk of chronic disease, above and beyond their basic nutritional functions' [3].

* Certain sections of this chapter has been published in Food Science and Product Technology (Ed. T. Nakano, and L. Ozimek) Research Signpost, India, 127-139 (2002)

Functional foods have been the center of attention for the last few decades by food producers as well as consumers. A lot of functional food products have been launched to the market to supply the demand of consumers for delicious, attractive, convenient yet healthy food. This results in the dynamic and increasing growth of functional foods market. The global functional food market is between US$30-50 billion [4] and estimated to grow steadily with the rate of 8% per annum. Dairy products occupy a major share in the functional food markets of Japan, Europe and Australia. It is estimated to account for 60% of functional food sale in Europe [5]. Functional dairy products contained various active components which can contribute to physiological regulations in the body.

Despite the rapid growth of functional food market, there is a scientific challenge in design and development of functional food products. Functional food products have to contain active component(s) in a sufficient amount to exhibit physiological effect. Identification and efficacy of the active component(s) have to be validated. Dose of the products has to be clearly identified. And being food, functional foods have to be safe according to all food safety regulations.

1.2 BIOACTIVE PEPTIDES

Bioactive peptides are defined as peptides that in addition to their nutritional values have a physiological effect in the body. The peptides are encrypted within the parent proteins and remained inactive until they are liberated from their native proteins and consequently, exert regulative activity through a hormone-like effect [6]. The release of these peptides from the native food proteins usually occurred during gastrointestinal digestion in the body or during food processing including enzymatic hydrolysis and fermentation [7]. Numerous research has been studied on the production and isolation of bioactive peptides derived from different food protein sources including caseins and whey proteins (see ref [7], [8] for review). Bioactive peptides that have been extensively studied are listed below.

1.2.1 Opiate peptides

Opiate peptides exhibit opiate-like activity. These peptides have affinity for an opiate receptor, which are located in the nervous, endocrine and immune system. Opiate peptides have been found to modulate social behaviour, produce analgesia, modulate intestinal transport of amino acids and suppress the intestinal motility and respiration [9]. β-casomorphins (β-casein f60-70) are major opiate peptides. Fragments of α-casein, α-lactalbumin and β-lactoglobulin namely, α-casomorphins, α-lactorphin and β-lactorphin, respectively are also identified as opiate peptides [10].

1.2.2 Antimicrobial peptides

Lactoferrin, an iron-binding glycoprotein present in mammal fluid including milk has been considered to be a defensive agent against microbial infection. Lactoferricin, peptides derived from a peptic digest of lactoferrin showed stronger antimicrobial activity than its parent protein [11]. The antimicrobial property of these peptides appears to be correlated with the net positive charge of the peptides [12]. Peptides with bactericidal activity against *Escherichia coli* and *Staphylococcus carnosus* have also been identified from α_{S2}-casein [13].

1.2.3 Antihypertensive / Angiotensin converting enzyme inhibitory peptides

Peptides exhibited antihypertensive activity were commonly found to inhibit angiotensin converting enzyme (ACE) [14], [15], [16]. ACE is a multifunctional enzyme present in the rennin-angiotensin system. ACE has a prominent role in the regulations of blood pressure as well as water and electrolyte balance. ACE elevates blood pressure by generating vasoconstrictor, angiotensin II, in conjunction with degrading vasodilator, bradykinin. Inhibition of ACE results in antihypertensive activity. More details concerning this enzyme and ACE inhibitory/antihypertensive peptides are further discussed in section 1.4-1.6.

1.2.4 Antithrombotic peptides

Peptides derived from caseinoglycomacropeptides of bovine κ-casein was found to inhibit the aggregation of ADP-activated platelets as well as the binding

of human fibrinogen γ-chain to a specific receptor on the platelet surface [17]. The peptide, namely casoplatelins corresponding to Met-Ala-Ile-Pro-Pro-Lys-Lys-Asn-Gln-Asp-Lys (κ-casein f106-116) or its smaller fragments (f106-112, 112-116, 113-116) have shown antithrombotic activity [18].

1.2.5 Immunomodulatory peptides

Immunomodulatory peptides contribute to an overall immunostimulatory response as well as cell proliferation. Research demonstrated that peptides derived from casein and whey proteins stimulate phagocytosis of sheep red blood cell and protect against *Klebsiella pneumoniae* infection in mice [19]. Peptides derived from β-casein (f1-25, f1-28, f59-79) enhanced the immunoglobulin IgG production in mouse spleen cell culture [20], [21]. Stimulatory and suppressive activities of β-casomorphin have also been observed [22].

1.3 ANGIOTENSIN CONVERTING ENZYME (ACE)

ACE (EC 3.4.15.1) also called kinase II is a metalloprotease with two zinc active catalytic sites [23] (Fig. 1.1). ACE plays a prominent role in the regulation of blood pressure and pathology of hypertension [14], [15]. ACE, as its name suggests, converts angiotensin I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu) to angiotensin II, Asp-Arg-Val-Tyr-Ile-His-Pro-Phe, by catalyzing cleavage between Phe and His to eliminate a dipeptide (His-Leu) from the carboxyl terminal of the angiotensin I [16]. Angiotensin II directly stimulates vascular smooth muscle contraction, resulting in an increase in systemic vascular resistance and an elevation in blood pressure. It also stimulates the secretion of aldosterone from adrenal cortex. Aldosterone produces kaliuresis and increases sodium and water retention in the distal tubule. Sodium and water retention elevates blood pressure. In addition, ACE can degrade bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg), an arteriolar vasodilator, present in the kallikrein-kinin system, into inactive peptides, resulting in a decrease in the vasodilatory effect of bradykinin [24]. Production of angiotensin II and degradation of bradykinin, both catalyzed by ACE, thus contribute to the cause of high blood pressure (Fig. 1.2). However, angiotensin II can be hydrolysed into a weaker vasoconstrictor,

angiotensin III (Arg-Val-Tyr-Ile-His-Pro-Phe) by aminopeptidase and then into inactive peptides by angiotensinases.

Hypertension, the medical term given to high blood pressure, is defined in an adult as a blood pressure greater than or equal to 140 mm Hg systolic pressure and greater than or equal to 90 mm Hg diastolic pressure. It is a high-risk disease that forms the cause of 20 to 50 percent of natural deaths [25]. High blood pressure is known to greatly increase the risk of developing heart disease, renal disease, hardening of the arteries (arteriosclerosis), eye damage, and stroke [26], [27]. According to the American Heart Association's 2001 Heart and Stroke Statistical Update, more than 30 percent of Americans with high blood pressure are unaware that they have this potentially life threatening condition; another 26 percent are on medication, but do not have their blood pressure under control [28]. Hypertension is estimated to have caused 1-8% of deaths in developing countries, being the highest in Latin America and the Caribbean [29]. Globally, almost 3 million deaths (6% of the world total) in 1990 were attributable to the condition [29]. Several forms of medication have been prescribed to alleviate this disease and subsequently bring about the control of blood pressure. A majority of research has directly addressed the primary cause(s) of the elevations in blood pressure with the objective of finding feasible means to eliminate them at the root level, in the least, to substantially reduce their effects.

1.4 ACE INHIBITORS

The inhibition of the activity of ACE or its blockage lowers the levels of angiotensin II, greatly aiding in diminishing the hypertensive effects, and therefore leading to the treatment of hypertension [23], [30]. The concept of blocking ACE forms the central idea of the research that has been carried out in this area over the past few decades. Clearly, the focus of the research has been on finding a potential ACE inhibitor either by natural or artificial means.

The first significant breakthrough in this direction took place in the 1960's with the discovery of an ACE inhibitor that was extracted from the venom of a Brazilian snake, *Bothrops jararaca* [31]. The crude mixture of peptides was extracted

and first described as a bradykinin-potentiating factor. Amino acid sequences of this factor were determined and named bradykinin potentiating peptides [32]. However, due to the fact that these peptides were present in low quantities in the snake venom, the focus of the research shifted to finding alternative sources of ACE inhibitors. Since the introduction of bioactive peptides as ACE inhibitors derived from bacterial collagenase treated gelatin [33], food proteins have proved to be a major alternative source for ACE inhibitory peptides [34]. Subsequent research revealed the occurrence of ACE inhibitory peptides in various different food proteins including sardine muscle [35], [36], zein [37], [38], bonito muscle [39], squid liver [40], caseins [41], [42], and whey proteins [43],[44]. In addition, it is interesting to note that the ACE inhibitory peptides have also been reported in a variety of fermented foods such as soy sauce [45], fermented milk [46], sake [47], cheese [48], fish sauce [49], and fermented soybean paste [50]. Among these sources, milk proteins namely caseins and whey proteins constitute the largest group that can provide high potential ACE inhibitors. Inhibitors of ACE derived from caseins are known as casokinin [51] whereas those from whey proteins are known as lactokinin [52]. The amino acid sequences of casokinin are listed in Tables 1.1-1.3.

1.5 ACE INHIBITORY PEPTIDES FROM MILK PROTEINS

1.5.1 Production of ACE inhibitory peptides

ACE inhibitory peptides, like other bioactive peptides, are latent in the native or parent proteins. These peptides are liberated with the onset of enzymatic hydrolysis of their parent proteins. In the ensuing sections, we discuss the characteristics of peptides that are isolated from two main sources including the enzymatic hydrolysate of caseins and fermented milk products. An extensive amount of research has been carried out for the peptides from these two sources.

The first source that we consider is the enzymatic hydrolysate of caseins with digestive enzymes and/or bacterial enzymes (Tables 1.1-1.3). Peptides corresponding to α_{s1}-casein sequence 23-34 (Phe-Phe-Val-Ala-Pro-Phe-Pro-Glu-Val-Phe-Gly-Lys) [41], sequence 194-199 (Thr-Thr-Met-Pro-Leu-Trp) [53], and β-casein

sequence 177-183 (Ala-Val-Pro-Tyr-Pro-Gln-Arg) [42] were isolated from tryptic hydrolysate of caseins and exhibited strong ACE inhibitory effects. Experimental results obtained from various reports suggested that hydrolysate of caseins digested by trypsin can yield more effective ACE inhibitors when compared to those derived from hydrolysate of caseins digested by other mammalian digestive enzymes [41], [42], [53]. Peptides fractionated from proteinase K digest of β-casein show mild inhibitory activity [44]. A peptide corresponding to α_{s1}-casein (sequence 23-27), Phe-Phe-Val-Ala-Pro shows a very potent ACE inhibitory activity [42]. This peptide was obtained from the casein hydrolysate, which was obtained by digestion of casein with trypsin and subsequently with proline-specific endopeptidase. It is interesting to note that this peptide has much stronger inhibitory activity than peptide Phe-Phe-Val-Ala-Pro-Phe-Pro-Glu-Val-Phe-Gly-Lys, which has 7 more amino acids in the sequence.

Enzymes produced by bacteria are also used to digest milk proteins to yield ACE inhibitors. Extracellular proteinase purified from *Lactobacillus helveticus* CP790 can degrade α_{s1}-, α_{s2}- and β- caseins into several ACE inhibitory peptides [54], [55]. Most of these peptides have long chains and hold a strong to mild ACE inhibitory activity. The most potent ACE inhibitor derived from *Lactobacillus helveticus* proteinase digest is the peptide Asp-Glu-Leu-Gln-Asp-Lys-Ile-His-Pro-Phe-Ala-Gln-Thr-Gln-Ser-Leu-Val-Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro-Asn-Ser (β-casein sequence 43-69) [54].

The second source of ACE inhibitory peptides that we discuss here, is from several fermented milk products such as Calpis sour milk (the commercial fermented milk available in Japan) [56], yogurt-like product [57], and cheeses [58], [59], [60]. In fact, the ACE inhibitors are formed during the fermentation process. The amino acid sequences of these ACE inhibitory peptides are illustrated in Table 1.4.

Two types of ACE inhibitory peptides are purified from Calpis sour milk, which is fermented by *Lactobacillus helveticus and Saccharomyces cerevisiae*. These peptides corresponding to β-casein sequence 74-76 (Ile-Pro-Pro) and sequence 84-86 (Val-Pro-Pro) show a very potent ACE inhibitory activity [56]. The dipeptide, Tyr-Pro was isolated from a yogurt-like product fermented by *Lactobacillus helveticus* [57].

This peptide, found in α_{s1}-casein (sequence 146-147, and sequence 159-160), β-casein (sequence 114-115), and κ-casein (sequence 58-59), was reported to have mild ACE inhibitory effect. Based on the evidence that the proteinase of *Lactobacillus helveticus* CP790 preferably hydrolyses the casein molecules at the C-terminal region where the dipeptide Tyr-Pro is present, the release of Tyr-Pro peptide is believed to be due to the proteolytic action at the beginning of bacterial growth in the yogurt-like product [57].

It is interesting to note that although identical lactic acid bacteria (*Lactobacillus helveticus*) are used to produce Calpis sour milk and yogurt-like product, the occurrence of ACE inhibitory peptides in both products is different. Tripeptides Ile-Pro-Pro and Val-Pro-Pro present in Calpis sour milk are not found in the yogurt-like product, which is produced over a shorter fermentation period. This is a clear indication that varying fermentation conditions result in different ACE inhibitory peptide productions. Moreover, it is noteworthy that these tripeptides are not detected in the casein hydrolysate prepared by digesting casein with proteinase, which is produced by *Lactobacillus helveticus* (the lactic acid bacteria used to produce Calpis sour milk) [54]. As a matter of fact, the undetected tripeptides (β-casein sequences 74-76 and 84-86) are hidden in the sequence of peptides that are obtained from the proteinase digest including β-casein sequences 16-97.

Milk fermented by different species of lactic acid bacteria such as *Lactobacillus delbrueckii* ssp. *bulgaricus* SS1, and *Lactobacillus lactis* ssp. *cremoris* FT4 [61] shows the occurrence of other kinds of ACE inhibitory peptides. *Lactobacillus* sp. are used more widely than other lactic acid bacteria in producing ACE inhibitory peptides due to the fact that Lactobacilli can produce remarkable ACE inhibitory peptides compared to others [46]. The occurrence of ACE inhibitors is also reported in ultra-heat-treated (UHT) milk fermented by *Lactobacillus casei* ssp. *rhamnosus* and subsequently digested by pepsin and trypsin [62]. Milk fermented by *Lactococcus lactis* ssp.*lactis*, *Lactococcus lactis* ssp.*cremoris*, *Lactococcus lactis* ssp. *lactis* biovar. *diacetylactis*, and *Leuconostoc mesenteroides* ssp. *cremoris*, and subsequently hydrolysed by pepsin, and trypsin contains strong ACE inhibitory peptides including α_{s1}-casein sequences 142-147 (Leu-Ala-Tyr-Phe-Tyr-Pro), 157-164 (Asp-Ala-Tyr-Pro-Ser-Gly-Ala-Trp), and 194-199 (Thr-Thr-Met-Pro-Leu-Trp) [63].

Occurrence of ACE inhibitory peptides is also reported in several kinds of cheese including Roquefort [48], Leerdam [48], Gouda [48], [59], Mozzarella [58], Italica [58], Crescenza [58] and Gorgonzola [58]. Peptide Leu-Val-Tyr-Pro-Phe-Pro-Gly-Pro-Ile-His-Asn-Ser-Leu-Pro-Gln was isolated from Crescenza cheese [58]. This peptide corresponds to β-casein sequence 58-72 and exhibits a strong ACE inhibitory effect. Peptides Arg-Pro-Lys-His-Pro-Ile-Lys-His-Gln (α_{s1}-casein sequence 1-9) and Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro-Asn (β-casein sequence 60-68) were isolated from Gouda cheese [59]. These peptides show powerful ACE inhibitory activity. A peptide Arg-Pro-Lys-His-Pro-Ile-Lys-His-Gln was also found in Festivo cheese [60]. Meisel *et al.* [48] (Fig. 1.3) and Ryhanen *et al.* [60] showed that during cheese ripening, ACE inhibitory activity increased with developing proteolysis. In addition, low levels of proteolysis found in fresh cheese, Quarg cheese, and Harz cheese were associated with low ACE inhibitory index values [48]. However, ACE inhibitory activity decreased when the proteolysis exceeded a certain level during cheese maturation. The ACE inhibitory peptides present naturally in cheese remain active for a limited period before splitting into inactive fragments with the gradual progress of ripening.

1.5.2 Physiological significance

The antihypertensive effect of peptides with ACE inhibitory activity *in vitro* comes into role with the ingestion of these peptides, followed by the absorption of the peptides from the intestine, and finally with the arrival at their target organs. A number of peptides with ACE inhibitory activity *in vitro* have been shown to be able to decrease blood pressure in spontaneously hypertensive rats (SHR), and their antihypertensive effects have been demonstrated to be dose-dependent when orally administered to SHR [54], [55], [57], [64].

It is interesting to address here that the antihypertensive effect of peptides with ACE inhibitory activity *in vitro* may be influenced by the action of gastrointestinal enzymes. Maeno *et al.* [55] reported that peptide Tyr-Lys-Val-Pro-Gln-Leu (IC_{50} = 22 μM) had low antihypertensive activity in SHR because the peptide was decomposed to Tyr-Lys-Val-Pro with weaker ACE inhibitory activity ($IC_{50} \geq$ 1000 μM) by pancreatin or carboxypeptidase A. This indicates that in order to exert

antihypertensive effect, a peptide with ACE inhibitory activity *in vitro* has to be resistant to gastrointestinal enzymatic digestion. In contrast, a degradation of a peptide by gastrointestinal enzyme may give rise to stronger ACE inhibitory activity. For instance, peptide Lys-Val-Leu-Pro-Val-Pro-Gln had a low inhibitory strength with an IC_{50} value of 1000 μM. However, once this peptide was hydrolysed by pancreatin into Lys-Val-Leu-Pro-Val-Pro, the potency increased by a factor of 200 to an IC_{50} value of 5 μM [55]. Moreover, both peptides exhibited similar potent antihypertensive activity in SHR. These results suggest that the actual strength of an ACE inhibitor should be assessed based on its activity during post-ingestion rather than its *in vitro* potency.

Peptide Phe-Pro, and Val-Tyr-Pro from proteinase K digest of β-casein (Table 1.2) significantly reduced systolic blood pressure 6 hours after a gas intubation [44]. Milk digested by proteinase produced by *Lactobacillus helveticus* CP790 also showed antihypertensive effect [54].

Milk fermented by several strains of *Lactobacillus helveticus* or by *Lactobacillus delbrueckii* ssp. *bulgaricus* exhibited antihypertensive effect in SHR. However, milk fermented by other lactic acid bacteria including *Lactobacillus debrueckii* ssp. *lactis, Lactobacillus casei, Lactobacillus acidophilus,* and *Lactococcus lactis* ssp. *lactis* did not show notable antihypertensive property [46].

The tripeptides (Ile-Pro-Pro and Val-Pro-Pro) isolated from Calpis sour milk (Table 1.4) exhibited strong antihypertensive effect in SHR reflecting their high ACE inhibitory activity *in vitro* [64]. Masuda *et al.* [65] reported that these peptides were absorbed directly in the intestine without being disintegrated by digestive enzymes, reached the abdominal aorta and inhibited ACE, to show antihypertensive effect. In contrast, the dipeptide Tyr-Pro obtained from the yogurt-like product (Table 1.4), which showed low ACE inhibitory activity *in vitro* exerted strong antihypertensive effect on a similar level as did the tripeptides Ile-Pro-Pro and Val-Pro-Pro [57]. The mechanism for the high antihypertensive effect of the dipeptide is not clear.

The decrease in systolic blood pressure was statistically significant in SHR introduced with crude peptide extracts prepared from Italian cheeses (Blue, Edam, Havarti and 8-month-aged Gouda cheeses) [59]. The strongest effect was

noticed in the SHR orally administered with the extract from Gouda cheese. However, peptides purified from this cheese (Arg-Pro-Lys-His-Pro-Ile-Lys-His-Gln and Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro-Asn) showed mild antihypertensive effect in SHR in spite of their high ACE inhibiting activity *in vitro* (Table 1.4). This is suggestive of a significant contribution from other peptides besides the two peptides to the high antihypertensive activity of Gouda cheese.

A clinical study revealed a significant reduction of systolic and diastolic blood pressure in hypertensive patients who had an intake of 10 g of tryptic hydrolysate of casein given twice a day for 4 weeks [66]. Hata *et al.* [67] observed a remarkable decrease in systolic and diastolic blood pressure in hypertensive patients who ingested daily 95 ml of Calpis sour milk containing tripeptides Ile-Pro-Pro and Val-Pro-Pro (Fig. 1.4). The blood pressure remained at low levels for 4 weeks after the daily ingestion of Calpis sour milk ended. No adverse effects on serum lipid metabolism were observed in the hypertensive subjects.

It is worth noting that a number of ACE inhibitors derived from caseins possessing other biological activities have been reported. β-Casomorphin-7 (Tyr-Pro-Phe-Pro-Gly-Pro-Ile), not only showed ACE inhibitory activity (IC_{50} = 500 μM) but also had an opioid activity [51]. Peptide Leu-Val-Tyr-Pro-Phe-Pro-Gly-Pro-Ile-His-Asn-Ser-Leu-Pro-Gln (β-casein sequence 58-72) having an ACE inhibitory activity (IC_{50} = 15 μM) is effective in inhibiting the activity of amino- and endo-peptidase of *Pseudomonas fluorescens* ATCC948 [58]. In addition, β-lactorphin (Tyr-Gly-Leu-Phe) derived from whey protein, which inhibits ACE with an IC_{50} value of 733 μM possesses an opioid activity [68]. The understanding of the multi-functionality of these ACE inhibitors can therefore greatly supplement the study of their uses in activating various biological or physiological effects.

1.6 ADVERSE EFFECTS OF ACE INHIBITORS

The most common side effects observed in patients prescribed synthetic ACE inhibitors are dry cough, elevated plasma potassium levels (hyperkalemia), low blood pressure (hypotension), headache, drowsiness, dizziness, abnormal taste (metallic or salty taste), and rash. The most serious, although rare, adverse effects of

ACE inhibitors are renal failure, a decrease in white blood cells, and swelling of tissues (angioedema). Pregnant women or individuals with severe kidney problems are recommended to avoid using ACE inhibitors [69], [70], [71]. There has been no report on adverse effect of ACE inhibitors derived from food proteins.

1.7 REMARKS

Over the last few decades, a rigorous study of various pharmaceuticals has been conducted to determine their ability towards the reduction of blood pressure levels, which primarily lead to the synthesis of those compounds that act as ACE inhibitors such as Captopril and Enalapril [69]. While such products are effective in decreasing elevated blood pressure levels, their use can often result in negative side effects. Consequently, a number of studies have focused on the development of ACE inhibitors extracted from natural sources. These products have been proposed as alternative antihypertensive agents for practical uses with the expectation that being derived from natural substances, these ACE inhibitors would not result in negative side effects [72]. However, such a hypothesis needs the support of substantial scientific evidence that can prove the safety of using the products on a long-term basis. One of the commonly available commercial products of this kind is Calpis sour milk largely marketed in Japan, which contains a wide variety of peptides including peptides Ile-Pro-Pro and Val-Pro-Pro. Two other examples of this kind are beverage rich in peptides Ile-Pro-Pro and Val-Pro-Pro available in Japan; and Festivo cheese, a low fat cheese containing peptide Arg-Pro-Lys-His-Pro-Ile-Lys-His-Gln produced and marketed in Finland.

1.8 RESEARCH OUTLINE AND OBJECTIVES

High blood pressure or hypertension is a major risk factor for cardiovascular diseases (CVD), which are a leading cause of mortality in developed countries. Modification in life style such as exercise, no smoking, diet control including DASH (Dietary Approaches to Stop Hypertension) has been practiced in the prevention and treatment of hypertension. Functional dairy foods containing ACE inhibitory peptide may become another alternative in the management of hypertension.

In addition, ACE inhibitory peptides isolated from milk proteins can be incorporate into foods as an antihypertensive ingredient.

The objectives of this research, therefore, were to investigate the production of ACE inhibitory peptides derived from bovine casein by pancreatin as well as to explore the ability to generate ACE inhibitory peptides by some strains of probiotics. The research also focused on applying membrane technology and chromatographic techniques to isolate such peptides. Peptides characterized in fractions obtained from chromatography were also compared to previously described ACE inhibitory peptides. More specifically, the research was aimed:

- To optimize the production of ACE inhibitory peptides derived from bovine casein by pancreatin
- To enrich the ACE inhibitory peptides derived from casein by ultrafiltration and column chromatography
- To characterize the ACE inhibitory peptides derived from pancreatic digest of casein by mass spectrometry and compare them with previously known ACE inhibitory peptides
- To release ACE inhibitory peptides in fermented milk by fermentation with lactic acid bacteria, which were classified as probiotics.
- To optimize the production of ACE inhibitory peptides by the fermentation with probiotics
- To enrich the ACE inhibitory peptides released by the fermentation of probiotic
- To characterize the ACE inhibitory peptides produced by probiotics and compare them with previously described ACE inhibitory peptides

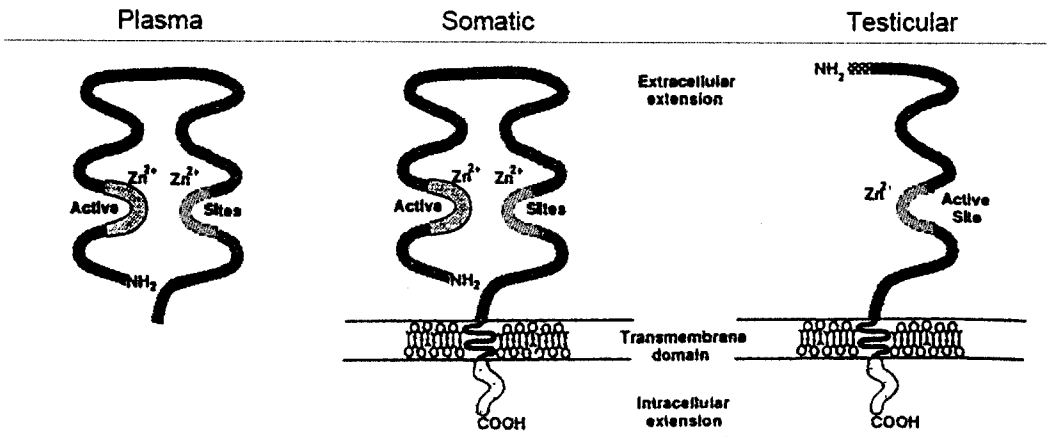

Figure 1.1: Structure and conformation of ACE derived from plasma, somatic cell origin or testis, showing the active catalytic sites, zinc dependency and the N- and C- terminal ends [23]

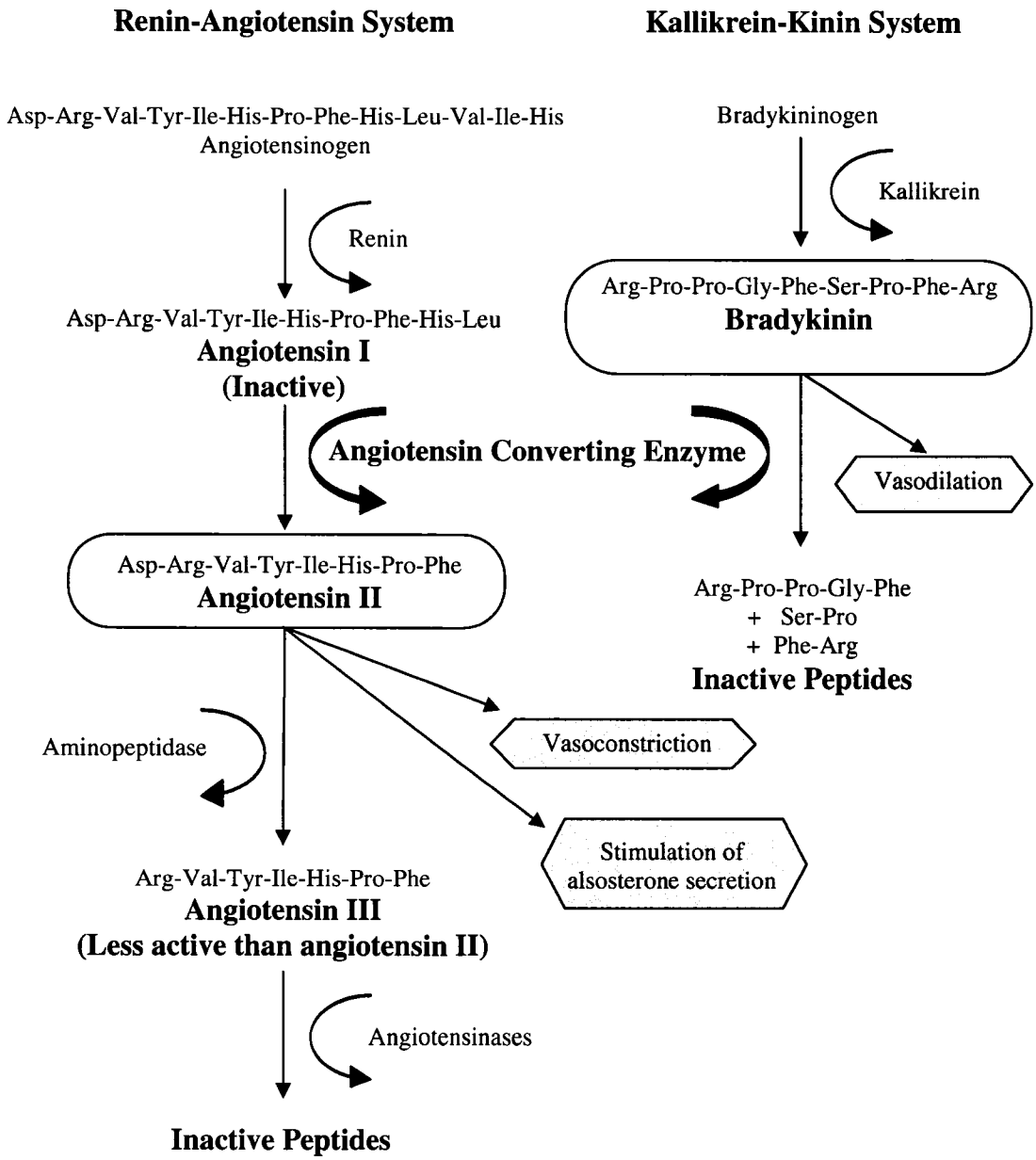

Figure 1.2: Regulation of blood pressure by angiotensin converting enzyme.

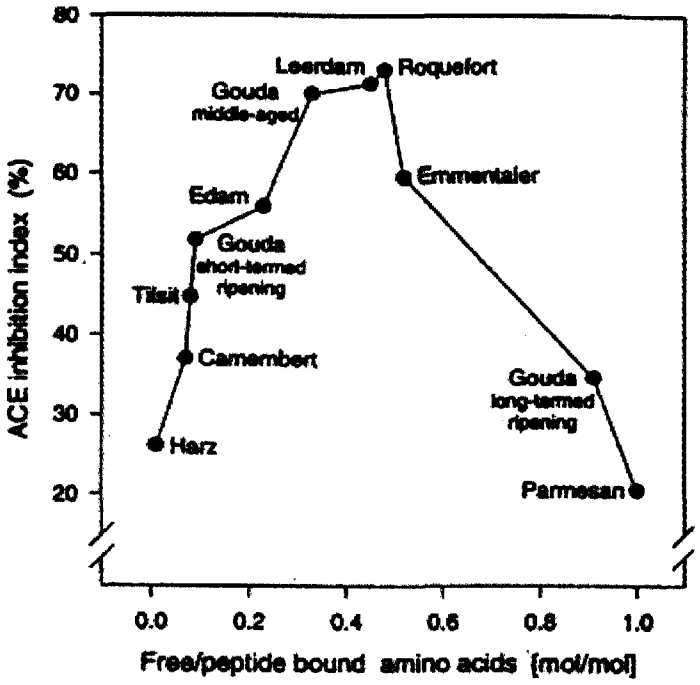

Figure 1.3: ACE inhibition of extracts from ripened cheese in dependence on proteolysis as measured by the ratio of free to peptide-bound amino acids [48]

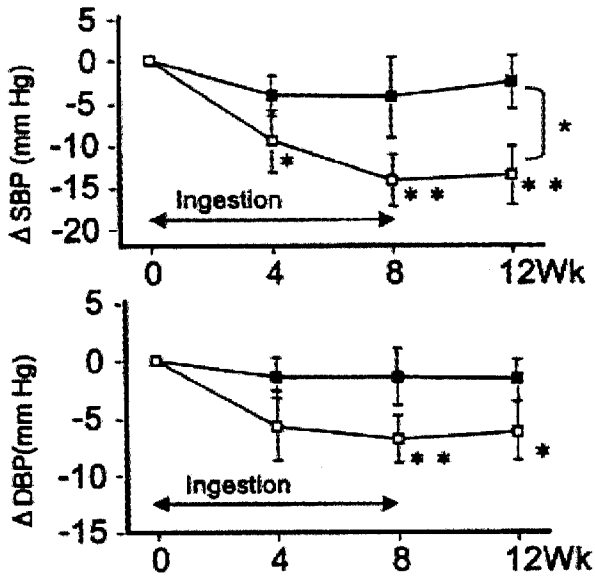

Figure 1.4: Changes in systolic blood pressure (SBP) and diastolic blood pressure (DBP) of hypertensive subjects by treatment for 8 weeks with Calpis sour milk (white) and artificial acidified milk as placebo (black). [64]

* Significant difference from initial value at $p \leq 0.05$

** Significant difference from initial value at $p \leq 0.01$

Table 1.1: ACE inhibitory peptides derived from α_{S1}-casein

Peptide Sequence	Position	Treatment	IC_{50}[1] (μM)	Ref.
Phe-Phe-Val-Ala-Pro-Phe-Pro-Glu-Val-Phe-Gly-Lys	23-34	Trypsin	77	[41]
Phe-Phe-Val-Ala-Pro	23-27	Trypsin + Proline-specific-endopeptidase	6	[42]
Thr-Thr-Met-Pro-Leu-Trp	194-199	Trypsin	16	[53]
Ala-Tyr-Phe-Tyr-Pro-Glu	143-148	Proteinase from *Lactobacillus helveticus* CP790	106	[54]
Gly-Thr-Gln-Tyr-Thr-Asp-Ala-Pro-Ser-Phe-Ser-Asp-Ile-Pro-Asn-Pro-Ile-Gly-Ser-Glu-Asn-Ser-Glu-Lys-Thr-Thr-Met-Pro-Leu-Trp	170-199	Proteinase from *Lactobacillus helveticus* CP790	346	[54]
Tyr-Lys-Val-Pro-Gln-Leu	104-109	Proteinase from *Lactobacillus helveticus* CP790	22	[55]

[1] The peptide concentration needed to inhibit 50% of the ACE activity

Table 1.2: ACE inhibitory peptides derived from β-casein

Peptide Sequence	Position	Treatment	IC_{50}[1] (μM)	Ref.
Ala-Val-Pro-Tyr-Pro-Gln-Arg	177-183	Trypsin	15	[42]
Val-Tyr-Pro-Phe-Pro-Gly	59-64	Proteinase K	221	[44]
Val-Tyr-Pro	59-61	Proteinase K	288	[44]
Phe-Pro	62-63, 157-158, 205-206	Proteinase K	315	[44]
Thr-Pro-Val-Val-Val-Pro-Pro-Phe-Leu-Gln-Pro	80-90	Proteinase K	749	[44]
Pro-Pro-Gln-Ser-Val-Leu-Ser-Leu-Ser-Gln-Ser-Lys-Val-Leu-Pro-Val-Pro-Gln	158-175	Proteinase from *Lactobacillus helveticus* CP790	25	[54]
Ser-Lys-Val-Leu-Pro-Val-Pro-Gln	168-175	Proteinase from *Lactobacillus helveticus* CP790	39	[54]
Lys-Tyr-Pro-Val-Gln-Pro-Phe-Thr-Glu-Ser-Gln-Ser-Leu-Thr-Leu	113-127	Proteinase from *Lactobacillus helveticus* CP790	93	[54]
Arg-Asp-Met-Pro-Ile-Gln-Ala-Phe	183-190	Proteinase from *Lactobacillus helveticus* CP790	209	[54]
Try-Gln-Glu-Pro-Val-Leu-Gly-Pro-Val-Arg-Gly-Pro-Phe-Pro-Ile-Ile-Val	193-209	Proteinase from *Lactobacillus helveticus* CP790	101	[54]
Leu-Pro-Gln-Asn-Ile-Pro-Pro-Leu-Thr-Gln-Thr-Pro-Val-Val-Val-Pro-Pro-Phe-Leu-Gln-Pro-Glu-Val-Met-Gly-Val-Ser-Lys	80-97	Proteinase from *Lactobacillus helveticus* CP790	144	[54]

Peptide Sequence	Position	Treatment	IC_{50}[1] (μM)	Ref.
Leu-Ser-Ser-Ser-Glu-Glu-Ser-Ile-Thr-Arg-Ile-Asn-Lys-Lys-Ile-Glu-Lys-Phe-Gln-Ser-Glu-Glu-Gln-Gln-Gln-Tyr-Glu-Asp-Glu-Leu-Gln-Asp-Lys-Ile-His-Pro-Phe-Ala-Gln-Thr-Gln-Ser-Leu-Val-Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro-Asn-Ser-Leu-Pro-Gln-Asn-Ile-Pro-Pro-Leu-Thr-Gln-Thr-Pro-Val-Val-Val-Pro-Pro- Phe-Leu-Gln-Pro-Glu-Val-Met-Gly-Val-Ser-Lys	16-97	Proteinase from *Lactobacillus helveticus* CP790	108	[54]
Leu-Leu-Tyr-Gln-Gln-Pro-Val-Leu-Gly-Pro-Val-Arg-Gly-Pro-Phe-Pro-Ile-Ile-Val	191-209	Proteinase from *Lactobacillus helveticus* CP790	21	[54]
Asp-Glu-Leu-Gln-Asp-Lys-Ile-His-Pro-Phe-Ala-Gln-Thr-Gln-Ser-Leu-Val-Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro-Asn-Ser	43-69	Proteinase from *Lactobacillus helveticus* CP790	4	[54]
Lys-Val-Leu-Pro-Val-Pro-Gln	169-175	Proteinase from *Lactobacillus helveticus* CP790	1000	[55]
Lys-Val-Leu-Pro-Val-Pro	169-174	Proteinase from *Lactobacillus helveticus* CP790 + Pancreatin	5	[55]
Lys-Val-Leu-Pro-Val-Pro	169-174	Proteinase from *Lactobacillus helveticus* CP790+ Carboxypeptidase A	5	[55]

[1] The peptide concentration needed to inhibit 50% of the ACE activity

Table 1.3: ACE inhibitory peptides derived from α_{S2}- caseins

Peptide Sequence	Position	Treatment	IC_{50}[1] (μM)	Ref.
Thr-Lys-Val-Ile-Pro	198-202	Proteinase from *Lactobacillus helveticus* CP790	400	[55]

[1] The peptide concentration needed to inhibit 50% of the ACE activity

Table 1.4: ACE inhibitory peptides present in fermented milk products

Peptide Sequence	Position	Treatment	IC_{50}[1] (μM)	Ref.
Ile-Pro-Pro	β-casein sequence 74-76	Calpis sour milk *(Lactobacillus helveticus + Saccharomyces cerevisiae)*	5	[56]
Val-Pro-Pro	β-casein sequence 84-86	Calpis sour milk *(Lactobacillus helveticus + Saccharomyces cerevisiae)*	9	[56]
Tyr-Pro	α_{S1}-casein sequence 146-147, α_{s1}-casein sequence 159-160	Yogurt-like product (*Lactobacillus helveticus* CPN4)	720	[57]
Tyr-Pro	β-casein sequence 114-115	Yogurt-like product (*Lactobacillus helveticus* CPN4)	720	[57]
Tyr-Pro	κ-casein sequence 58-59	Yogurt-like product (*Lactobacillus helveticus* CPN4)	720	[57]

Peptide Sequence	Position	Treatment	IC_{50}[1] (μM)	Ref.
Leu-Val-Tyr-Pro-Phe-Pro-Gly-Pro-Ile-His-Asn-Ser-Leu-Pro-Gln	β-casein sequence 58-72	Crescenza cheese (*Streptococcus thermophilus* + *Lactobacillus debrueckii* ssp. *bulgaricus*)	15	[58]
Arg-Pro-Lys-His-Pro-Ile-Lys-His-Gln	α_{S1}-casein sequence 1-9	Gouda cheese (*Lactococcus lactis* ssp.*cremoris* + *Lactobacillus debrueckii* ssp. *bulgaricus*)	13	[59]
Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro-Asn	β-casein sequence 60-68	Gouda cheese (*Lactococcus lactis* ssp.*cremoris* + *Lactobacillus debrueckii* ssp. *bulgaricus*)	15	[59]
Arg-Pro-Lys-His-Pro-Ile-Lys-His-Gln	α_{S1}-casein sequence 1-9	Festivo cheese (*Lactococus* sp., *Leuconostoc* sp. *Propionibacterium* sp., *Lactobacillus* sp., *Lactobacillus acidophilus*, and *Bifidobacterium* sp.)	nd	[60]

Peptide Sequence	Position	Treatment	IC_{50}[1] (μM)	Ref.
Leu-Asn-Val-Pro-Gly-Glu-Ile-Val-Glu	β-casein sequence 6-14	*Lactobacillus delbrueckii* ssp. *bulgaricus* SS1	300	[61]
Asn-Ile-Pro-Pro-Leu-Thr-Gln-Thr-Pro-Val	β-casein sequence 73-82	*Lactobacillus delbrueckii* ssp. *bulgaricus* SS1	173	[61]
Asp-Lys-Ile-His-Pro-Phe	β-casein sequence 47-52	*Lactobacillus lactis* ssp. *cremoris* FT4	257	[61]
Ala-Val-Pro-Tyr-Pro-Gln-Arg	β-casein sequence 177-183	*Lactobacillus casei* ssp. *rhamnosus* + Pepsin + Trypsin	nd	[62]
Tyr-Gln-Glu-Pro-Val-Leu-Gly-Pro-Val-Arg	β-casein sequence 193-202	*Lactobacillus casei* ssp. *rhamnosus* + Pepsin + Trypsin	nd	[62]
Asp-Ala-Tyr-Pro-Ser-Gly-Ala-Trp	α_{S1}-casein sequence 157-164	Lactococcus lactis ssp.lactis, Lactococcus lactis ssp.cremoris, Lactococcus lactis ssp. lactis biovar. diacetylactis, and Leuconostoc mesenteroides ssp. cremoris + pepsin + trypsin	98	[63]

Peptide Sequence	Position	Treatment	IC_{50}[1] (μM)	Ref.
Thr-Thr-Met-Pro-Leu-Trp	α_{S1}-casein sequence 194-199	Lactococcus lactis ssp.lactis, Lactococcus lactis ssp.cremoris, Lactococcus lactis ssp. lactis biovar. diacetylactis, and Leuconostoc mesenteroides ssp. cremoris + pepsin + trypsin	51	[63]
Leu-Ala-Tyr-Phe-Tyr-Pro	α_{S1}-casein sequence 142-147	Lactococcus lactis ssp.lactis, Lactococcus lactis ssp.cremoris, Lactococcus lactis ssp. lactis biovar. diacetylactis, and Leuconostoc mesenteroides ssp. cremoris + pepsin + trypsin	65	[63]
Ala-Val-Pro-Tyr-Pro-Gln-Arg	β-casein sequence 177-183	Lactococcus lactis ssp.lactis, Lactococcus lactis ssp.cremoris, Lactococcus lactis ssp. lactis biovar. diacetylactis, and Leuconostoc mesenteroides ssp. cremoris + pepsin + trypsin	274	[63]

Peptide Sequence	Position	Treatment	IC_{50}[1] (μM)	Ref.
Glu-Met-Pro-Phe-Pro-Lys	β-casein sequence 108-113	Lactococcus lactis ssp.lactis, Lactococcus lactis ssp.cremoris, Lactococcus lactis ssp. lactis biovar. diacetylactis, and Leuconostoc mesenteroides ssp. cremoris + pepsin + trypsin	423*	[63]
Tyr-Gln-Gln-Pro-Val-Leu	β-casein sequence 193-198	Lactococcus lactis ssp.lactis, Lactococcus lactis ssp.cremoris, Lactococcus lactis ssp. lactis biovar. diacetylactis, and Leuconostoc mesenteroides ssp. cremoris + pepsin + trypsin	280	[63]

[1] The peptide concentration needed to inhibit 50% of the ACE activity

* Expressed as μg/mL

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CHAPTER 2*

ENRICHMENT OF ANGIOTENSIN CONVERTING ENZYME INHIBITORY PEPTIDES IN HYDROLYSATES BY ULTRAFILTRATION MEMBRANE

2.1 INTRODUCTION

Angiotensin converting enzyme (ACE; EC 3.4.15.1), a peptidyl dipeptidase found in the rennin-angiotensin system, plays a prominent role in the regulation of blood pressure by converting angiotensin I to angiotensin II [1]. The latter is an effective vasoconstrictor causing high blood pressure. In addition, ACE degrades bradykinin, a vasodilator into inactive peptides, resulting in a decrease in the vasodilatory effect. The inhibition of ACE activity may lower the level of angiotensin II and potentiate the activity of bradykinin, leading to antihypertensive effect.

Recently, many researchers have reported that food proteins contain biologically active peptides [2], [3], [4], [5] including ACE inhibitory peptides from animal [6], [7], [8] and plant [9], [10], [11] proteins. These peptides are liberated with the onset of enzymatic hydrolysis of their parent proteins. ACE inhibitory peptides have been reported in casein hydrolysates prepared with trypsin [6], [12] , proteinase K [13], and pepsin .

* Certain sections of this chapter has been accepted for publication in Milchwissenschaft/Milk Science International (2006).

* Conference Proceedings: Certain sections of this research was presented at the 10th World Congress of Clinical Nutrition. Phuket, Thailand. November 30th – December 3rd, 2004. Oral Presentation: Angiotensin converting enzyme inhibitor in pancreatin hydrolysate of bovine casein isolated by ultrafiltration membrane.

Many ACE inhibitory peptides have been chromatographically isolated from protein hydrolysates. It has previously demonstrated that peptides with high ACE inhibitory activity were made up of 2 to 25 amino acid residues whereas peptides with lower ACE inhibitory activity composed of more amino acid residues (see refs. [15] and [16] for review). We speculated that chain length or molecular weight of the peptides can be prerequisite to the potency of ACE inhibitory peptides. A potential method to isolate the peptides of a desired molecular weight range is ultrafiltration (UF), a technique adopted for many purposes (e.g., cheese making, whey processing, and cheese whey fractionation) in the dairy industry. Due to different molecular weight cut-off value of the membrane equipped in the UF system, peptides with different chain length can be collected in fractions obtained. There is, however, limited information available concerning separation of ACE inhibitory peptides by UF. This study was, therefore, undertaken to separate ACE inhibitory peptides from pancreatin digest of bovine casein by UF.

2.2 MATERIALS AND METHODS

2.2.1 Materials

Bovine casein, pancreatin from porcine pancreas (4x U.S.P), hippuryl-L-histidyl-L-leucine (HHL), ACE from rabbit lung, o-phthaldialdehyde (OPA), dithiothreitol, and standard amino acids were purchased from Sigma-Aldrich Canada Ltd. (Ontario, Canada). Sodium dodecyl sulfate (SDS) was acquired from Bio-Rad Laboratories Canada Ltd. (Ontario, Canada). Other chemicals were obtained from Fisher Scientific Ltd. (Edmonton, Canada). Water was purified by reverse osmosis followed by deionization with a MILLI-Q water purification system (Millipore Corporation, Ontario, Canada).

2.2.2 Preparation of casein hydrolysate

To 6% (w/v) casein solution, prepared by adjusting pH to 7.6 with 1 N NaOH, pancreatin was added at an enzyme to substrate ratio of 1 to 100, and the mixture was incubated at 37 °C for 2, 4, 6, 8, 10, 12 or 18 h. Three replicates were

prepared for incubation mixture at each hydrolysis time. The pH of the mixture was maintained at 7.6 with 1 N NaOH throughout the incubation period. The mixture containing casein hydrolysate, referred to as casein pancreatin hydrolysate (CPH), was then adjusted to pH 4.6 with 1 N HCl, and centrifuged at 12,000 g and 20 °C for 15 min. The supernatant collected was passed through a hollow fiber ultrafiltration membrane with 2,000 Da molecular weight cut-off (MWCO) (Supelco Inc. Bellefonte, PA, USA) under the pressure of 20 psi. Both permeate and retentate obtained were then lyophilized and stored at –20 °C for further analysis.

2.2.3 Determination of ACE inhibitory activity

The method of Cushman and Cheung [17], modified by Nakamura *et al.* [18] was used. The 300 µl assay mixture prepared was comprised of 200 µl of 5 mM HHL dissolved in 0.1 M sodium borate buffer (pH 8.3) containing 0.3 M NaCl, 80 µl of peptide solution and 20 µl of 2 mU ACE. Increasing concentrations (0 – 2500 µg/ml) of peptide were prepared in each assay. After incubation at 37 °C for 30 min, the ACE activity in the assay mixture was stopped by addition of 250 µl of 1 N HCl. The hippuric acid released by the action of ACE was extracted with 1.75 ml of ethyl acetate. Following the removal of ethyl acetate by evaporation at 80 °C under vacuum, the dried hippuric acid obtained was dissolved in water and its absorbance was determined at 228 nm. The percentage of inhibition was calculated using the formula:

$$\% \, Inhibition = \frac{B - A}{B - C} \times 100 \ ,$$

where

A represents the optical density in the presence of HHL, ACE and an inhibitor,

B represents the optical density in the presence of HHL and ACE

and *C* represents the optical density in the presence of HHL.

Zero percent ACE activity inhibition was defined as the activity observed in the presence of HHL and ACE. One hundred percent ACE activity inhibition was defined as the activity observed when ACE and an inhibitor were absent.

The inhibitory activity was expressed as the concentration of peptide required for 50% inhibition of the enzyme activity (IC_{50}). IC_{50} value of each fraction was determined by fitting data to the sigmoidal equation correlated between % inhibition and peptide concentration given below. IC_{50} was the value of x when y equaled 50.

$$y = \frac{ab + cx^d}{b + x^d},$$

where,

y is % inhibition,

x is peptide concentration (μg/ml),

and a,b,c, and d are constants, which may have different values for different fractions. These constants were obtained from a curve fitting software, CurveExpert, version 1.3 copyright©1995-1997 by Daniel Hyams. R^2 (coefficient of determination) of each correlation was ranged from 0.85 to 0.99.

2.2.4 *Determination of the degree of hydrolysis*

The degree of hydrolysis (DH) was determined by the OPA reaction as described by Nielsen *et al.* [19]. The OPA reagent was prepared by dissolving 0.10 M disodium tetraborate decahydrate, 3.47 mM SDS, 5.96 mM OPA (dissolved in ethanol prior to mixing) and 5.70 mM dithiothreitol. Three milliliters of OPA reagent was added to 400 μl of sample solution in water. After mixing for 5 s and standing for exactly 2 min, the optical density of the reaction mixture was read at 340 nm. Serine was used as a standard. The percentage of degree of hydrolysis (% DH) was calculated using the following formula:

$$\%\,DH = \frac{\left[\left(\frac{OD_{Sample} - OD_{Blank}}{OD_{Standard} - OD_{Blank}}\right) \times \frac{0.9516 \times 10}{S \times P}\right] - \beta}{\alpha \times h_{tot}} \times 100,$$

in which

OD represents the optical density observed

S represents percentage of sample solution concentration,

P represents percentage of protein content in the sample,

and α, β, and h_{tot} are 1.039, 0.383, and 8.2, respectively given by Adler-Nissen [20] for casein

2.2.5 *Determination of nitrogen content*

A LECO Nitrogen/Protein Determinator model FP-428 (LECO Corporation, St. Joseph, MI, USA) was used to determine the nitrogen content. A 20 mg sample was placed in the sample holder and the instrument was operated following the procedure described in the company manual. The protein content was calculated using a factor of 6.38 to convert from nitrogen content.

2.2.6 *Amino acid analysis*

Tryptophan was analyzed by a reverse-phase HPLC method described by Sedgwick *et al.* [21]. A sample was weighed in a 1.3 × 10 cm glass screw cap culture tube, to which 4 ml of 4.2 N NaOH and 0.8 ml of 25% (w/v) pyrogallol were added. The sample was hydrolyzed in the presence of nitrogen at 110 °C for 20 h. After hydrolysis, 100 µl of 0.075 % (w/v) amino-n-caproic acid and 100 µl of water were added to the hydrolysate, and the mixture was centrifuged at 770 g for 15 min. An aliquot (200 µl) of the supernatant obtained was then mixed with 30 µl of glacial acetic acid and 600 µl of saturated potassium tetraborate tetrahydrate. This was mixed with a fluoraldehyde reagent in an approximate ratio of 1 to 1 prior to reverse-phase HPLC analysis. The fluoraldehyde reagent consisted of 0.25 g of OPA first dissolved in 6 ml methanol, 56 ml of 0.04 M sodium borate buffer, pH 9.5, 0.25 ml of 2-mercaptoethanol and 2 ml of Brij 35. The Varian 5000 HPLC system (Varian, Inc., Walnut Creek, CA, USA) was equipped with a Supelcosil 3 µm LC-18 reverse phase column (4.6 x 150 mm; Supelco, Inc. Bellefonte, PA, USA.) and a Fluorichrom fluorescence detector (Varian, Inc., Walnut Creek, CA, USA). Fluorescence was monitored with excitation wavelength of 340 nm and emission wavelength of 450 nm.

Analysis of amino acids other than tryptophan was performed by using a Beckman model 6300 amino acid analyzer on samples hydrolyzed in the presence of nitrogen at 110 °C for 24 h in glass-distilled 6 N HCl containing 0.1% (w/v) phenol.

2.2.7 *Statistical analysis*

Comparison of ACE inhibitory effect among CPH fractions within the same and with different hydrolysis time was carried out using an analysis of covariance (ANCOVA) with peptide concentration as a covariate. If a significant difference was found on ANCOVA, a Least Significant Difference (LSD) test was performed.

Yields of CPH, and its fractions, and amino acid compositions in permeates with different hydrolysis time were compared by using analysis of variance (ANOVA) to detect significant difference between means. The Tukey's Honest Significant Difference test was carried out for the post hoc testing.

All statistical analysis was performed on a data analysis software SPSS, version 13.0 (SPSS Inc., Chicago, Ill, USA). All differences were considered significant at $p \leq 0.05$. Statistical analysis results were shown in details in Appendix A.

2.3 RESULTS AND DISCUSSION

The DH and ACE inhibitory activity determined in CPH prepared with different hydrolysis time are shown in Fig.2.1. The DH (estimated as percentage of cleaved peptide bonds) rapidly increased during the first 4 h with the rate of increase being relatively gradual thereafter. Similarly, the ACE inhibitory activity rapidly increased during the first 2 h, indicating occurrence of peptides with ACE inhibitory activity. No measurable ACE inhibitory activity was observed in casein without pancreatin treatment. This is in accordance with previous reports that enzymatic hydrolysis of casein released ACE inhibitory peptides [6],[12],[13],[14]. The rate of increase in the inhibitory activity was apparently small after 2 h of incubation. The inhibitory activity then dropped at 12 h and increased at 18 h. The decrease in the ACE inhibitory activity at 12 h was probably because some peptides with ACE inhibitory effect were degraded and thus lost their activity as previously reported in the hydrolysis of whey proteins with digestive enzyme [8] or in the further enzymatic hydrolysis of Emmental cheese water-soluble extracts [22]. However, further hydrolysis could result in an increase in ACE inhibition as well. Literature review

[16] has shown that peptide **Ile-Pro-Pro** (β-casein, f 74-76) had exceptionally higher inhibitory activity than peptide Asn-**Ile-Pro-Pro**-Leu-Thr-Gln-Thr-Pro-Val (β-casein, f 73-82). Or peptide **Thr-Thr-Met-Pro-Leu-Trp** (α_{s1}-casein, f 194-199) had remarkably higher ACE inhibitory activity than peptide Gly-Thr-Gln-Tyr-Thr-Asp-Ala-Pro-Ser-Phe-Ser-Asp-Ile-Pro-Asn-Pro-Ile-Gly-Ser-Glu-Asn-Ser-Glu-Lys-**Thr-Thr-Met-Pro-Leu-Trp** (α_{s1}-casein, f 170-199). This could explain the increase in ACE inhibitory activity at 18 h was due to further hydrolysis.

The yield of dry matter of CPH and its fractions including supernatant and UF permeate are given in Table 2.1. The yield of CPH was relatively constant among samples obtained with different hydrolysis time, and averaged 98.3% of dry weight of casein used for pancreatin digestion, indicating minor losses of peptides during hydrolysis. The yield of dry matter of supernatant was already high at 2 h (81% of recovered CPH), increased with increasing hydrolysis time, and reached 88% at 18 h. This indicated that most (> 80%) of casein was degraded after 2 h of incubation with pancreatin, and thus not precipitated at pH 4.6. The yield of permeate obtained with 2,000 Da MWCO membrane also increased with increasing incubation time and was the highest in the sample prepared with 18 h hydrolysis, accounting for approximately 50% of CPH recovered. Statistical analysis revealed that there was no significant difference ($p > 0.05$) of the yields of CPH, and permeate among different hydrolysis times (Appendix A; Table A1, A2). However, yield of supernatant at different time were different ($p \leq 0.05$) with the lowest at 2 and 4 h, the highest at 18 h and similar at 6, 8, 10, and 12 h (Table A3). Hydrolysis time greater than 18 h was not attempted in this study. Nonetheless, our preliminary study (S. Kusump, T. Nakano, and L. Ozimek, unpublished data) showed that a commercial sample of CPH (Sigma-Aldrich Canada Ltd.), an intensively hydrolysed casein with no detectable Coomassie blue stained protein band on SDS-gel electrophoresis had ACE inhibitory activity with IC_{50} of 600 μg/ml comparable to that of CPH permeate in this study (Table 2.2). This suggests that the yield of permeate is likely increased if the casein-pancreatin mixture is incubated longer but the increase of ACE inhibitory activity may be small, if any. Further study is needed to determine the hydrolysis time providing the highest yield of permeate without losing ACE inhibitory activity.

ACE inhibitory activities determined in CPH fractions are shown in Fig. 2.2-2.8 and Table 2.2. The ACE inhibitory activity was higher ($p \leq 0.05$) in the permeate than in the corresponding supernatant, and lower ($p \leq 0.05$) in the retentate than in the corresponding supernatant in all samples examined (Table A4 - A10). The IC_{50} value ranged from 728 to 1097 μg peptide/ml in the supernatant, 464 to 814 μg peptide/ml in the permeate, and 1106 to 1588 μg peptide/ml in the retentate. According to IC_{50} values, the ACE inhibitory activity of the permeate was 1.1-1.7 fold stronger than that of the supernatant and the ACE inhibitory activity of the supernatant was 1.4-1.7 fold higher than that of the retentate. These results demonstrated that UF could separate peptides of higher ACE inhibitory activity from those of lower ACE inhibitory activity. This is consistent with the report of Pihlanto-Leppala *et al.* [8] who sequentially digested whey proteins, β-lactoglobulin and α-lactalbumin with pepsin, trypsin and chymotrypsin, fractionated the hydrolysates by UF with 1,000 Da MWCO membrane, and found higher ACE inhibitory activity in the permeate than in the supernatant.

In addition, IC_{50} of the permeate at 2 h was akin to that of the supernatant at 18 h (Table 2.2). This indicated that with UF, production time of ACE inhibitory peptide could be reduced.

When compared only the ACE inhibitory activity of the permeates obtained at different hydroysis time, permeate at 8 and 18 h exhibited the highest ($p \leq 0.05$) activity, permeate at 2, 4, 6, 10 and 12 h exerted lowest ($p \leq 0.05$) activity (Fig. 2.9 and Table A11). Nonetheless, permeate at 6 and 18 h had similar activity ($p > 0.05$). IC_{50} was the lowest in the permeate at 8 h (501 μg peptide/ml). Therefore, the permeate obtained from the UF of CPH at 8 h was chosen for further purification of ACE inhibitory peptides in the experiments described in the next chapter.

Results of amino acid analysis (Table 2.3) showed that serine, glutamine/ glutamic acid, leucine, lysine and proline are the major amino acids in both casein and CPH permeate. In the latter, the contents of serine and glutamine/ glutamic acid apparently decreased and those of leucine and tyrosine increased with increasing hydrolysis time. There was no appreciable difference in the contents of remaining amino acids among the permeates with different hydrolysis time. Permeates compared

to casein tended to have a lower content of glutamine/glutamic acid and higher contents of tyrosine, phenylalanine, lysine, arginine and tryptophan. Amino acids were also categorized into aromatic, basic, aliphatic and cyclic amino acids according to their side chains, and the amino acid content in each category was compared between casein and permeates (Fig. 2.10). The content of aromatic amino acid (Phe, Trp and Tyr) or basic amino acid (Arg, Lys and His) was higher ($p \leq 0.05$) (Table A12, A13) and that of cyclic amino acid (Pro) was lower ($p \leq 0.05$) (Table A14) in all permeates examined. The content of aliphatic amino acid (Ala, Val, Met, Leu, and Ile) was, however, similar ($p > 0.05$) between casein and permeates at 2, 4, and 6 h and higher ($p \leq 0.05$) in permeates at 8, 10, 12 and 18 h (Table A15). This is consistent with previous reports [23],[24],[25] demonstrating that peptides with high ACE inhibitory activity are rich in aromatic or aliphatic or basic amino acids.

High blood pressure is a worldwide problem causing several million deaths every year [26]. ACE inhibitors are thought to be one of the preferred remedies for this condition [27]. The UF used in the present study appears to be an efficient technique for preparation of low molecular size (< 2,000 Da) peptides that can inhibit ACE activity. In order to exhibit ACE inhibitory properties *in vivo*, the peptides have to be small enough to be absorbed in gastrointestinal tracts and delivered to their target organ [28][29]. Roberts and others [30] fed rats with peptides composed of 3 to 51 amino acids, and reported absorption of these compounds through the guts as intact peptides. It is interesting to test whether the ACE inhibitory peptides present in the CPH permeate in this study can be absorbed intact through the intestinal wall and produce their physiological action.

2.4 CONCLUSION

We postulated that molecular weight of the peptides can be used as a criterion for isolating peptides with high ACE inhibitory activity and UF can be a process to enrich the ACE inhibitory peptides in the permeate fraction. With a selection of appropriate membrane module, UF can yield a desired molecular weight distribution of the fractions. The present results demonstrate for the first time the production of ACE inhibitory peptide from CPH using UF. The technique is simple

and straightforward for large-scale production. It may contribute to the development of economical procedures for industrial production of ACE inhibitory peptides.

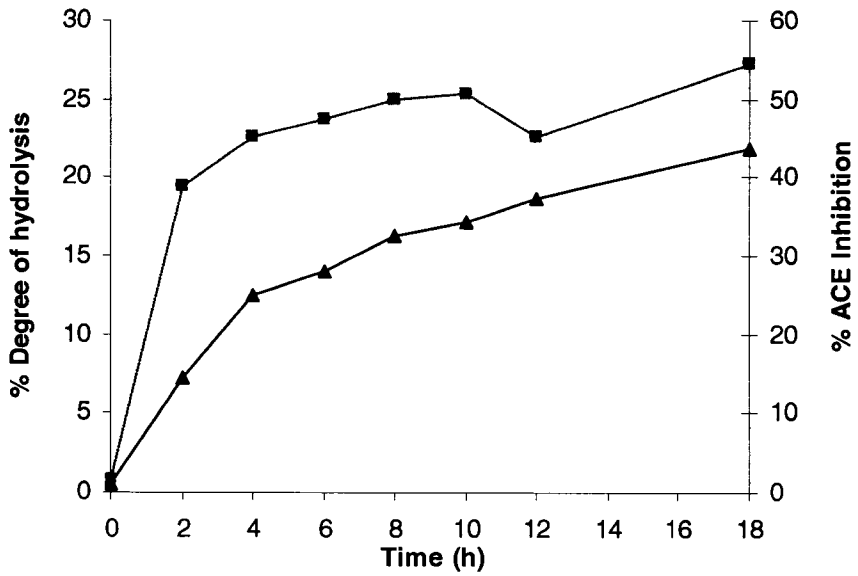

Figure 2.1:.Hydrolysis of casein with pancreatin as a function of time.

—▲— Degree of hydrolysis

—■— ACE inhbiitory effect

The ACE inhibitory activity was determined in 80 µl of supernatant fraction (1 mg dry weight/ml) at each hydrolysis time. See Methods for other details.

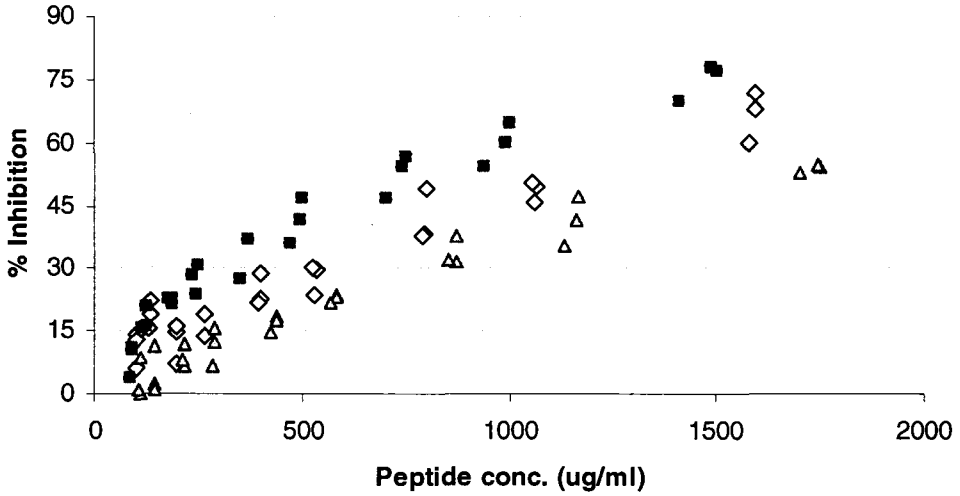

Figure 2.2:.ACE inhibitory activity of CPH fractions at the hydrolysis time of 2 h.

◇ Supernatant obtained by centrifugation of CPH

■ Permeate obtained by UF of supernatant

△ Retentate obtained by UF of supernatant

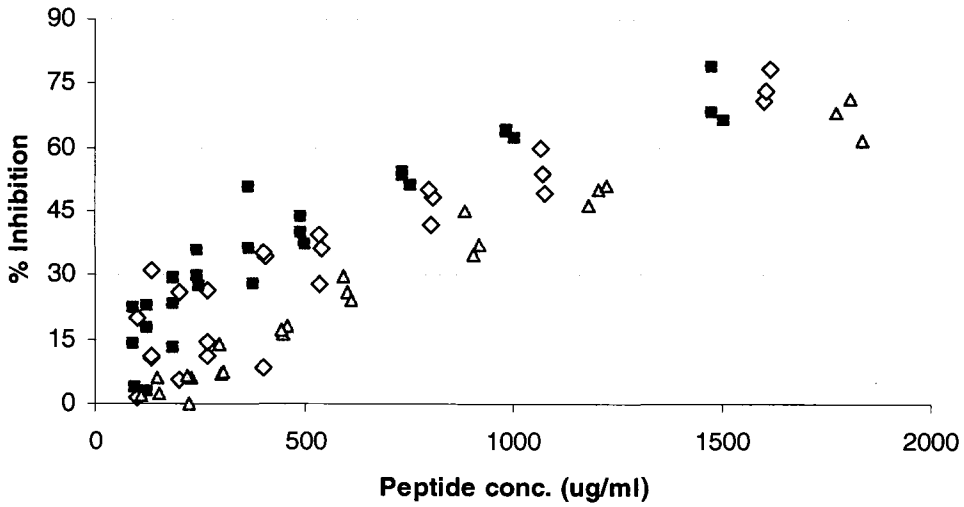

Figure 2.3: ACE inhibitory activity of CPH fractions at the hydrolysis time of 4 h.

◇ Supernatant obtained by centrifugation of CPH

■ Permeate obtained by UF of supernatant

△ Retentate obtained by UF of supernatant

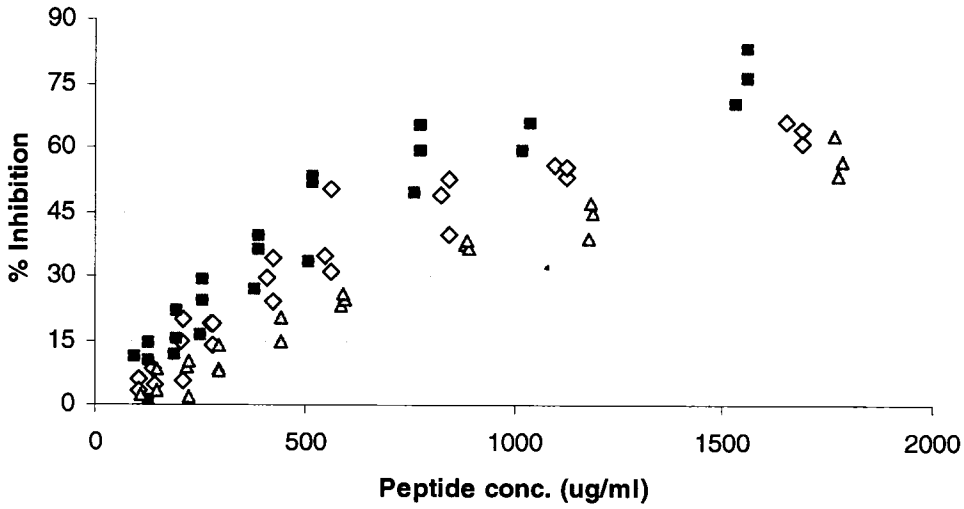

Figure 2.4: ACE inhibitory activity of CPH fractions at the hydrolysis time of 6 h.

◇ Supernatant obtained by centrifugation of CPH

■ Permeate obtained by UF of supernatant

△ Retentate obtained by UF of supernatant

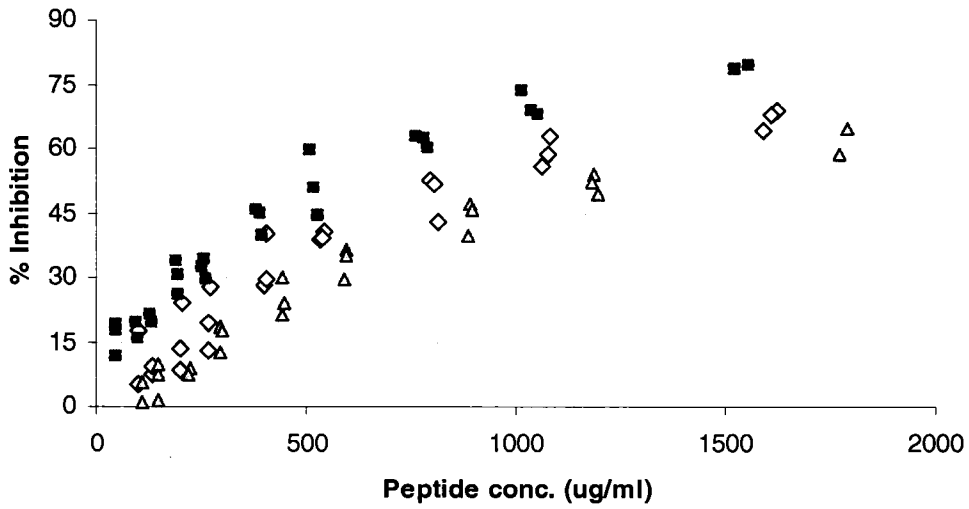

Figure 2.5: ACE inhibitory activity of CPH fractions at the hydrolysis time of 8 h.

◇ Supernatant obtained by centrifugation of CPH

■ Permeate obtained by UF of supernatant

△ Retentate obtained by UF of supernatant

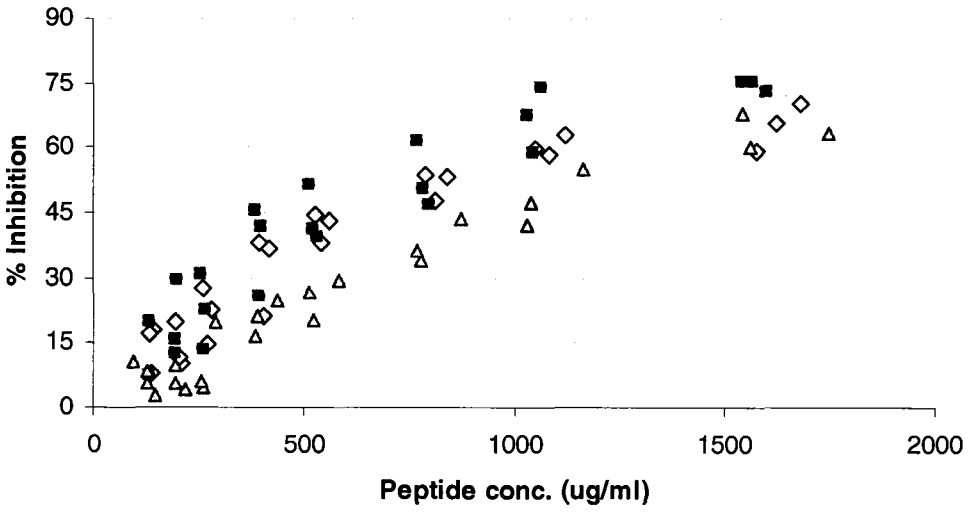

Figure 2.6: ACE inhibitory activity of CPH fractions at the hydrolysis time of 10 h.

◇ Supernatant obtained by centrifugation of CPH

■ Permeate obtained by UF of supernatant

△ Retentate obtained by UF of supernatant

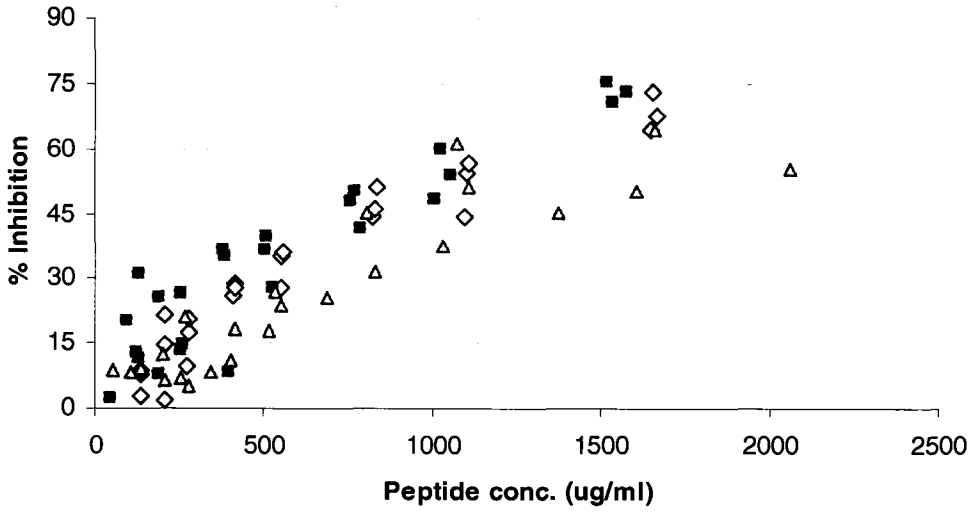

Figure 2.7: ACE inhibitory activity of CPH fractions at the hydrolysis time of 12 h.

◇ Supernatant obtained by centrifugation of CPH

■ Permeate obtained by UF of supernatant

△ Retentate obtained by UF of supernatant

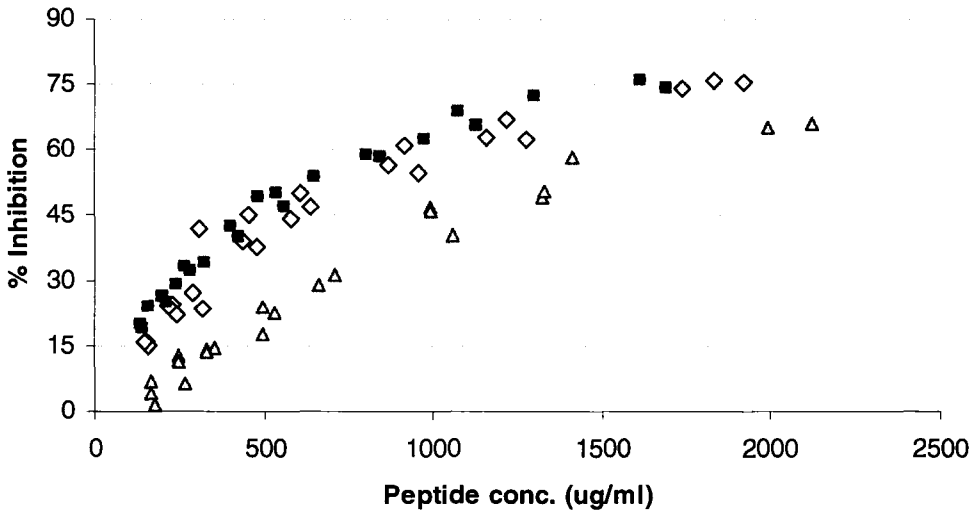

Figure 2.8: ACE inhibitory activity of CPH fractions at the hydrolysis time of 18 h.

◇ Supernatant obtained by centrifugation of CPH

■ Permeate obtained by UF of supernatant

△ Retentate obtained by UF of supernatant

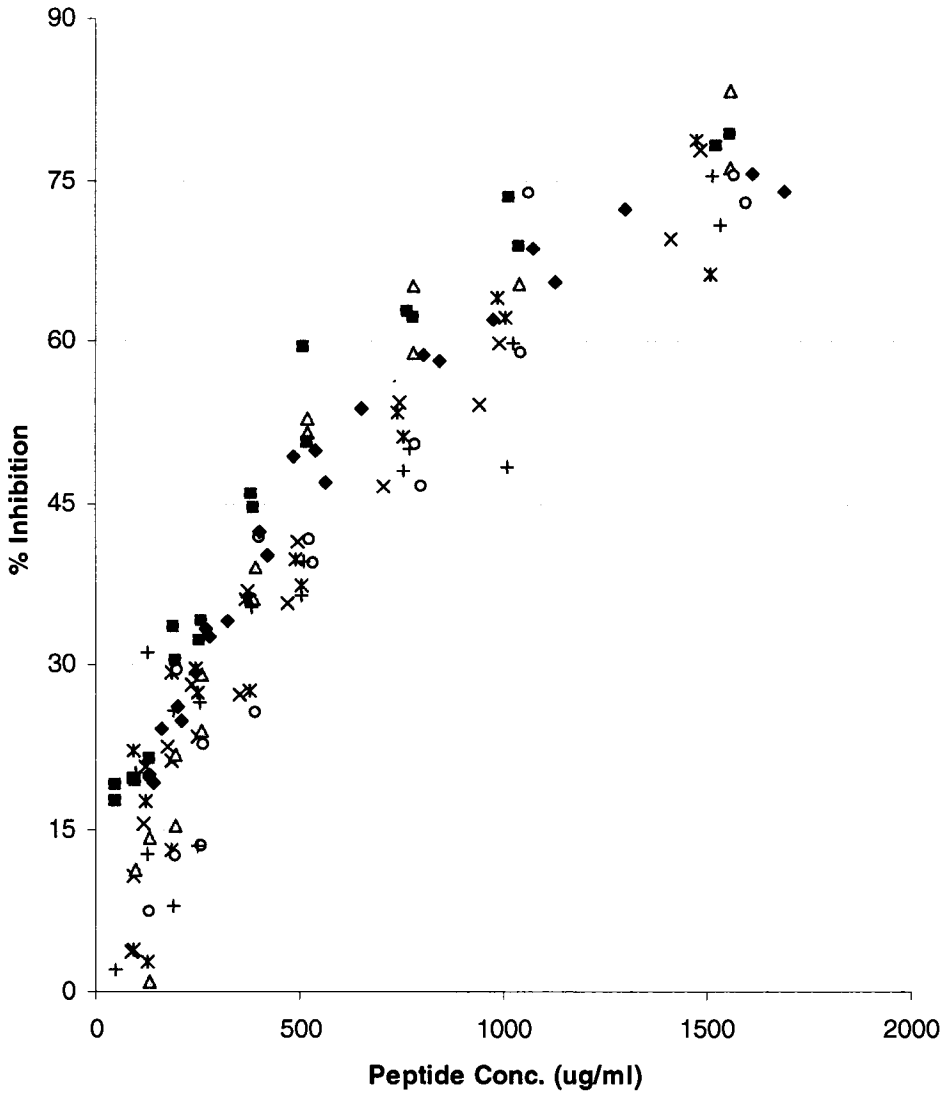

Figure 2.9: ACE inhibitory activity of permeates obtained from supernatant of CPH at different hydrolysis times.

× 2 ✱ 4 △ 6 ■ 8 ○ 10 + 12 ◆ 18

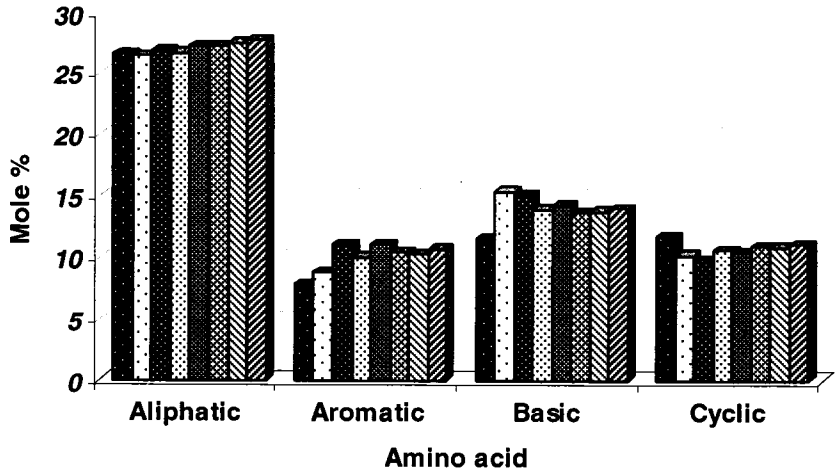

Figure 2.10: Amino acid contents of casein and permeates categorized by amino acid side chain.

■ Casein □ 2 ■ 4 ▨ 6 ■ 8 ▩ 10 ▧ 12 ▨ 18

Table 2.1: Yield of CPH and its fractions at different hydrolysis time

Time (h)	Yield[1]		
	CPH	Supernatant	Permeate
2	98 ± 0.2	79 ± 1.0[b]	39 ± 2.9
4	98 ± 2.6	80 ± 2.6[b]	41 ± 4.0
6	99 ± 2.1	82 ± 1.7[a,b]	45 ± 1.1
8	98 ± 3.3	82 ± 2.1[a,b]	47 ± 3.6
10	99 ± 5.4	83 ± 1.5[a,b]	48 ± 5.2
12	98 ± 2.2	83 ± 0.9[a,b]	49 ± 4.5
18	98 ± 6.2	86 ± 5.0[a]	50 ± 7.1

[1] Percentage of dry weight of casein used for pancreatin digestion expressed as mean ± SD

Different letters in the same column indicate significantly different ($p \leq 0.05$)

Table 2.2: ACE inhibitory activity of CPH fractions

Hydrolysis time (h)	Concentration (μg peptide/ml) for 50% inhibition (IC_{50})*		
	Supernatant	Permeate	Retentate
2	1096.9 ± 79.7	729.4 ± 70.2	1588.0 ± 59.5
4	852.4 ± 60.6	690.7 ± 48.3	1191.1 ± 24.3
6	817.2 ± 94.5	531.0 ± 23.3	1406.0 ± 27.1
8	803.4 ± 23.5	464.0 ± 51.1	1141.1 ± 65.9
10	749.0 ± 4.7	730.2 ± 60.0	1105.6 ± 44.0
12	906.3 ± 30.7	814.4 ± 53.9	1311.8 ± 161.6
18	751.3 ± 60.3	579.0 ± 38.6	1244.8 ± 33.4

* Expressed as mean ± SD

Table 2.3: Amino acid compositions of casein and CPH permeates obtained at different hydrolysis time

Amino acid	Casein	Permeate						
		Hydrolysis time (h)						
		2	4	6	8	10	12	18
		(moles/100 moles of total amino acids)						
Asparagine and aspartic acid	6.6	5.7	5.5	5.9	5.6	5.8	5.8	5.6
Threonine	5.0	4.2	4.0	4.3	4.2	4.3	4.4	4.4
Serine	8.8	8.7	8.0	8.2	7.6	7.6	7.5	7.0
Glutamine and glutamic acid	18.5	17.8	16.8	17.4	16.5	16.8	16.7	16.2
Glycine	3.1	2.6	2.7	2.9	2.9	3.0	3.1	3.2
Alanine	4.3	4.6	4.7	4.5	4.5	4.5	4.5	4.5
Valine	6.6	6.2	5.9	6.2	6.2	6.3	6.3	6.3
Methionine	2.3	2.3	2.2	2.0	2.3	2.3	2.3	2.3
Isoleucine	4.5	4.2	4.0	4.2	4.0	4.1	4.2	4.1
Leucine	8.9	9.2	10.1	9.7	10.4	10.2	10.3	10.6
Tyrosine	3.9	4.4	5.8	5.2	5.9	5.5	5.4	5.6
Phenylalanine	3.9	4.3	5.0	4.7	5.0	4.8	4.8	4.9
Histidine	2.3	2.1	1.9	2.1	2.0	2.1	2.2	2.4
Lysine	6.6	9.0	8.9	8.2	8.4	8.0	8.1	8.2
Arginine	2.7	4.2	4.4	3.6	3.8	3.5	3.5	3.5
Proline	11.8	10.2	9.7	10.6	10.5	11.0	10.8	11.1
Tryptophan	0.1	0.2	0.2	0.2	0.2	0.2	0.2	0.2

Note: Cysteine was not analysed.

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CHAPTER 3

PURIFICATION AND IDENTIFICATION OF ANGIOTENSIN CONVERTING ENZYME INHIBITORY PEPTIDES FROM CASEIN HYDROLYSATES

3.1 INTRODUCTION

Food proteins have been regarded as nutritional values for centuries until lately researchers have found that food proteins also exhibit functional properties such as antimicrobial [1], immunomodulatory [2], antihypertensive [3] antithrombotic [4] properties. These properties are the results of the liberation of bioactive peptides from food proteins by enzymatic reaction. Angiotensin converting enzyme inhibitory peptides are one of the bioactive peptides latent in food proteins, which exhibit antihypertensive property. Angiotensin converting enzyme (ACE; EC 3.4.15.1), a peptidyl dipeptidase found in the rennin-angiotensin system, plays a prominent role in the regulation of blood pressure by converting angiotensin I to angiotensin II [6], an effective vasoconstrictor causing high blood pressure. In addition, ACE degrades bradykinin, a vasodilator into inactive peptides, resulting in a decrease in the vasodilatory effect. The inhibition of ACE activity lowers the level of angiotensin II and potentiates the activity of bradykinin, leading to antihypertensive effect.

ACE inhibitory peptides have been isolated from many food proteins including caseins [3], whey [5], zein [6], wheat [7], pea [8], and fish [9] proteins. We have employed ultrafiltration to isolate ACE inhibitory peptides from the pancreatic hydrolysate of casein (CPH) in our previous study [10]. Chromatographic techniques are widely used to purify ACE inhibitory peptides. Identification of ACE inhibitory peptides indicate that ACE inhibitory peptides comprise of 2 to 25 amino acid residues and most of the residues are hydrophobic [3],[11]. Two to three step gel filtration chromatography has been reported for isolating ACE inhibitory peptides from casein hydrolysate [12], [13]. However, the method was time consuming and requires a large volume of buffer.

In the present study, a single step separation method with hydrophobic interaction chromatography (HIC) on phenyl-agarose or gel filtration chromatography on Bio-Gel P2 was used to separate ACE inhibitory peptides from the permeate of CPH. Amino acid sequence of ACE inhibitory peptides was then identified by using on-line HPLC-tandem mass spectrometry (LC/MS/MS).

3.2 MATERIALS AND METHODS

3.2.1 Materials

Bovine casein, pancreatin from porcine pancreas (4x U.S.P), hippuryl-L-histidyl-L-leucine (HHL), ACE from rabbit lung, trifluoroacetic acid (TFA), bovine serum albumin (BSA), o-phthaldialdehyde (OPA), Brij 35, standard amino acids and phenyl-agarose were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Bio-Gel P2 was acquired from Bio-Rad Laboratories Canada Ltd. (Mississauga, ON, Canada). Other chemicals were obtained from Fisher Scientific Ltd. (Edmonton, AB, Canada). Water was purified by reverse osmosis followed by deionization with a MILLI-Q water purification system (Millipore Corporation, Mississauga , ON, Canada).

3.2.2 Preparation of casein hydrolysate

To 6% (w/v) casein solution, prepared by adjusting pH to 7.6 with 1 N NaOH, pancreatin was added at an enzyme to substrate ratio of 1 to 100, and the mixture was incubated at 37 °C for 8 h. The pH of the mixture was maintained at 7.6 with 1 N NaOH throughout the incubation period. The mixture containing casein hydrolysate, referred to as casein pancreatin hydrolysate (CPH), was then adjusted to pH 4.6 with 1 N HCl, and centrifuged at 12,000 g and 20 °C for 15 min. The supernatant collected was passed through a hollow fiber ultrafiltration membrane with 2,000 Da molecular weight cut-off (MWCO) (Supelco Inc. Bellefonte, PA, USA) under the pressure of 20 psi. The permeate obtained was then lyophilized and stored at – 20 °C for further purification steps.

3.2.3 Determination of ACE inhibitory activity

The method of Cushman and Cheung [14], modified by Nakamura *et al.* [15] was used. The 300 µl assay mixture prepared was comprised of 200 µl of 5 mM HHL dissolved in 0.1 M sodium borate buffer (pH 8.3) containing 0.3 M NaCl, 80 µl of peptide solution and 20 µl of 2 mU ACE. After incubation at 37 °C for 30 min, the ACE activity in the assay mixture was stopped by addition of 250 µl of 1 N HCl. The hippuric acid released by the action of ACE was extracted with 1.75 ml of ethyl acetate. Following the removal of ethyl acetate by evaporation at 80 °C under vacuum, the dried hippuric acid obtained was dissolved in water and its absorbance was determined at 228 nm. The percentage of inhibition was calculated using the formula: $(B-A)/(B-C) \times 100$, where *A* represents the absorbance in the presence of HHL, ACE and an inhibitor, and *B* represents the absorbance in the presence of HHL and ACE. *C* represents the absorbance in the presence of HHL. The inhibitory activity was expressed as the concentration of peptide required for 50% inhibition of the enzyme activity (IC_{50})

3.2.4 Hydrophobic interaction chromatography (HIC)

HIC was carried out at either pH 6.0 or 6.8. A sample of 150 mg of permeate of CPH was mixed in 10 ml of 0.01 M sodium phosphate buffer containing 5 M NaCl (buffer A). prior to mixing, buffer A was adjusted to an appropriate pH. This mixture was passed through a 0.45 µm Millipore filter unit (Millipore, Corporation, Mississauga, ON, Canada). The filtrate obtained was applied to a 1.5×6.5 cm column of phenyl-agarose equilibrated with buffer A adjusted to an appropriate pH value. The column was washed with buffer A having the same pH and eluted stepwise with decreasing concentrations of NaCl from 5 to 1 M, and then with water. Fractions of 2 ml were collected at a flow rate of 18 ml/h and monitored for peptide contents by measuring absorbance at 210 and 230 nm. Fractions from a major peptide peak eluted with different concentration of NaCl or water were pooled and desalted using a 9×1 cm column of Bio-Gel P2. The desalted eluate was then concentrated to 1 ml, and its 80 µl portion was tested for ACE inhibitory activity. Eluates with high ACE inhibitory activity were lyophilized and determined for IC_{50}.

3.2.5 Gel filtration chromatography

A sample of 100 mg of permeate of CPH was dissolved in 1 ml of 50 mM ammonium bicarbonate (buffer B). This preparation was passed through a 0.45 μm Millipore filter unit. A 700 μl portion of the filtrate obtained was applied to a 55.5 cm × 1 cm column of Bio-Gel P2 equilibrated and eluted with buffer B. Fractions of 1 ml were collected at a flow rate of 11 ml/h and monitored for peptide contents by measuring absorbance at 210 and 230 nm. A portion (80 μl) of peptide-containing fraction was tested for ACE inhibitory activity. Eluates with high ACE inhibitory activity were pooled, lyophilized and determined for IC_{50}.

3.2.6 Reverse phase HPLC (RP-HPLC)

Preparative RP-HPLC was performed on a Varian 9010 HPLC system (Varian, Inc., Walnut Creek, CA, USA) equipped with a Hewlett Packard 1050 autosampler (Hewlett-Packard, Palo Alto, CA, USA) and a UV Monitor 1305 (Bio-Rad Laboratories Canada Ltd., Ontario, Canada). A sample of 0.0031 g of the fraction with the highest IC_{50} from Bio-Gel P2 was dissolved in 1 ml of 0.1% TFA in water (solvent A). After filtration through a 0.22 μm Millipore filter unit, a 80 μl portion of the filtrate was applied to a Sephasil Peptide C18 5 μm reverse phase column (4.6 × 100 mm, Amersham Biosciences AB, Uppsala, Sweden) with C18 cartridge as a guard column. The peptides were eluted at a flow rate of 1.0 ml/min starting with solvent A for 10 min and a linear gradient from 0 to 70% of 0.1% TFA in acetonitrile (solvent B) for 60 min. The eluates were monitored for absorbance at 214 nm and those corresponding to a single peptide peak were pooled for further studies.

3.2.7 Identification of peptides by HPLC/MS/MS

Fractions obtained from RP-HPLC were subjected to LC- MS/MS analysis on a Waters Q-Tof Premier Mass spectrometer (Waters, Milford, MA, USA) equipped with a Waters nanoAquity UPLC system (Waters, Milford, MA, USA). The RP-HPLC fractions were concentrated in a speed vacuum drying device (Savant Speed Vac® plus SC11A, TeleChem International, Sunnyvale, CA, USA) to about 10% of original volumes. Two microliters of concentrated peptide solution (with overloading

factor of 2) were loaded onto a peptide trap (180μm × 20mm, Symmetry® C18 nanoAcquity™ column, Waters, Milford, MA, USA) sequentially equipped with an analytical column (75 μm × 100 mm, Atlantis™ dC18 nanoAcquity™ column, Waters, Milford, MA, USA). Desalting on the peptide trap was achieved by flushing trap with 2% acetonitrile, 0.1% formic acid (solvent C) at a flow rate of 4 μl/min for 1.5 min. Peptides were separated with a gradient of 2 - 95% of 0.1% formic acid in acetronitrile (solvent D) over 35 min at a flow rate of 300 nl/min. The column was connected to a Waters NanoEase™ emitter with tip size of 90 μm O.D. and 20 μm I.D. (Waters, Milford, MA, USA) for ESI-MS/MS analysis.

The MS/MS data obtained were analyzed through MS/MS Ion Search, one of the Mascot search engines (Matrix Science Inc., Boston, MA). Databases were established by grouping all possible sequences with variants of the expected proteins. Peptides were identified by searching against these homemade databases. Settings for a database search were as follows: parent ion and MS/MS tolerance were set to 80 ppm and 0.4 Da respectively; no enzyme was specified; 2 missed cleavage sites per peptide were allowed; commonly possible modifications, deamidation (NQ), N-acetyl (protein), N-formyl (protein), oxidation (M), phospho (ST) and phospho (Y), were set as the variable modifications.

Peptide identifications were further confirmed by examining the scores and manual inspection of the original MS/MS spectra. Good spectra with significant numbers of matched high intensity peaks were considered important for the identification.

3.2.8 Determination of peptide content

Peptide content was spectrophotometrically measured at the wavelength of 210 nm as described by Stoscheck [16] using BSA as a standard.

3.2.9 Amino acid analysis

Tryptophan was analyzed by a RP-HPLC method described by Sedgwick *et al.* [17]. A sample was weighed in a 1.3 × 10 cm glass screw cap culture tube, to which 4 ml of 4.2 N NaOH and 0.8 ml of 25% (w/v) pyrogallol were added. The sample was hydrolyzed in the presence of nitrogen at 110 °C for 20 h. After

hydrolysis, 100 µl of 0.075 % (w/v) amino-n-caproic acid and 100 µl of water were added to the hydrolysate, and the mixture was centrifuged at 770 g for 15 min. An aliquot (200 µl) of the supernatant obtained was then mixed with 30 µl of glacial acetic acid and 600 µl of saturated potassium tetraborate tetrahydrate. This was mixed with a fluoraldehyde reagent (see below) in an approximate ratio of 1 to 1 prior to RP-HPLC analysis. The fluoraldehyde reagent consisted of 0.25 g of OPA first dissolved in 6 ml methanol, 56 ml of 0.04 M sodium borate buffer, pH 9.5, 0.25 ml of 2-mercaptoethanol and 2 ml of Brij 35. The Varian 5000 HPLC system (Varian, Inc., Walnut Creek, CA, USA) was equipped with a Supelcosil 3 µm LC-18 reverse phase column (4.6 x 150 mm; Supelco, Inc. Bellefonte, PA, USA.) and a Fluorichrom fluorescence detector (Varian, Inc., Walnut Creek, CA, USA). Fluorescence was monitored with excitation wavelength of 340 nm and emission wavelength of 450 nm.

Analysis of amino acids other than tryptophan was performed by using a Beckman model 6300 amino acid analyzer on samples hydrolyzed in the presence of nitrogen at 110 °C for 24 h in glass-distilled 6 N HCl containing 0.1% (w/v) phenol.

3.3 RESULTS AND DISCUSSION

3.3.1 HIC

Elution profile of CPH permeate on phenyl-agarose column at pH 6.0 and 6.8 are shown in Fig. 3.1, and the ACE inhibitory activities determined in fractions from the column are given in Table 3.1. ACE inhibitory activities eluted from phenyl-agarose was very weak at pH 6.0, and slightly increased at pH 6.8. However, the IC_{50} determined at this pH was much lower than that for the CPH permeate (IC_{50} of 464 µg peptide/ml) applied to the column. These observations suggested that most of the ACE inhibitory peptides did not bind to the phenyl-agarose.

It was previously reported that peptides with ACE inhibitory activity were rich in hydrophobic amino acid residues [18], [19]. This formed the idea of using phenyl-agarose column to separate such peptides. However, results from this study indicated that the phenyl-agrose column with 0.01 M sodium phosphate containing NaCl as eluent may not be suitable for isolating ACE inhibitory peptides from CPH.

Changes in the HIC medium along with elution buffer or pH may yield an improved result. However, those changes were not attempted in this study.

3.3.2 Gel filtration chromatography

Elution profile of CPH permeate on Bio-Gel P2 column is shown in Fig. 3.2, and analytical data for eluates from the column is given in Table 3.2. High ACE inhibitory activities were seen in three fractions (I-III) accounting for , respectively 25, 20, 23 % of permeate used for purification. The IC_{50} value was the lowest in Fraction II and lower in fraction III than in fraction I. The value for fraction II was 1.5 fold less than that for the CPH (see above), suggesting that column chromatography on Bio-Gel P2 is an appropriate technique to isolate ACE inhibitory peptides.

Amino acid analysis (Table 3.3) of fractions collected from gel filtration chromatography revealed that major amino acids in fractions I were glutamine and glutamic acid, proline and leucine. While leucine, glutamine and glutamic acid and serine were major amino acids in fraction II. Fraction III had lysine, leucine and phenylalanine as major amino acids. Amino acids were also categorized according to their side chains (Fig. 3.3). Fraction II contained higher contents of aliphatic (Ala, Val, Met, Leu, and Ile), aromatic (Phe, Trp and Tyr), and basic (Arg, Lys and His) amino acids, and lower contents of cyclic (Pro) amino acids than did fraction I. Fraction III had higher contents of aromatic, and basic amino acids and lower contents of aliphatic and cyclic amino acids than did fraction I or II. However, when the contents of hydrophobic amino acids (Ala, Val, Met, Leu, Ile, Phe, Trp, Tyr and Pro) were compared, it was the highest in fraction II, and higher in fraction I than in fraction III. This is in a good agreement with previous report [18],[19], which demonstrated that peptides with high ACE inhibitory effect were rich in hydrophobic or basic amino acids. However, the present result indicated that amount of basic amino acids may not play a crucial role in ACE inhibitory effect.

3.3.3 RP-HPLC

Fraction II from Bio-Gel P2 gel filtration column was further fractionated by RP-HPLC and its chromatogram is illustrated in Fig. 3.4. Seventeen peak fractions collected were determined for ACE inhibitory activity (Fig. 3.5). Most of hydrophobic

fractions exhibited stronger ACE inhibitory activity compared to relatively hydrophilic fractions with the exception of fraction 4. Fraction 13 exhibited the highest ACE inhibitory activity per peak area unit whereas fraction 1 exerted the lowest activity. Some of the fractions obtained by RP-HPLC were subjected to LC/MS/MS for peptide identification.

3.3.4 LC/MS/MS

Peptides corresponding to fraction numbers 3-5, 9, 11-17 from RP-HPLC (Fig. 3.4) were identified by LC/MS/MS (Table 3.4). Nine to seventeen peptides were identified in each fraction. Most of them were derived from α_{S1}-casein and β-casein. Same sequences of peptide were also identified in more than one fraction. Among the identified peptides, only two (LQSW and EMPFPK) had been previously described as ACE inhibitors. Peptide LQSW exhibited ACE inhibitory activity with IC_{50} of 500 μM [24] and peptide EMPFPK had IC_{50} of 423 μg/ml [31].

Some of the peptides identified in this study had C-terminal amino acid sequence similar to that of ACE inhibitory peptides previously described. Peptide SERYL (peptide number 1) shared structure at the C-terminal end with peptide VP**SERYL** previously described with IC_{50} of 233 μM [21]. Peptide number 16 (VLPVPQ) was present at the C-terminal position of peptide SK**VLPVPQ**, which was previously suggested a reduction of blood pressure of 32 mm Hg at the dose of 1 mg/kg body weight of spontaneously hypertensive rat (SHR) [26] and peptide K**VLPVPQ,** whose IC_{50} was 5 μM [26]. Peptide number 25 (SDKI**AKY**) had peptide AKY (IC_{50} of 20 μM) as its C-terminal end. Similarly, peptide number 74 (V**IPY**) and 132 (VQQKP**VAL**) contained peptides that had been previously described as its own C-terminal end [25], [28]. Studies of structure and ACE inhibition relationships using computer-aided molecular modeling indicate the prominent role of amino acid sequence at the C-terminal end especially, the last three residues on ACE inhibitory activity [32]. Peptides with sequence homology at the C-terminal end may exhibit similar ACE inhibitory effect. However, the synthesis of the identified peptides included in the present report may be needed for further study to verify the prediction.

Some of the peptides identified in this study shared some of amino acid sequence with ACE inhibitory peptides previously reported (Table 3.4). For example,

peptide V**IPY**V (peptide number 12) had sequence homology with peptide IPY, which was reported to be a strong ACE inhibitor, however, IC_{50} of the peptide IPY was not determined [25]. Peptide Y**LGY**LEQ (peptide number 40) shared structure with peptide LGY previously described with IC_{50} of 104 μM [28].

ACE prefers substrate containing three hydrophobic amino acid residues at the C-terminal end [32]. More than 60% of the peptides identified in this study contained aromatic or aliphatic or cyclic amino acid at the C-terminal tripeptide residues for examples, peptides NNQFLPYPY (peptide numbers 8), GPFPI (peptide number 9) and VIPYV (peptide number 12). These peptides may exhibit strong ACE inhibitory activity and probably contributed to the strong ACE inhibitory activity of the fraction.

Meisel [32] suggested that di-, tri- and short chain ACE inhibitory peptides had tyrosine, phenylalanine, tryptophan or proline residue at the C-terminal end. Some of the peptides included in the Table 3.4 (for examples, peptides LQSW, FLLY, VPVEPF and PEVIEGP) contained one of the residue mentioned above at the C-terminal end.

Arginine and lysine at the C-terminal position also contribute to the ACE inhibitory activity due to their positive charge on the guanidine and the ε-amino group, respectively [3], [18], [33]. Four peptides with lysine at the C-terminal end (PFPK, EMPFPK, PVPEK, VLPVPEK) were identified in this study.

Some bioactive peptides have more than one physiological effect. For example, β-casomorphin, an opiate peptide derived from β-casein was found to exhibit ACE inhibitory activity [19]. Some of the peptide sequences found in this study may suggest other physiological effects of the peptide. For example, peptide VLPVPQ (number 16) may exert antioxidant property as did peptide **VLPVPQ**K (β-CN f170-176) [34]. Peptide TTMPL (number 128) as a part of peptide **TTMPL**W (α_{S1}-CN f194-199), which stimulated microphages [2], may have immunomodulatory activity.

In conclusion, Bio-Gel P2 can be employed for the isolation of ACE inhibitory peptides from the permeate of CPH whereas phenyl-agarose is not suitable to separate such peptides. The gel filtration method is simple and environmentally friendly. It can easily be scaled up for mass production. One of the fractions from Bio-

Gel P2 with the highest ACE inhibitory activity was further studied by using RP-HPLC and LC/MS/MS. RP-HPLC gave 17 peaks of peptide with varying ACE inhibitory activity. LC/MS/MS analysis of major ACE inhibitory peptides demonstrated that more than 60% of the peptides identified contained hydrophobic amino acid residues at the C-terminal end, half of which had hydrophobic residues at the C-terminal tripeptide.

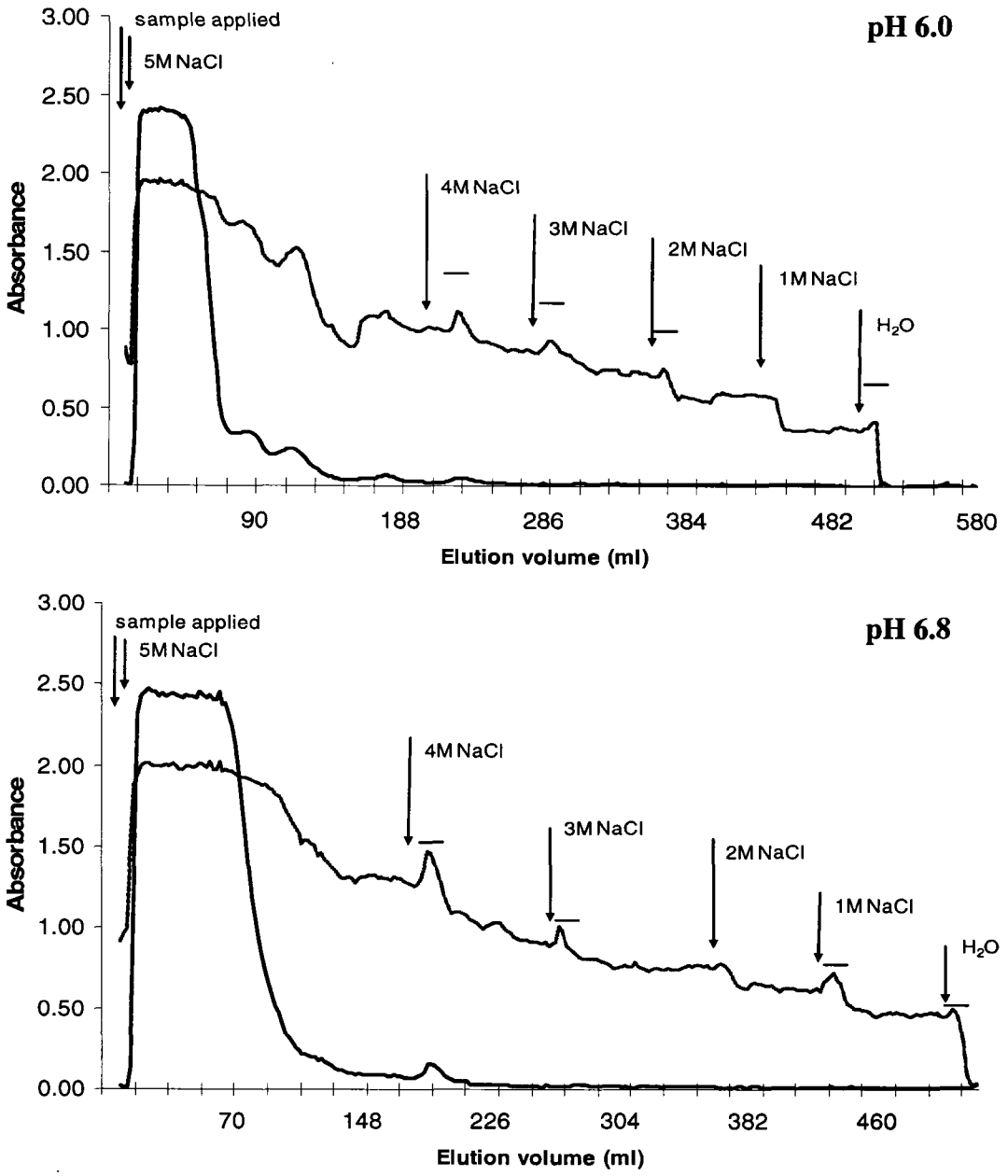

Figure 3.1: Hydrophobic interaction chromatography of CPH permeate at different pH values. A sample prepared from 15 mg of CPH permeate was applied to a 1.5 × 6.5 cm column of phenyl-agarose equilibrated with 0.01 M sodium phosphate/5 M NaCl (buffer A) adjusted to an appropriate pH. The column was washed with buffer A and eluted stepwise with decreasing concentrations of NaCl from 5 to 1 M, and then with water. Fractions (2 ml) collected at a flow rate of 18 ml/h were monitored for peptide contents by measuring absorbance at 210 and 230. Horizontal bars denote fractions pooled for further study. See methods for other details

—— A 210 —— A 230

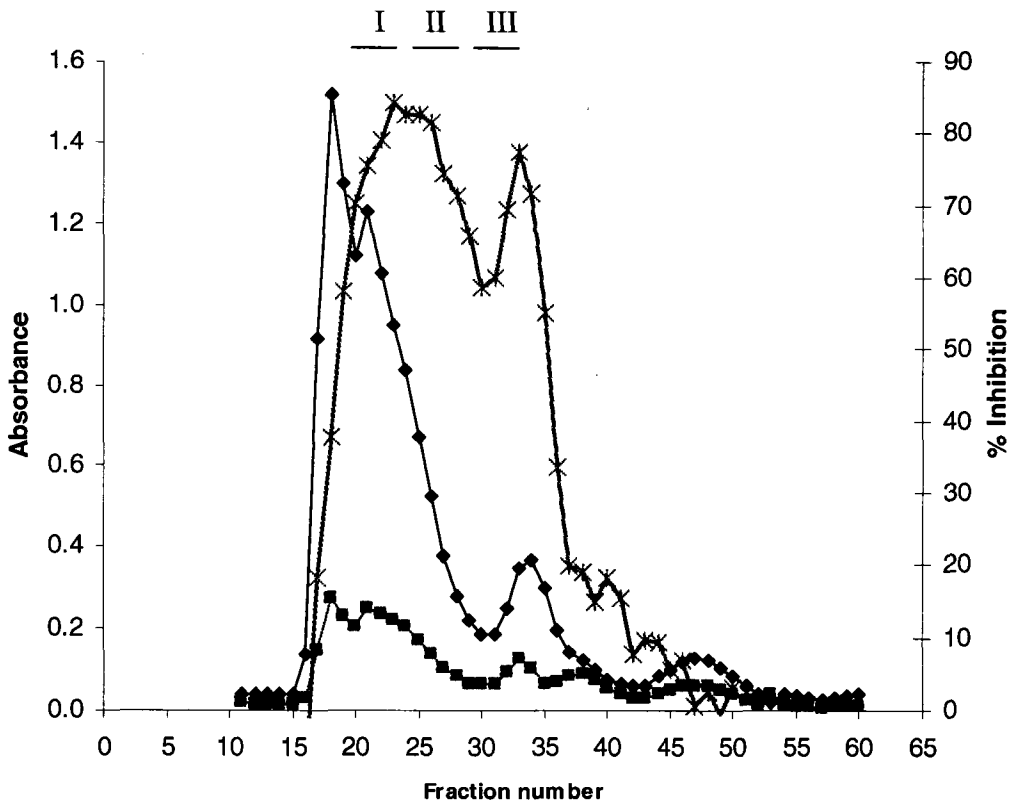

Figure 3.2: Gel filtration chromatography of CPH permeate on a 55.5 × 1 cm column of Bio-Gel P2. Factions (1ml) collected at a flow rate of 11 ml/h were monitored for absorbance at 210 and 230 nm and for ACE inhibitory activity. Horizontal bars denote fractions pooled for further study. See methods for other details.

—◆— A210 —■— A230 —✳— ACE inhibition

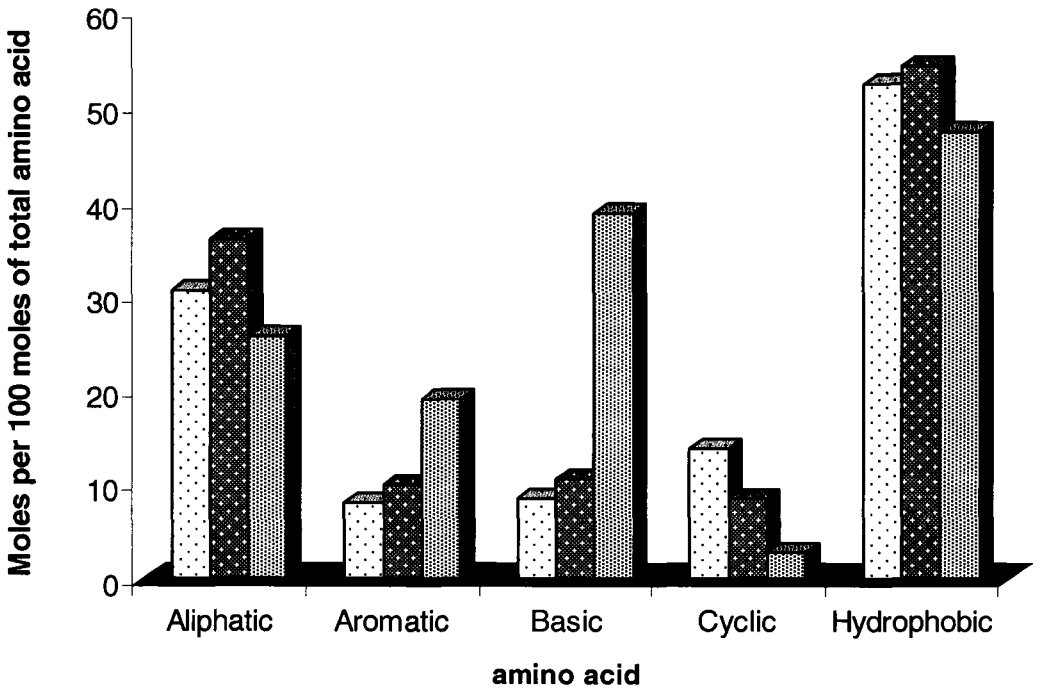

Figure 3.3: Amino acid contents of fractions obtained from Bio-Gel P2 gel filtration column. Amino acids were categorized according to their side chains

Aliphatic amino acid: Ala, Val, Met, Leu, and Ile

Aromatic amino acid: Phe, Trp and Tyr

Basic amino acid: Arg, Lys and His

Cyclic amino acid: Pro

Hydrophobic amino acid: Ala, Val, Met, Leu, Ile, Phe, Trp, Tyr and Pro

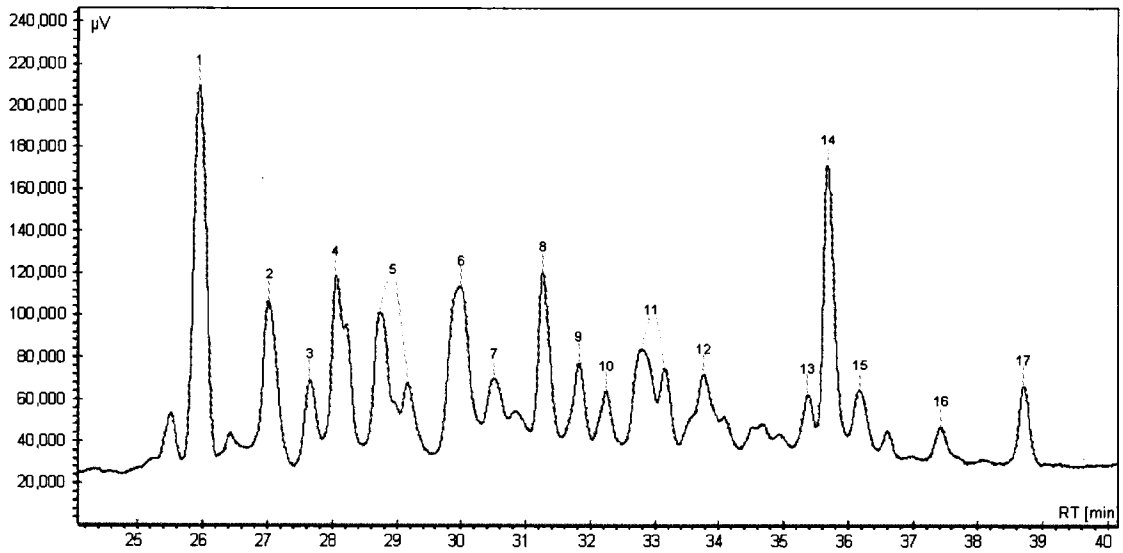

Figure 3.4: Fractionation by preparative RP-HLPC on a Sephasil Peptide C18 5 μm reverse phase column (4.6 × 100 mm, Amersham Biosciences AB, Uppsala, Sweden) with C18 cartridge as a guard column. An aliquot (80 μl) of 0.0031 g/ml 0.1% TFA in water (solvent A) of the CPH permeate fraction from Biogel P2 column was injected to the HPLC system (the Varian 9010 HPLC system equipped with Hewlett Packard 1050 autosampler and a Bio-Rad UV Monitor 1305. The peptides were eluted at a flow rate of 1.0 ml/min starting with solvent A for 10 min and a linear gradient from 0 to 70% of 0.1% TFA in acetonitrile (solvent B) for 60 min.

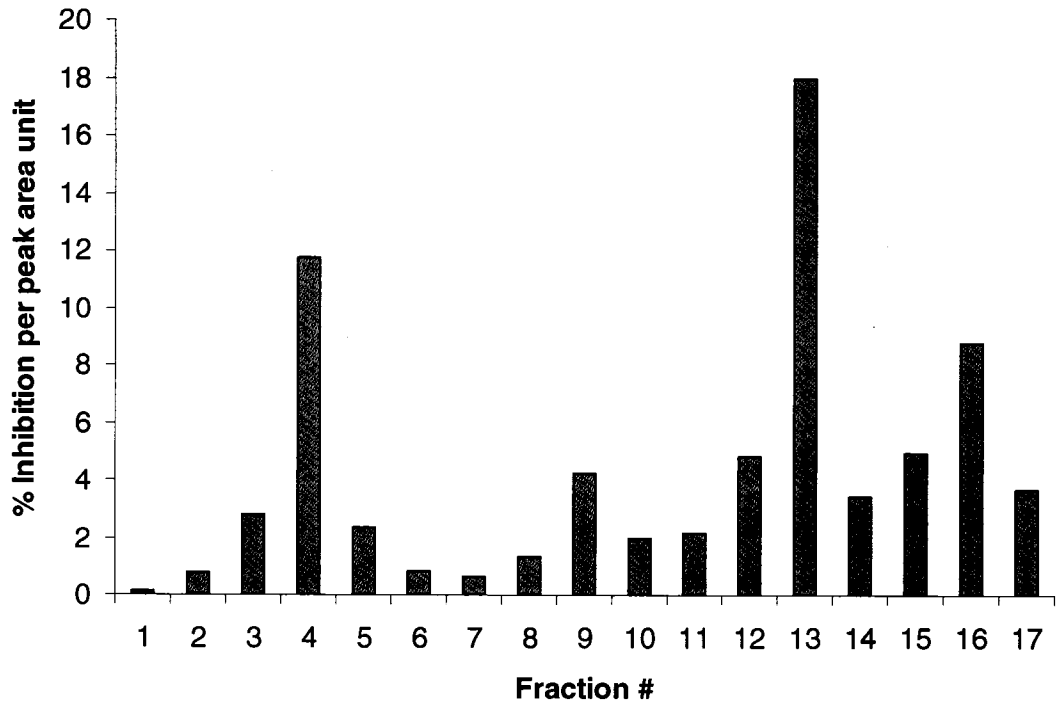

Figure 3.5: ACE inhibitory activity of fractions obtained by RP-HPLC. The percentage of ACE inhibition obtained from the ACE inhibitory activity assay using a dry collected fraction from one run of RP-HPLC dissolved in 80 µl of 0.1 M sodium borate buffer, pH 8.3 containing 0.3 M NaCl as a sample.

Table 3.1: ACE inhibitory activity of fractions of the permeate of CPH obtained from phenyl agarose column

Eluent		% ACE inhibition	IC_{50} (μg peptide/ml)
Phosphate buffer pH 6.0 with	4 M NaCl	0	nd
	3 M NaCl	4	nd
	2 M NaCl	3	nd
H_2O		0	nd
Phosphate buffer pH 6.8 with	4 M NaCl	14	> 1000
	3 M NaCl	11	> 1000
	1 M NaCl	4	> 1000
H_2O		7	> 1000

nd, not determined

Table 3.2: ACE inhibitory activity, peptide content and yield of fractions obtained from Bio-Gel P2 gel filtration column

Fraction	IC_{50} (μg peptide/ml)	Yield[1]
I	598.4 ± 116.5	24.7 ± 6.4
II	319.3 ± 4.8	20.3 ± 4.8
III	435.0 ± 132.5	23.0 ± 9.5

[1] Percentage of dry weight of permeate used for the purification
All data are expressed as mean ± SD

Table 3.3: Amino acid composition of fractions obtained from Bio-Gel P2 gel filtration column

Amino acid (Mole %)	Fraction		
	I	II	III
	Moles/100 moles of total amino acids		
Asparagine and aspartic acid	6.0	3.9	1.2
Threonine	4.8	4.4	1.4
Serine	6.9	10.1	3.3
Glutamine and glutamic acid	18.3	13.3	6.0
Glycine	3.2	3.5	2.0
Alanine	6.5	5.6	2.0
Valine	7.7	6.3	1.7
Methionine	2. 8	2.2	3.2
Isoleucine	4.4	4.9	1.6
Leucine	9.2	17.0	17.1
Tyrosine	4.3	5.8	5.3
Phenylalanine	3.7	4.0	13.4
Histidine	1.8	2.8	1.6
Lysine	6.1	7.0	31.6
Tryptophan	0.0	0.1	0.1
Arginine	0.59	0. 7	5.6
Proline	13.8	8.5	2.8

Cysteine was not analyzed

Table 3.4: Peptides identified by LC/MS/MS in the RP-HPLC purified fractions

No.	Fraction[a]	Observed mass	Calculated mass[b]	Protein fragment[c]	Sequence	ACE inhibitory peptide[d]	IC_{50}[e] (μM)	Blood pressure reduction (mm Hg) at {mg/kg body weight}[f]	Ref
1	13	667.35	666.33	α_{S1}-CN f(88-92)	SERYL	RY VPSERYL	10 233		[20] [21]
2		718.31	717.30	α_{S1}-CN f(144-148)	YFYPE	YP	720		[22]
3		831.40	830.39	α_{S1}-CN f(144-149)	YFYPEL	YP	720		[22]
4		885.44	884.43	α_{S1}-CN f(91-97)	YLGYLEQ				
5		687.32	1372.65	α_{S1}-CN f(161-173)	TDAPSFSDIPNPI				
6		560.28	559.30	κ-CN f(160-164)	NTVQV				
7		633.37	632.35	κ-CN f(25-29)	YIPIQ				
8		578.26	1154.54	κ-CN f(52-60)	NNQFLPYPY	YP	720		[22]
9		530.30	529.29	β-CN f(203-207)	GPFPI	FP	315	27 {8}	[23]
10		533.27	532.26	β-CN f(140-143)	LQSW	LQSW	500	2 {1}	[24]
11		664.30	663.31	β-CN f(140-144)	LQSWM	LQSW	500	2 {1}	[24]
12		590.36	589.36	α_{S2}-CN f(200-204)	VIPYV	TKVIP IPY	400 n.d.	9 {1}	[24] [25]
13		704.41	703.39	α_{S2}-CN f(175-180)	ALPQYL				
14		795.41	794.40	α_{S2}-CN f(92-97)	FPQYLQ	FP	315	27 {8}	[23]
15	4	533.26	532.26	β-CN f(140-143)	LQSW	LQSW	500	2 {1}	[24]

No.	Fraction[a]	Observed mass	Calculated mass[b]	Protein fragment[c]	Sequence	ACE inhibitory peptide[d]	IC_{50}[e] (μM)	Blood pressure reduction (mm Hg) at {mg/kg body weight}[f]	Ref
16		652.39	651.40	β-CN f(170-175)	VLPVPQ	SKVLPVPQ KVLPVPQ	39 5	32 {1}	[26] [24]
17		674.35	673.34	β-CN f(177-182)	AVPYPQ	YP AVPYP AVP	720 80 340		[22] [27] [27]
18		477.24	476.23	α_{S1}-CN f(195-198)	TMPL				
19		555.25	554.24	α_{S1}-CN f(145-148)	FYPE	YP	720	27 {1}	[22]
20		777.40	776.41	α_{S1}-CN f(166-172)	YVPLGTQ				
21		454.23	906.46	α_{S1}-CN f(8-15)	HQGLPQEV				
22		515.20	514.17	κ-CN f(79-82)	LSNT				
23		633.35	632.35	κ-CN f(25-29)	YIPIQ				
24		813.38	812.37	κ-CN f(35-41)	YPSYGLN	YP	720	27 {1}	[22]
25		904.41	903.41	κ-CN f(19-25)	SDKIAKY	AKY	20		[28]
26		473.77	945.53	κ-CN f(43-50)	YQQKPVAL	VAL	2		[28]
27		491.28	490.28	α_{S2}-CN f(200-203)	VIPY	TKVIP IPY	400 n.d.	9 {1}	[24] [25]
28		591.30	590.31	α_{S2}-CN f(175-179)	ALPQY				
29	16	488.29	487.28	β-CN f(110-113)	PFPK	FP	315	27 {8}	[23]
30		530.30	529.29	β-CN f(203-207)	GPFPI	FP	315	27 {8}	[23]
31		533.27	532.26	β-CN f(140-143)	LQSW	LQSW	500	2 {1}	[24]

No.	Fraction[a]	Observed mass	Calculated mass[b]	Protein fragment[c]	Sequence	ACE inhibitory peptide[d]	IC_{50}[e] (μM)	Blood pressure reduction (mm Hg) at {mg/kg body weight}[f]	Ref
32		555.32	554.31	β-CN f(190-193)	FLLY				
33		664.31	663.31	β-CN f(140-144)	LQSWM	LQSW	500	2 {1}	[24]
34		751.37	750.36	β-CN f(114-119)	YPVEPF	YP	720	27 {1}	[22]
35		633.36	632.35	κ-CN f(25-29)	YIPIQ				
36		585.32	1168.62	κ-CN f(69-78)	SPAQILQWQV				
37		740.39	739.38	κ-CN f(150-156)	PEVIEGP				
38		718.31	717.30	α_{S1}-CN f(144-148)	YFYPE	YP	720	27 {1}	[22]
39		831.40	830.39	α_{S1}-CN f(144-149)	YFYPEL	YP	720	27{1}	[22]
40		885.44	884.43	α_{S1}-CN f(91-97)	YLGYLEQ	LGY	104		[28]
41		962.48	961.49	α_{S1}-CN f(25-33)	VAPFPEVFG	FP PFPE FPE	315 >1000 n.d.	27 {8}	[23] [30] [25]
42	15	488.29	487.28	β-CN f(110-113)	PFPK	FP	315	27 {8}	[23]
43		533.27	532.26	β-CN f(140-143)	LQSW	LQSW	500	2 {1}	[24]
44		555.31	554.31	β-CN f(190-193)	FLLY				
45		664.32	663.31	β-CN f(140-144)	LQSWM	LQSW	500	2 {1}	[24]
46		689.44	688.43	β-CN f(134-139)	HLPLPL	HLPLP	41		[29]
47		751.35	750.36	β-CN f(114-119)	YPVEPF	YP	720	27 {1}	[22]
48		560.29	559.30	κ-CN f(160-164)	NTVQV				
49		633.36	632.35	κ-CN f(25-29)	YIPIQ				

No.	Fraction[a]	Observed mass	Calculated mass[b]	Protein fragment[c]	Sequence	ACE inhibitory peptide[d]	IC_{50}[e] (μM)	Blood pressure reduction (mm Hg) at {mg/kg body weight}[f]	Ref
50		813.39	812.37	κ-CN f(36-42)	PSYGLNY				
51		634.81	1267.62	κ-CN f(51-60)	INNQFLPYPY	YP	720	27 {1}	[22]
52		628.32	627.33	α_{S1}-CN f(91-95)	YLGYL				
53		668.32	667.32	α_{S1}-CN f(145-149)	FYPEL	YP	720	27 {1}	[22]
54		718.32	717.30	α_{S1}-CN f(144-148)	YFYPE	YP	720	27 {1}	[22]
55		831.38	830.39	α_{S1}-CN f(144-149)	YFYPEL	YP	720	27{1}	[22]
56		885.44	884.43	α_{S1}-CN f(91-97)	YLGYLEQ				
57		902.41	901.42	α_{S1}-CN f(143-149)	AYFYPEL	AYFYPE YP	106 720	 27 {1}	[26] [22]
58		795.41	794.40	α_{S2}-CN f(92-97)	FPQYLQ	FP	315	27 {8}	[23]
59	12	530.30	529.29	β-CN f(203-207)	GPFPI	FP	315	27{8}	[23]
60		533.26	532.26	β-CN f(140-143)	LQSW	LQSW	500	2 {1}	[24]
61		664.32	663.31	β-CN f(140-144)	LQSWM	LQSW	500	2 {1}	[24]
62		751.37	750.36	β-CN f(114-119)	YPVEPF	YP	720	27 {1}	[22]
63		633.37	632.35	κ-CN f(25-29)	YIPIQ				
64		813.37	812.37	κ-CN f(35-41)	YPSYGLN	YP	720	27{1}	[22]
65		496.75	991.48	κ-CN f(52-59)	NNQFLPYP	YP	720	27 {1}	[22]
66		628.34	627.33	α_{S1}-CN f(91-95)	YLGYL				
67		668.33	667.32	α_{S1}-CN f(145-149)	FYPEL	YP	720	27 {1}	[22]

No.	Fraction[a]	Observed mass	Calculated mass[b]	Protein fragment[c]	Sequence	ACE inhibitory peptide[d]	IC_{50}[e] (μM)	Blood pressure reduction (mm Hg) at {mg/kg body weight}[f]	Ref
68		718.30	717.30	α_{S1}-CN f(144-148)	YFYPE	YP	720	27 {1}	[22]
69		885.44	884.43	α_{S1}-CN f(91-97)	YLGYLEQ				
70		590.36	589.35	α_{S2}-CN f(200-204)	VIPYV	TKVIP IPY	400 n.d.	9 {1}	[24] [25]
71		704.39	703.39	α_{S2}-CN f(175-180)	ALPQYL				
72		795.41	794.40	α_{S2}-CN f(92-97)	FPQYLQ	FP	315	27 {8}	[23]
73	9	378.19	377.20	α_{S2}-CN f(202-204)	PYV				
74		491.29	490.28	α_{S2}-CN f(200-203)	VIPY	TKVIP IPY	400 n.d.	9 {1}	[24] [25]
75		590.32	589.35	α_{S2}-CN f(200-204)	VIPYV	TKVIP IPY	400 n.d.	9 {1}	[24] [25]
76		795.40	794.40	α_{S2}-CN f(92-97)	FPQYLQ	FP	315	27 {8}	[23]
77		819.50	818.49	α_{S2}-CN f(198-204)	TKVIPYV	TKVIP IPY	400 n.d.	9 {1}	[24] [25]
78		607.81	1213.61	α_{S2}-CN f(89-97)	YQKFPQYLQ	FP	315	27 {8}	[23]
79		633.36	632.35	κ-CN f(25-29)	YIPIQ				
80		746.36	745.34	κ-CN f(6-11)	EQPIRC				
81		796.42	795.42	κ-CN f(25-30)	YIPIQY				
82		813.38	812.37	κ-CN f(35-41)	YPSYGLN	YP	720	27 {1}	[22]
83		718.31	717.30	α_{S1}-CN f(144-148)	YFYPE	YP	720	27 {1}	[22]

No.	Fraction[a]	Observed mass	Calculated mass[b]	Protein fragment[c]	Sequence	ACE inhibitory peptide[d]	IC_{50}[e] (μM)	Blood pressure reduction (mm Hg) at {mg/kg body weight}[f]	Ref
84		757.37	756.37	α_{S1}-CN f(91-96)	YLGYLE				
85		510.78	1019.54	α_{S1}-CN f(8-16)	HQGLPQEVL				
86		533.28	532.26	β-CN f(140-143)	LQSW	LQSW	500	2 {1}	[24]
87		664.32	663.31	β-CN f(140-144)	LQSWM	LQSW	500	2 {1}	[24]
88	17	488.29	487.28	β-CN f(110-113)	PFPK	FP	315	27 {8}	[23]
89		533.26	532.26	β-CN f(140-143)	LQSW	LQSW	500	2 {1}	[24]
90		664.32	663.31	β-CN f(140-144)	LQSWM	LQSW	500	2 {1}	[24]
91		742.44	741.44	β-CN f(203-209)	GPFPIIV	FP	315	27 {8}	[23]
92		668.32	667.32	α_{S1}-CN f(145-149)	FYPEL	YP	720	27 {1}	[22]
93		718.30	717.30	α_{S1}-CN f(144-148)	YFYPE	YP	720	27 {1}	[22]
94		813.40	830.39	α_{S1}-CN f(144-149)	YFYPEL	YP	720	27 {1}	[22]
95		885.44	884.43	α_{S1}-CN f(91-97)	YLGYLEQ				
96		633.36	632.35	κ-CN f(25-29)	YIPIQ				
97	14	488.29	487.28	β-CN f(110-113)	PFPK	FP	315	27 {8}	[23]
98		533.26	532.26	β-CN f(140-143)	LQSW	LQSW	500	2 {1}	[24]
99		555.32	554.31	β-CN f(190-193)	FLLY				
100		664.32	663.31	β-CN f(140-144)	LQSWM	LQSW	500	2 {1}	[24]
101		689.42	688.43	β-CN f(134-139)	HLPLPL	HLPLP	41		[29]
102		668.33	667.32	α_{S1}-CN f(145-149)	FYPEL	YP	720	27 {1}	[22]
103		718.30	717.30	α_{S1}-CN f(144-148)	YFYPE	YP	720	27 {1}	[22]

No.	Fraction[a]	Observed mass	Calculated mass[b]	Protein fragment[c]	Sequence	ACE inhibitory peptide[d]	IC_{50}[e] (μM)	Blood pressure reduction (mm Hg) at {mg/kg body weight}[f]	Ref
104		831.38	830.37	α_{S1}-CN f(144-149)	YFYPEL	YP	720	27 {1}	[22]
105		885.43	884.43	α_{S1}-CN f(91-97)	YLGYLEQ				
106		560.28	559.30	κ-CN f(160-164)	NTVQV				
107		633.36	632.35	κ-CN f(25-29)	YIPIQ				
108		813.37	812.37	κ-CN f(36-42)	PSYGLNY				
109		634.82	1267.62	κ-CN f(51-60)	INNQFLPYPY	YP	720	27 {1}	[22]
110		506.29	505.29	α_{S2}-CN f(161-164)	LNFL				
111		795.40	794.40	α_{S2}-CN f(92-97)	FPQYLQ	FP	315	27 {8}	[23]
112	3	534.31	533.31	β-CN f(124-128)	SLTLT				
113		569.33	568.32	β-CN f(172-176)	PVPEK	SKVLPVPE	39		[26]
114		674.32	673.31	β-CN f(184-189)	DMPIQA				
115		664.32	663.31	β-CN f(140-144)	LQSWM	LQSW	500	2 {1}	[24]
116		391.24	780.47	β-CN f(170-176)	VLPVPEK				
117		555.25	554.24	α_{S1}-CN f(145-148)	FYPE	YP	720	27 {1}	[22]
118		649.36	648.35	α_{S1}-CN f(166-171)	YVPLGT				
119		777.42	776.41	α_{S1}-CN f(166-172)	YVPLGTQ				
120		633.36	632.35	κ-CN f(25-29)	YIPIQ				

No.	Fraction[a]	Observed mass	Calculated mass[b]	Protein fragment[c]	Sequence	ACE inhibitory peptide[d]	IC_{50}[e] (μM)	Blood pressure reduction (mm Hg) at {mg/kg body weight}[f]	Ref
121		591.31	590.31	α_{S2}-CN f(175-179)	ALPQY				
122	5	488.28	487.28	β-CN f(110-113)	PFPK	FP	315	27 {8}	[23]
123		533.26	532.26	β-CN f(140-143)	LQSW	LQSW	500	2 {1}	[24]
124		674.36	673.34	β-CN f(177-182)	AVPYPQ	YP AVPYP AVP	720 80 340	27 {1}	[22] [27] [27]
125		748.36	747.36	β-CN f(108-113)	EMPFPK	FP EMPFPK	315 423 μg/ml	27 {8}	[23] [31]
126		521.25	520.25	α_{S1}-CN f(146-149)	YPEL	YP	720	27 {1}	[22]
127		555.24	554.24	α_{S1}-CN f(145-148)	FYPE	YP	720	27 {1}	[22]
128		562.25	561.28	α_{S1}-CN f(194-198)	TTMPL	TTMPLW	51	14 {100}	[20]
129		649.34	648.35	α_{S1}-CN f(166-171)	YVPLGT				
130		718.31	717.30	α_{S1}-CN f(144-148)	YFYPE	YP	720	27 {1}	[22]
131		777.41	776.40	α_{S1}-CN f(166-172)	YVPLGTQ				
132		633.36	632.35	κ-CN f(25-29)	YIPIQ				
133		813.37	812.37	κ-CN f(36-42)	PSYGLNY				
132		473.76	945.53	κ-CN f(43-50)	YQQKPVAL	VAL	2		[28]

No.	Fraction[a]	Observed mass	Calculated mass[b]	Protein fragment[c]	Sequence	ACE inhibitory peptide[d]	IC_{50}[e] (μM)	Blood pressure reduction (mm Hg) at {mg/kg body weight}[f]	Ref
135	11	497.23	496.24	κ-CN f(103-106)	LSFM				
136		633.36	632.35	κ-CN f(25-29)	YIPIQ				
137		813.38	812.37	κ-CN f(36-42)	PSYGLNY				
138		533.27	532.26	β-CN f(140-143)	LQSW	LQSW	500	2 {1}	[24]
139		751.32	750.36	β-CN f(114-119)	YPVEPF	YP	720	27 {1}	[22]
140		491.29	490.28	α_{S1}-CN f(166-169)	YVPL				
141		654.34	653.34	α_{S1}-CN f(165-169)	YYVPL				
142		718.32	717.30	α_{S1}-CN f(144-148)	YFYPE	YP	720	27 {1}	[22]
143		885.44	884.43	α_{S1}-CN f(91-97)	YLGYLEQ				
144		590.34	589.35	α_{S2}-CN f(200-204)	VIPYV	TKVIP IPY	400 n.d.	9 {1}	[24] [25]
145		704.37	703.39	α_{S2}-CN f(175-180)	ALPQYL				
146		795.41	794.40	α_{S2}-CN f(92-97)	FPQYLQ	FP	315	27 {8}	[23]

n.d., not determined

[a] Fraction present in a decreasing order of the ACE inhibitory activity as shown in Figure 3.4 (b).

[b] Calculated monoisotopic mass.

[c] Peptide sequence was obtained from a Mascot search engine (Matrix Science Inc., Boston, MA).

[d] Previously reported ACE inhibitory peptides.

[e] Peptide concentration needed to inhibit 50% ACE activity.

[f] maximum decrease in systolic blood pressure after oral administration in spontaneously hypertensive rats.

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CHAPTER 4 *

PROBIOTIC *LACTOBACILLUS CASEI* ADA03 AS A STARTER CULTURE RELEASED ANGIOTENSIN CONVERTING ENZYME INHIBITORS FROM BOVINE MILK

4.1 INTRODUCTION

Since the distinguished work of Metchnikoff [1] who suggested the benefit of lactic acid bacteria (LAB) on increasing human longevity by reducing the toxic substance formation in the colon, numerous research has been focused on the health benefit harvested from microorganisms. In the light of this discovery, the word probiotics has been used and variously defined. Definition of probiotics given by Fuller [2] was "a live microbial feed supplement, which beneficially affects the host animal by improving its intestinal microbial balance". Salminen [3] extended the definition to "a live microbial food ingredient that is beneficial to health". In order to deliver the health benefit, probiotics have to be able to endure gastric and bile acids in gastrointestinal tract, to colonize on intestinal epithelial cells, to survive during food processing and storage, and to be safe to human [4], [5], [6]. All these abilities are suggested for the criteria of probiotics. Studies from our research group showed that *Lactobacillus casei* ADA03, *Lactobacillus casei* ADA05, *Lactobacillus acidophilus* MR100, and *Lactobacillus bulgaricus* MR110, which were considered Generally Recognized as Safe (GRAS), could survive and function in the presence of gastric and bile acids [7], adhere to intestinal cell surface [7] as well as exhibited immunostimulatory [8], cytotoxic [9], and apoptotic activities [9], suggesting that they are probiotics. It is interesting to investigate whether these probiotics have other physiological properties such as anti-hypertensive and anti-thrombotic activities.

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* a version of this chapter has been submitted for publication in Milchwissenschaft/Milk Science International (2006)

* Conference Proceedings. Certain sections of this research was presented at European Conference on Probiotics and their Applications, Krakow, Poland (2005), Poster Presentation: Probiotics released angiotensin converting enzyme inhibitory peptides in fermented milk.

Hypertension is one of the main health concerns in the western countries. This abnormally high blood pressure along with its complications such as kidney failure, heart failure, stroke, etc. is the leading cause of mortality worldwide [10]. Many treatments have been developed to mitigate the condition. Angiotensin converting enzyme inhibitor is one of the recommended remedies for hypertension [11]. Angiotensin converting enzyme (ACE; EC 3.4.15.1) converts angiotensin I into angiotensin II, a strong vasoconstrictor, in conjunction with inactivating bradykinin, a vasodilator, and consequently, elevates blood pressure [12]. LAB including *lactobacillus* sp. have been reported to produce ACE inhibitors and lower high blood pressure *in vivo* [13], [14], [15], [16], [17], [18], [19]. However, abilities to produce ACE inhibitory peptides and exhibit antihypertensive activity as well as those, which belong to probiotics appeared to be strain-dependent. There is limited information available concerning the production of ACE inhibitory peptides by bacteria strains, which have been proved to be probiotics. This study was, therefore, undertaken to determine whether ACE inhibitory peptides can be released in bovine milk fermented with the four strains of probiotics including *Lactobacillus casei* ADA03, *Lactobacillus casei* ADA05, *Lactobacillus acidophilus* MR100, and *Lactobacillus bulgaricus* MR110 and to explore the possibility to improve ACE inhibitory activity if there is any, by ultrafiltration.

4.2 MATERIALS AND METHODS

4.2.1 Materials

Skim milk powder was purchased from Canada Safeway (Calgary, AB, Canada). Glycerol, hippuryl-L-histidyl-L-leucine (HHL), angiotensin converting enzyme (ACE) from rabbit lung, o-phthaldialdehyde (OPA), and dithiothreitol were obtained from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Sodium dodecyl sulfate was acquired from Bio-Rad Laboratories (Hercules, CA, USA.). Bacto® Lactobacilli MRS broth and agar were from Difco (Detroit, MI, USA). Other chemicals were obtained from Fisher Scientific Ltd. (Edmonton, AB, Canada). Water was purified by reverse osmosis followed by deionization with a MILLI-Q water

purification system (Millipore Corporation, Mississauga , ON, Canada). Four strains of probiotics, *Lactobacillus acidophilus* MR100 (MR100), *Lactobacillus bulgaricus* MR110 (MR110), *Lactobacillus casei* ADA03 (ADA03), *Lactobacillus casei* ADA05 (ADA05) were obtained from stock cultures available in Alberta Dairy Association Research Unit, Department of Agricultural, Food and Nutritional Science, University of Alberta.

4.2.2 Bacterial propagation and growth conditions

Each probiotic was stored at – 80 °C in MRS broth containing 20% (v/v) sterilized glycerol until required for use in experiments. Prior to experimental use, probiotic cultures were subcultured twice in MRS broth at 37 °C for 24 h.

4.2.3 Preparation of fermented milk

Reconstituted skim milk (12% w/v) was autoclaved at 121 °C for 15 min and cooled down to 37 °C prior to the addition of a 1% (v/v) inoculum of probiotic from MRS broth. The mixture of milk and single strain of probiotic was then incubated at 37 °C for 18, 24 or 48 h. Three replicates were prepared for each incubation period. The mixture was then centrifuged at 14,300 g and 20 °C for 30 min. Half of supernatant collected was passed through a hollow fiber ultrafiltration membrane with 2,000 Da molecular weight cut-off (Supelco, Inc. Bellefonte, PA, USA.) under the pressure of 20 psi. Supernatant and permeate obtained were then lyophilized and stored at – 20 °C for further analysis.

4.2.4 Determination of ACE inhibitory activity

The method of Cushman and Cheung [20], modified by Nakamura *et al.* [21] was used. The 300 µl assay mixture prepared was comprised of 200 µl of 5 mM HHL dissolved in 0.1 M sodium borate buffer (pH 8.3) containing 0.3 M NaCl, 80 µl of peptide solution and 20 µl of 2 mU ACE. Increasing concentrations (0 – 3000 µg/ml) of peptide were prepared in each assay. After incubation at 37 °C for 30 min, the ACE activity in the assay mixture was stopped by addition of 250 µl of 1 N HCl. The hippuric acid released by the action of ACE was extracted with 1.75 ml of ethyl

acetate. Following the removal of ethyl acetate by evaporation at 80 °C under vacuum, the dried hippuric acid obtained was dissolved in water and its absorbance was determined at 228 nm. The percentage of inhibition was calculated using the formula: $100 \times (B-A)/(B-C)$, where *A* represents the absorbance in the presence of HHL, ACE and an inhibitor, and *B* represents the absorbance in the presence of HHL and ACE. *C* represents the absorbance in the presence of HHL. The inhibitory activity was expressed as the concentration of peptide required for 50% inhibition of the enzyme activity (IC_{50}).

4.2.5 Determination of viable plate count

Viable plate counts of probiotics were performed using the pour plate technique [22]. A number of 10-fold serial dilutions of the fermented milk were prepared. Bacto® Lactobacilli MRS agar was used for enumeration of probiotics. Plates were incubated at 37 °C for 48 h in an aerobic atmosphere supplemented with 10% carbon dioxide. Large, white colonies embedded in or on the MRS agar were counted.

4.2.6 Determination of titrable acidity

Organic acids were quantified by a titrimetric method [23]. To 20 ml of fermented milk, 40 ml of CO_2-free water and 2 ml of phenolphthalein were added. The mixture was titrated with 0.1 N NaOH to the first persistent pink color. Acid content of the fermented milk was reported as percentage (w/v) of lactic acid. (A ml of 0.1 N NaOH is equivalent to 0.0090 g of lactic acid)

4.2.7 Determination of proteolytic activity

The proteolytic activity was determined by the OPA reaction as described by Church et al. [24] modified by Nielsen and others [25]. The OPA reagent was prepared by dissolving 0.10 M disodium tetraborate decahydrate, 3.47 mM SDS, 5.96 mM OPA (dissolved in ethanol prior to mixing) and 5.70 mM dithiothreitol. Three milliliters of OPA reagent was added to 400 µl of sample solution in water. After mixing for 5 s and standing for exactly 2 min, the absorbance of the reaction mixture

was read at 340 nm. Serine was used as a standard. The proteolytic activity was expressed as the milliequivalent (mEq) of serine-NH_2 per gram protein. A difference in mEq of serine-NH_2 per gram protein between fermented and non-fermented skim milk samples was reported.

4.2.8 Determination of protein/peptide content

A LECO Nitrogen/Protein Determinator model FP-428 (LECO Corporation, St. Joseph, MI, USA) was used to determine the nitrogen content. A 20 mg sample was placed in the sample holder and the instrument was operated following the procedure described in the company manual. The protein content was calculated using a factor of 6.38 to convert from the nitrogen content.

4.2.9 Amino acid analysis

Tryptophan was analyzed by a reverse-phase HPLC method described by Sedgwick *et al.* [26]. A sample was weighed in a 1.3 × 10 cm glass screw cap culture tube, to which 4 ml of 4.2 N NaOH and 0.8 ml of 25% (w/v) pyrogallol were added. The sample was hydrolyzed in the presence of nitrogen at 110 °C for 20 h. After hydrolysis, 100 µl of 0.075 % (w/v) amino-n-caproic acid and 100 µl of water were added to the hydrolysate, and the mixture was centrifuged at 770 g for 15 min. An aliquot (200 µl) of the supernatant obtained was then mixed with 30 µl of glacial acetic acid and 600 µl of saturated potassium tetraborate tetrahydrate. This was mixed with a fluoraldehyde reagent in an approximate ratio of 1 to 1 prior to reverse-phase HPLC analysis. The fluoraldehyde reagent consisted of 0.25 g of OPA first dissolved in 6 ml methanol, 56 ml of 0.04 M sodium borate buffer, pH 9.5, 0.25 ml of 2-mercaptoethanol and 2 ml of Brij 35. The Varian 5000 HPLC system (Varian, Inc., Walnut Creek, CA, USA) was equipped with a Supelcosil 3 µm LC-18 reverse phase column (4.6 x 150 mm; Supelco, Inc. Bellefonte, PA, USA.) and a Fluorichrom fluorescence detector (Varian, Inc., Walnut Creek, CA, USA). Fluorescence was monitored with excitation wavelength of 340 nm and emission wavelength of 450 nm.

Analysis of amino acids other than tryptophan was performed by using a Beckman model 6300 amino acid analyzer on samples hydrolyzed in the presence of nitrogen at 110 °C for 24 h in glass-distilled 6 N HCl containing 0.1% (w/v) phenol.

4.2.10 Statistical analysis

Viable plate count, pH, titrable acidity, and proteolytic activity of probiotics were compared by using analysis of variance. The Tukey's Honest Significant Difference test was performed for the post hoc testing. All statistical analysis was performed on a data analysis software SPSS, version 13.0 (SPSS Inc., Chicago, Ill, USA) to determine significant effect ($p \leq 0.05$) of the different treatments. All statistic results are shown in details in Appendix B.

4.3 RESULTS

Viable cell count of probiotics at different incubation time is shown in Table 4.1. All probiotics grew well in bovine milk at 37 °C, reaching cell counts above 10^8 colony-forming units (cfu)/ml at 18 h. Cell counts of ADA03 and MR110 were constant for 24 h ($p > 0.05$) and showed decline phase at 48 h while those of ADA05 and MR100 remained constant ($p > 0.05$) throughout the experiment. ADA03 and ADA05 proliferated faster than MR100 and MR110 at 18 h ($p \leq 0.05$). At 24 h ADA03 grew faster than MR100 or MR110 ($p \leq 0.05$). Cell counts were not different between ADA03 and ADA05, ADA05 and MR110, and MR100 and MR110 at 24 h. At 48 h similar cell counts were observed between ADA03 and ADA05 and among ADA03, MR100 and MR110 ($p > 0.05$).

The pH of milk fermented (Table 4.1) was affected by probiotic strain as well as incubation time ($p \leq 0.05$). At 18 h, all probiotics gave different ($p \leq 0.05$) values (4.4, 4.7, 4.0 and 3.6 for ADA03, ADA05, MR100, and MR110, respectively). Each value decreased ($p \leq 0.05$) thereafter. The pH value determined in each sample was, in general, consistent with its content of acid produced (see below).

Organic acid produced during fermentation was determined by the titrimetric method and reported as percentage of lactic acid (Table 4.1). The acid produced appeared to be strain- and time- dependent. In general, the amount of acid

produced increased significantly ($p \leq 0.05$) with increasing incubation time. At 18 h, the amount was the highest in the milk fermented with MR110, higher in the milk fermented with MR100 compared to that with ADA03 and the lowest in the milk fermented with ADA05 ($p \leq 0.05$). At 24 h and 48 h, the amount of acid produced remained to be the highest ($p \leq 0.05$) in the milk fermented with MR110, but relatively constant ($p > 0.05$) among the remaining fermented milk samples with the exception of a lower ($p \leq 0.05$) content observed in the sample with ADA05 than that with MR100.

Proteolytic activity of probiotics in fermented milk expressed as the mEq of serine-NH_2 is illustrated in Fig. 4.1. The proteolytic activity indicated that peptides present varied among probiotics. MR110 had the highest ($p \leq 0.05$) proteolytic activity throughout the experiment. MR100 had higher ($p \leq 0.05$) proteolytic activity than ADA03 and ADA05 at all time and similar activity to MR110 at 48 h. Proteolytic activities of ADA03 and ADA05 were similar throughout with an exception at 18 h. Incubation time had significant effect ($p \leq 0.05$) only on ADA03 and ADA05. In ADA03, the proteolytic activity at 24 h was higher ($p \leq 0.05$) than that at 18 or 48 h. However, the activity at 18 h and 48 h were similar ($p > 0.05$). In ADA05, the proteolytic activity decreased ($p \leq 0.05$) with increasing incubation time.

ACE inhibitory activities of supernatant and permeate of milk fermented with probiotics were shown in Table 4.2. ADA03 produced strong ACE inhibitory effect whereas ADA05, MR100, and MR110 produced weak ACE inhibitory effect. It appeared that ACE inhibition was strain- and time- dependent. In general, permeate obtained showed higher ACE inhibitory activity than did corresponding supernatant. The highest ACE inhibitory activity with IC_{50} of 252 μg peptide/ml was found in the permeate of milk fermented with ADA03 (referred to hereafter as 03FM) for 24 h. The ACE inhibitory activity was 4 to 10 fold stronger in the permeate fraction of 03FM than that of milk fermented with ADA05, MR100 or MR110.

Amino acid analysis for 03FM at 24 h (Table 4.3) revealed that major amino acids in the supernatant were glutamine/glutamic acid and proline, and those in the permeate were glutamine/glutamic acid, proline, and leucine. Compared to the supernatant, the permeate contained higher amount of tyrosine, leucine, arginine,

phenylalanine, and valine, similar contents of glycine, alanine, proline, tryptophan, methionine, histidine and lysine and lower contents of asparagine/aspartic acid, threonine, serine, glutamine/glutamic acid and isoleucine. This reflected higher contents of aliphatic, aromatic, basic, and cyclic amino acids in the permeate than in the supernatant (Fig. 4.2).

The yields of dry matter of 03FM and its fractions were relatively constant among samples obtained from different incubation time (Table 4.3) and averaged 85.7, 62.1, and 14.7% for 03FM, supernatant and permeate, respectively.

4.4 DISCUSSION

It has been suggested that probiotics present in a product should be maintained at the level of 10^6 cfu/ml to provide therapeutic properties [5]. All probiotics grew well in milk, and their quantity remained above the therapeutic level throughout the experiment. However, proliferation of ADA03 and MR110 decreased after 24 h, which could be explained by the high amount of acid present in the milk [27], [28], [29]. While ADA05 and MR100 took more time to reach decline phase probably due to less acid accumulated.

It has been known that proteinase synthesis of *Lactobacillus* species is strongly affected by bacteria strain [30], [31], [32]. ACE inhibitory activities of ADA03 were higher than those of ADA05 even though in general, their proteolytic activities were similar. In addition, compared to MR100 and MR110, ADA03 showed the lowest proteolytic activity, nonetheless, exhibited the strongest ACE inhibitory activity. This indicated that the difference in site-specific cleavage of the proteinases synthesis by different strain resulted in the difference in amino acid composition of the peptides, which in turn, yielded different ACE inhibitory effects.

The present findings suggested that significant amounts of ACE inhibitory peptides can be produced by fermentation of bovine milk with ADA03. However, there is little information available concerning proteinases produced by this strain. Purification and characterization of proteinases produced by ADA03 should facilitate further studies to understand the role of enzymes involved in the formation of ACE inhibitory peptides.

Various strains of LAB including *L. helveticus* strains CPN4 [15], R211 [17], R389 [17], *L. debrueckii* ssp. *bulgaricus* SS1 [16], *L. lactis* ssp. *cremoris* FT4 [16] have been reported to produce peptides, which can lower blood pressure *in vivo*. The antihypertensive property of these peptides was believed to mediate through ACE inhibitory effect. This suggests that ACE inhibitory peptides released by probiotics in the present study could lower blood pressure. However, the efficacy of these peptides needs further investigation. IC_{50} of the permeate of 03FM at 24 h (252 μg/ml) in the present study was comparative to that of the peptides released from milk fermented with *L. delbrueckii* subsp. *bulgaricus* SS1 (β-casein f6-14 with IC_{50} of 290 μg/ml) or with *L. lactis* subsp. *cremoris* FT4 (β-casein f47-52 with IC_{50} of 194 μg/ml) [16].

With the application of ultrafiltration, IC_{50} value was improved by the factor of 4.0 to 4.4 in the permeate of 03FM (Table 4.2). This indicated that peptides with ACE inhibitory activity were concentrated by ultrafiltration as membrane permeable compounds. This is in good accordance with our previous study [33] in which we employed ultrafiltration to enrich ACE inhibitory activity in the permeate of pancreatic digest of casein.

In this study, we found high contents of hydrophobic and basic amino acid residues in permeate of 03FM at 24 h (Table 4.3 and Fig. 4.2). This is in good agreement with previous reports [34], [35], which demonstrated that peptides with high ACE inhibitory activity are rich in hydrophobic or basic amino acids.

In conclusion, our findings suggested that probiotic *Lactobacillus casei* ADA03 could be a potential starter culture for producing ACE inhibitory peptide with the incubation time of 24 h at 37 °C. It was also suggested that ultrafiltration could be exploited for enriching ACE inhibitory peptides in the permeate fraction.

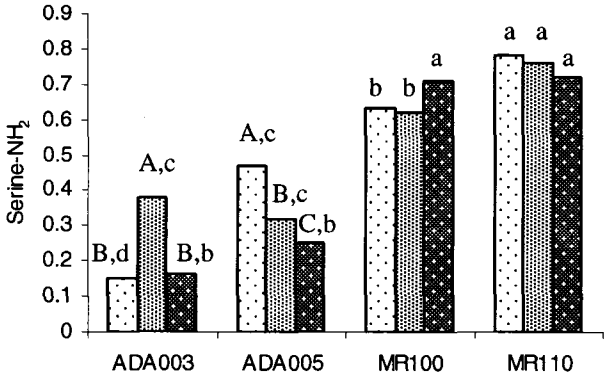

Figure 4.1: Proteolytic activity of probiotics in fermented milk at different incubation time. The activity was expressed as the mEq of serine-NH2 per gram protein.

□ 18 h ▨ 24 h ■ 48 h

A,B Capital letters within the same strain indicate significantly different effect of incubation time ($p \leq 0.05$).

a,b,c,d Lowercase letters at each incubation time indicate significantly different effect of strain within the same incubation time ($p \leq 0.05$).

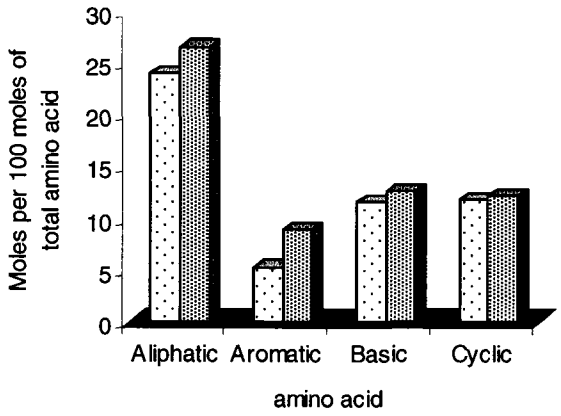

Figure 4.2: Contents of amino acids of supernatant and permeate of 003FM at 24 h. Amino acids were categorized according to their side chains.

□ Supernatant ▨ Permeate

Aliphatic amino acid: Ala, Val, Met, Leu, and Ile

Aromatic amino acid: Phe, Trp and Tyr

Basic amino acid: Arg, Lys and His

Cyclic amino acid: Pro

Table 4.1: Viable plate count, pH, and titrable acidity of milk fermented with probiotics at different incubation times

Parameter	Probiotic	Time (h)		
		18	24	48
Viable count ($\times 10^8$ cfu/ml)	ADA003	$20.0 \pm 1.7^{A,a}$	$25.0 \pm 5.0^{A,a}$	$13.0 \pm 1.5^{B,a,b}$
	ADA005	$18.0 \pm 2.1^{A,a}$	$19.0 \pm 4.0^{A,a,b}$	$18.0 \pm 5.9^{A,a}$
	MR100	$7.0 \pm 1.4^{A,b}$	$7.5 \pm 0.4^{A,c}$	$7.0 \pm 1.2^{A,b}$
	MR110	$11.0 \pm 0.0^{A,b}$	$11.0 \pm 0.8^{A,b,c}$	$6.5 \pm 0.5^{B,b}$
pH	ADA003	$4.41 \pm 0.11^{A,b}$	$3.97 \pm 0.09^{B,a,b}$	$3.66 \pm 0.07^{C,a}$
	ADA005	$4.70 \pm 0.02^{A,a}$	$4.14 \pm 0.16^{B,a}$	$3.77 \pm 0.11^{C,a}$
	MR100	$3.99 \pm 0.01^{A,c}$	$3.89 \pm 0.00^{B,b}$	$3.71 \pm 0.00^{C,a}$
	MR110	$3.63 \pm 0.00^{A,d}$	$3.57 \pm 0.00^{B,c}$	$3.43 \pm 0.00^{C,b}$
Titrable acidity (g of lactic acid/100 ml of fermented milk)	ADA003	$0.93 \pm 0.04^{C,c}$	$1.28 \pm 0.10^{B,b,c}$	$1.75 \pm 0.15^{A,b}$
	ADA005	$0.75 \pm 0.01^{C,d}$	$1.08 \pm 0.15^{B,c}$	$1.52 \pm 0.17^{A,b}$
	MR100	$1.21 \pm 0.02^{B,b}$	$1.37 \pm 0.02^{A,B,b}$	$1.52 \pm 0.12^{A,b}$
	MR110	$1.85 \pm 0.02^{B,a}$	$1.99 \pm 0.03^{A,B,a}$	$2.10 \pm 0.11^{A,a}$

Capital letters in the same row indicate significant difference ($p \leq 0.05$).

Lowercase letters within the same parameter in the same column indicate significant difference ($p \leq 0.05$).

All data are expressed as mean ± SD

Table 4.2: ACE inhibitory activity in fractions from milk fermented with probiotics

Probiotic	Fraction	Time (h)	IC_{50} [1] (μg peptide/ml)
ADA	Supernatant	18	2936
003		24	1118
		48	No activity
	Permeate	18	742
		24	252
		48	> 3000
ADA 005	Supernatant	18	No activity
		24	> 3000
		48	> 3000
	Permeate	18	> 3000
		24	> 3000
		48	2683
MR 100	Supernatant	18	> 3000
		24	> 3000
		48	> 3000
	Permeate	18	1653
		24	1592
		48	1334
MR 110	Supernatant	18	> 3000
		24	> 3000
		48	> 3000
	Permeate	18	> 3000
		24	2004
		48	1810

[1]Concentration of peptide that can inhibit 50% ACE activity.

Table 4.3: Amino acid composition of fractions of 03FM

Amino acid	Fraction	
	Supernatant	Permeate
	Moles per 100 moles of total amino acids	
Asparagine and Aspartic acid	7.0	5.5
Threonine	6.9	4.9
Serine	9.8	6.8
Glutamine and Glutamic acid	19.6	18.2
Glycine	3.9	4.3
Alanine	4.4	4.9
Valine	4.5	5.6
Methionine	2. 6	2.7
Isoleucine	5.3	3.9
Leucine	7.3	9.4
Tyrosine	2.0	4.4
Phenylalanine	3.3	4.5
Histidine	2.4	2.6
Lysine	7.1	6.5
Tryptophan	0.0	0.0
Arginine	2.1	3.6
Proline	11.8	12.2

Cysteine was not analyzed

Table 4.4: Yields of 03FM and its fractions

Time (h)	Yield[1]		
	03FM	Supernatant	Permeate
18	85.0 ± 6.0	61.1 ± 4.6	13.6 ± 4.8
24	83.9 ± 1.0	66.1 ± 2.5	14.0 ± 3.8
48	88.3 ± 2.2	59.2 ± 4.4	16.4 ± 2.4

[1] Percentage of dry weight of skim milk used for fermentation expressed as mean ± SD.

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CHAPTER 5

SEPARATION AND IDENTIFICATION OF ANGIOTENSIN CONVERTING ENZYME INHIBITORY PEPTIDES RELEASED BY PROBIOTIC *LACTOBACILLUS CASEI* ADA03

5.1 INTRODUCTION

Angiotensin converting enzyme (ACE; EC 3.4.15.1) plays a prominent role in the regulation of blood pressure by converting angiotensin I into angiotensin II, a strong vasoconstrictor, in association with inactivating bradykinin, a vasodilator [1]. Inhibition of ACE may lead to the efficient treatment of hypertention [2]. The production of ACE inhibitory peptides has been reported in soy [3], and milk [4], [5], [6] fermented with lactic acid bacteria. It has been demonstrated that *Lactobacillus helveticus* along with *Saccharomyces cerevisiae* produced highly effective ACE inhibitory peptides VPP and IPP derived from β-casein and κ-casein, respectively in Calpis™ sour milk [7]. The peptides showed remarkable antihypertensive effect in spontaneously hypertensive rats [8].

Our previous study showed that *Lactobacillus casei* ADA03 can release ACE inhibitory peptides from bovine milk [9]. However, little is known about the structure of these peptides. The present study was undertaken to isolate ACE inhibitory peptides from milk fermented with *Lactobacillus casei* ADA03 using gel filtration chromatography and to characterize such peptides using on-line HPLC-tandem mass spectrometry (LC/MS/MS).

5.2 MATERIALS AND METHODS

5.2.1 Materials

Skim milk powder was purchased from Canada Safeway (Calgary, AB, Canada). Glycerol, hippuryl-L-histidyl-L-leucine (HHL), ACE from rabbit lung,

trifluoroacetic acid (TFA), bovine serum albumin (BSA), o-phthaldialdehyde (OPA), Brij 35, standard amino acids and phenyl-agarose were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Bio-Gel P2 was acquired from Bio-Rad Laboratories Canada Ltd. (Mississauga, ON, Canada). Bacto® Lactobacilli MRS broth and agar were from Difco (Detroit, MI, USA). Other chemicals were obtained from Fisher Scientific Ltd. (Edmonton, AB, Canada). Water was purified by reverse osmosis followed by deionization with a MILLI-Q water purification system (Millipore Corporation, Mississauga, ON, Canada). Probiotic, *Lactobacillus casei* ADA03 (ADA03) was obtained from stock cultures available in Alberta Dairy Association Research Unit, Department of Agricultural, Food and Nutritional Science, University of Alberta.

5.2.2 Bacterial propagation and growth conditions

ADA03 was stored at – 80 °C in MRS broth containing 20% (v/v) sterilized glycerol until required for use in experiments. Prior to experimental use, probiotic cultures were subcultured twice in MRS broth at 37 °C for 24 h.

5.2.3 Preparation of fermented milk

Reconstituted skim milk (12% w/v) was autoclaved at 121 °C for 15 min and cooled down to 37 °C prior to the addition of a 1% (v/v) inoculum of ADA03 from MRS broth. The mixture containing ADA03 was incubated at 37 °C for 24 h. Fermented milk (referred to hereafter as 03FM) was then centrifuged at 14,300 g and 20 °C for 30 min. The supernatant collected was passed through a hollow fiber ultrafiltration membrane with 2,000 Da molecular weight cut-off (Supelco, Inc. Bellefonte, PA, USA.) under the pressure of 20 psi. The permeate obtained was then lyophilized and stored at – 20 °C for further analysis.

5.2.4 Determination of ACE inhibitory activity

The method of Cushman and Cheung [10], modified by Nakamura *et al.* [7] was used. The 300 µl assay mixture prepared was comprised of 200 µl of 5 mM HHL dissolved in 0.1 M sodium borate buffer (pH 8.3) containing 0.3 M NaCl, 80 µl

of peptide solution and 20 µl of 2 mU ACE. After incubation at 37 °C for 30 min, the ACE activity in the assay mixture was stopped by addition of 250 µl of 1 N HCl. The hippuric acid released by the action of ACE was extracted with 1.75 ml of ethyl acetate. Following the removal of ethyl acetate by evaporation at 80 °C under vacuum, the dried hippuric acid obtained was dissolved in water and its absorbance was determined at 228 nm. The percentage of inhibition was calculated using the formula: $(B-A)/(B-C) \times 100$, where *A* represents the absorbance in the presence of HHL, ACE and an inhibitor, and *B* represents the absorbance in the presence of HHL and ACE. *C* represents the absorbance in the presence of HHL. The inhibitory activity was expressed as the concentration of peptide required for 50% inhibition of the enzyme activity (IC_{50}).

5.2.5 Gel filtration chromatography

A sample of 270 mg of permeate of milk fermented with AD03 was dissolved in 1 ml of 50 mM ammonium bicarbonate (buffer A). This preparation was passed through a 0.45 µm Millipore filter unit (Millipore, Corporation, Mississauga, ON, Canada). The filtrate obtained was applied to a 55.5 cm × 1 cm column of Bio-Gel P2 equilibrated with buffer A. Fractions of 1 ml were collected at a flow rate of 11 ml/h and monitored for peptide content by measuring absorbance at 210 and 230 nm. A portion (80 µl) of peptide-containing fraction was tested for ACE inhibitory activity. Eluates with high ACE inhibitory activity were pooled, lyophilized and determined for IC_{50}.

5.2.6 Reverse phase HPLC (RP-HPLC)

Preparative RP-HPLC was performed on a Varian 9010 HPLC system (Varian, Inc., Walnut Creek, CA, USA) equipped with a Hewlett Packard 1050 autosampler (Hewlett-Packard, Palo Alto, CA, USA) and a UV Monitor 1305 (Bio-Rad Laboratories Canada Ltd., Mississauga, ON, Canada). A sample of 0.0052 g of the fraction with the highest IC_{50} from gel filtration was dissolved in 1 ml of 0.1% TFA in water (solvent A). After filtered through a 0.22 µm Millipore filter unit, a 80 µl portion of the filtrate was applied to a Sephasil Peptide C18 5 µm reverse phase

column (4.6 × 100 mm, Amersham Biosciences, Uppsala, Sweden) with C18 cartridge as a guard column. The peptides were eluted at a flow rate of 1.0 ml/min starting with solvent A for 10 min and a linear gradient from 0 to 70% of 0.1% TFA in acetonitrile (solvent B) for 60 min. The eluates were monitored for absorbance at 214 nm and the fractions were collected on a peak basis for further studies.

5.2.7 On-line RP-HPLC-tandem mass spectrometry (LC/MS/MS)

Fractions obtained from RP-HPLC were subjected to LC- MS/MS analysis on a Waters Q-Tof Premier Mass spectrometer (Waters, Milford, MA, USA) equipped with a Waters nanoAquity UPLC system (Waters, Milford, MA, USA). The RP-HPLC fractions were concentrated in a speed vacuum drying device (Savant Speed Vac® plus SC11A, TeleChem International, Sunnyvale, CA, USA) to about 10% of original volumes. 2 µl of concentrated peptide solution (with overloading factor of 2) was loaded onto a peptide trap (180µm × 20mm, Symmetry® C18 nanoAcquity™ column, Waters, Milford, MA, USA) sequentially equipped with an analytical column (75 µm × 100 mm, Atlantis™ dC18 nanoAcquity™ column, Waters, Milford, MA, USA). Desalting on the peptide trap was achieved by a flushing trap with 2% acetonitrile, 0.1% formic acid (solvent C) at a flow rate of 4 µl/min for 1.5 min. Peptides were separated with a gradient of 2 - 95% of 0.1% formic acid in acetronitrile (solvent D) over 35 min at a flow rate of 300 nl/min. The column was connected to a Waters NanoEase™ emitter with tip size of 90 µm O.D. and 20 µm I.D. (Waters, Milford, MA, USA) for ESI-MS/MS analysis.

The MS/MS data obtained were analyzed through MS/MS Ion Search, one of the Mascot search engines (Matrix Science Inc., Boston, MA). Databases were established by grouping all possible sequences with variants of the expected proteins. Peptides were identified by searching against these homemade databases. Settings for a database search were as follows: parent ion and MS/MS tolerance were set to 80 ppm and 0.4 Da respectively; no enzyme was specified; 2 missed cleavage sites per peptide were allowed; commonly possible modifications, deamidation (NQ), N-acetyl

(protein), N-formyl (protein), oxidation (M), phospho (ST) and phospho (Y), were set as the variable modifications.

Peptide identifications were further confirmed by examining the scores and manual inspection of the original MS/MS spectra. Good spectra with significant numbers of matched high intensity peaks were considered important for the identification.

5.2.8 Determination of peptide content

Peptide content was spectrophotometrically measured at the wavelength of 210 nm as described by Stoscheck [11] using BSA as a standard.

5.2.9 Amino acid analysis

Tryptophan was analyzed by a RP-HPLC method described by Sedgwick *et al.* [12]. A sample was weighed in a 1.3 × 10 cm glass screw cap culture tube, to which 4 ml of 4.2 N NaOH and 0.8 ml of 25% (w/v) pyrogallol were added. The sample was hydrolyzed in the presence of nitrogen at 110 °C for 20 h. After hydrolysis, 100 µl of 0.075 % (w/v) amino-n-caproic acid and 100 µl of water were added to the hydrolysate, and the mixture was centrifuged at 770 g for 15 min. An aliquot (200 µl) of the supernatant obtained was then mixed with 30 µl of glacial acetic acid and 600 µl of saturated potassium tetraborate tetrahydrate. This was mixed with a fluoraldehyde reagent (see below) in an approximate ratio of 1 to 1 prior to RP-HPLC analysis. The fluoraldehyde reagent consisted of 0.25 g of OPA first dissolved in 6 ml methanol, 56 ml of 0.04 M sodium borate buffer, pH 9.5, 0.25 ml of 2-mercaptoethanol and 2 ml of Brij 35. The Varian 5000 HPLC system (Varian, Inc., Walnut Creek, CA, USA) was equipped with a Supelcosil 3 µm LC-18 reverse phase column (4.6 x 150 mm; Supelco, Inc. Bellefonte, PA, USA.) and a Fluorichrom fluorescence detector (Varian, Inc., Walnut Creek, CA, USA). Fluorescence was monitored with excitation wavelength of 340 nm and emission wavelength of 450 nm.

Analysis of amino acids other than tryptophan was performed by using a Beckman model 6300 amino acid analyzer on samples hydrolyzed in the presence of nitrogen at 110 °C for 24 h in glass-distilled 6 N HCl containing 0.1% (w/v) phenol.

5.3 RESULTS AND DISCUSSION

5.3.1 Gel filtration chromatography

Elution profile of 03FM permeate on Bio-Gel P2 column is shown in Fig. 5.1, and analytical data for eluates from the column are given in Table 5.1. High ACE inhibitory activities were observed in three fractions (I-III) accounting for, respectively 7, 6 and 8% of 03FM permeate used for the purification. Fraction II had the lowest IC_{50} value (12.2 μg peptide/ml), which was approximately 21-fold less than that for the 03FM permeate chromatographed on Bio-Gel P2. Also the value for fraction II was approximately 3-fold lower than that for fraction I and 37-fold lower than that for fraction III. These results suggested that column chromatography on Bio-Gel P2 is an appropriate technique to isolate ACE inhibitory peptides. The IC_{50} value for fraction II was comparable to that for HPLC fractions from of Calpis™ sour milk reported by Nakamura and his colleagues [7].

Amino acid analysis of fractions from Bio-Gel P2 (Table 5.2) revealed that major amino acids in fraction I were glutamine/glutamic acid, proline and leucine while those in fraction II were glutamine/glutamic acid, proline, serine and leucine. Fraction III had glutamine/glutamic acid, alanine, leucine and proline as major amino acids. Amino acids were also categorized according to their side chains (Fig. 5.3). Fraction II contained lower amount of aliphatic amino acids (Ala, Val, Met, Leu, and Ile), and higher amount of aromatic (Phe, Trp and Tyr), basic (Arg, Lys and His) and cyclic (Pro) amino acids than did fraction I. Fraction III had more aliphatic and basic but less cyclic amino acid residues than did fraction I or II. The contents of aromatic amino acids were higher in fraction III than in fraction I but lower in fraction II than in fraction II. When amino acid hydrophobicity was compared, fraction II had the highest contents of hydrophobic (Ala, Val, Met, Leu, Ile, Phe, Trp, Tyr and Pro) amino acid residues. The content of hydrophobic amino acid was higher in fraction I compared to

fraction III. This is in a good agreement with previous report [14], [15] which demonstrated that peptides with high ACE inhibitory effect were rich in hydrophobic or basic amino acids.

5.3.2 RP-HPLC

Fraction II from Bio-Gel P2 chromatography was then studied using RP-HPLC. The chromatogram obtained is illustrated in Fig. 5.4, and the ACE inhibitory activity determined for each peak is shown in Fig. 5.5. The ACE inhibitory activity varied among the 17 fractions examined. Fraction 1 – 4 with relatively low hydrophobicity and fraction 11 scarcely had ACE inhibitory activity whereas the remaining fractions demonstrated varying activities of ACE inhibitory peptides present. The inhibitory activity was higher in fraction 7 and 14 – 17 compared to fraction 5, 8 – 10, 12 and 13. Fractions 5 – 10 and 12 – 17 containing ACE inhibitory peptides were further studied using LC/MS/MS.

5.3.3 LC/MS/MS

Peptides identified by LC/MS/MS are summarized in Table 5.3. Eleven to 15 peptides were identified in all fractions except fractions 5 and 6, in which less than six peptides were identified. A majority of the peptides identified were derived from β-casein. Minorities were from κ- and α_{S1}- caseins. Only a few peptides were from BSA, β-lactoglobulin or α_{S2}-casein. Same sequences of peptide such as VVVPP, PVPQK and IHPF were also identified in more than one fraction. Among the identified peptides, only one peptide LQSW (peptide number 66) derived from β-CN f(140-143), had been previously described as an ACE inhibitor with IC_{50} of 500 μM [22]. This peptide was also found in the casein pancreatin hydrolysate (Chapter 3, Table 3.3)

Some of the peptides identified in this study had a peptide previously reported as their C-terminal position. For example, peptide VV**VPP** (peptide number 1, 74, 98 and 108) contained peptide VPP identified in Calpis™ sour milk, which was well-known for its low IC_{50} and its efficacy to reduce blood pressure [7], [8]. Peptide FL**PYP** (peptide number 37 and 57) had peptide PYP, which was reported to have

IC_{50} of 220 μM [24] at its C-terminal site. Peptide VYP with IC_{50} of 288 μM [23] was found at the C-terminal position of peptide L**VYP** (peptide number 64, 86, 97 and 107). Studies of structure and ACE inhibition relationships using computer-aided molecular modeling indicated the prominent role of amino acid sequence at the C-terminal end especially, the last three residues [30]. Peptides with sequence homology at the C-terminal end may exhibit similar ACE inhibitory effect.

Some of the peptides identified in this study also shared some of amino acid sequence previously reported in ACE inhibitory peptides. For instance, peptide **PLPP**T (peptide number 2) and peptide QP**LPP**T (peptide number 4 and 78) had sequence homology with peptide LPP which had IC_{50} of 10 μM [16]. Peptide PSERY (peptide number 8) and peptide VPSERY (peptide number 11) shared an amino acid sequence with peptide **VPSERY**L whose IC_{50} was reported to be 233μM [18]. The similar amino acid sequence may have a similar effect on the inhibition of ACE.

It has been demonstrated that ACE prefers an inhibitor with hydrophobic amino acid at each of the three C-terminal position [30]. Half of the peptides identified in HPLC fractions with high to moderate ACE inhibitory activity (fractions 7 – 8 and 12 – 17 in Fig. 5.3 and 5.4) contained hydrophobic amino acids at the C-terminal end, of which more than 45% had hydrophobic amino acid as their C-terminal tripeptide. These included peptides VVVPP (peptide number 1), YYQQKPVA (peptide number 15), LPLPL (peptide number 17) and GPFPIIV (peptide number 42). Aromatic or cyclic amino acid at the C-terminal end was also demonstrated to play an important role in the ACE inhibition [30]. Some of the peptides identified in this study had an aromatic or cyclic residue at their C-terminal end. These included peptide LYQEP (peptide numbers 3), LAY (peptide number 5), IHPF (peptide number 16), LLQSW (peptide number 29). These peptides may exert strong ACE inhibitory activity and probably contributed to the ACE inhibitory activity of the fraction.

It has been reported that basic amino acids, arginine and lysine, contribute to the ACE inhibitory effect through their positive charges on the guanidine or the ε-amino group, repectively. The three peptides, PVPQK, VLNENLLR and SDK identified in the present study appear to be interesting peptides to be examined for their ACE inhibitory activity.

Some bioactive peptides have more than one physiological effect. For example, β-casomorphin, an opiate peptide derived from β-casein was found to exhibit ACE inhibitory activity [15]. Some of the peptide sequences found in this study may suggest physiological effect other than ACE inhibitory effect. For example, peptide PVPQK encrypted in peptide VL**PVPQK** (β-CN f170-176) may exert antioxidant property as did its native peptide [34]. Peptide GPFPIIV as a part of peptide LLYQQPVLGPVR**GPFPIIV** (α_{S1}-CN f191-199), which enhanced proliferation of rat lymphocytes, [(35)] may have immunomodulatory activity. However, synthesis of the peptide of interest should facilitate further study to explore physiological activities of the peptides as well as to demonstrate the effect of the sequence homology on ACE inhibition.

In conclusion, the ACE inhibitory activity found in the fraction obtained from Bio-Gel P2 was 21-fold higher than that in the permeate sample applied. This indicated that Bio-Gel P2 can be employed for the isolation of ACE inhibitory peptides from the permeate of milk fermented with *Lactobacillus casei* ADA03. The active fraction from Bio-Gel P2 with IC_{50} of 12.2 μg peptide/ml was applied to RP-HPLC for further fractionation. In general, high ACE inhibitory activity was found in purified HPLC peaks with high hydrophobicity. LC/MS/MS, a technique, which facilitates rapid characterization of peptides, showed that more than 22% of the identified ACE inhibitory peptides contained hydrophobic amino acid at each of the three C-terminal site. Peptide VVVPP, which is a precursor of peptide VPP present in Calpis™ sour milk, was also identified in the fractions from HPLC.

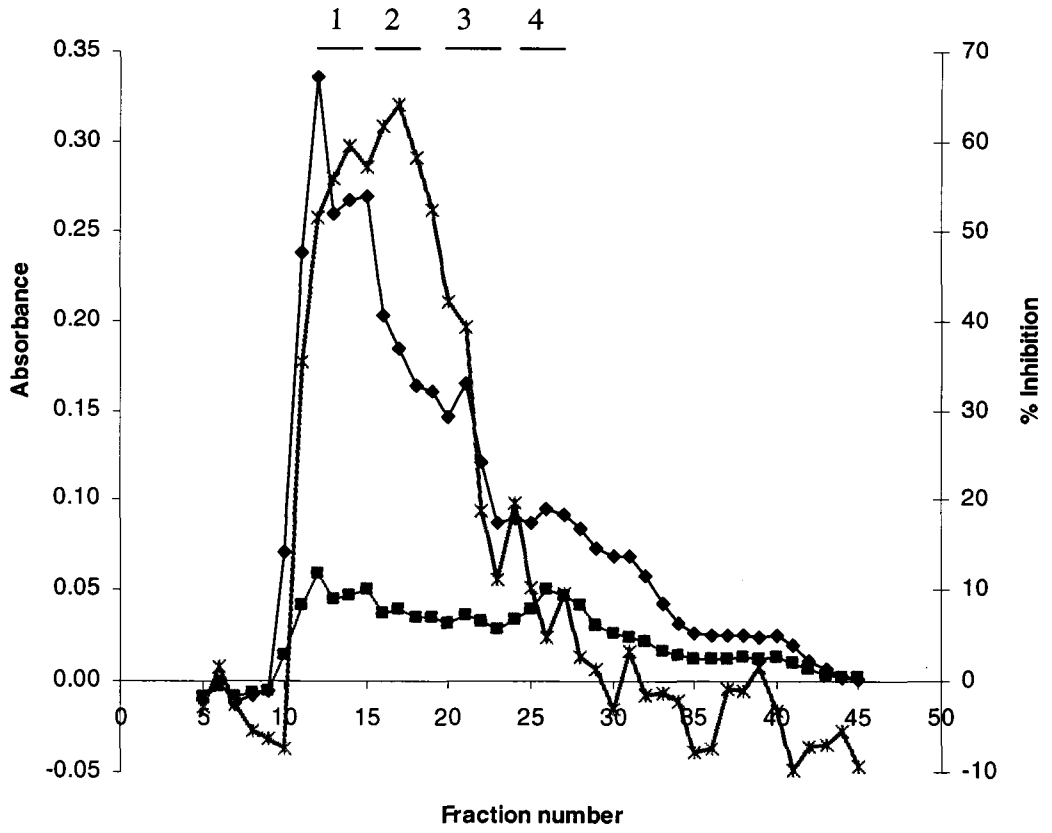

Figure 5.1: Gel filtration chromatography of 03FM permeate on Bio-Gel P2. Fraction of 1 ml were collected at a flow rate of 11 ml/h.

—◆— A210 —■— A230 —✳— ACE inhibition

Bars denote fractions pooled for further study.
See methods for other details

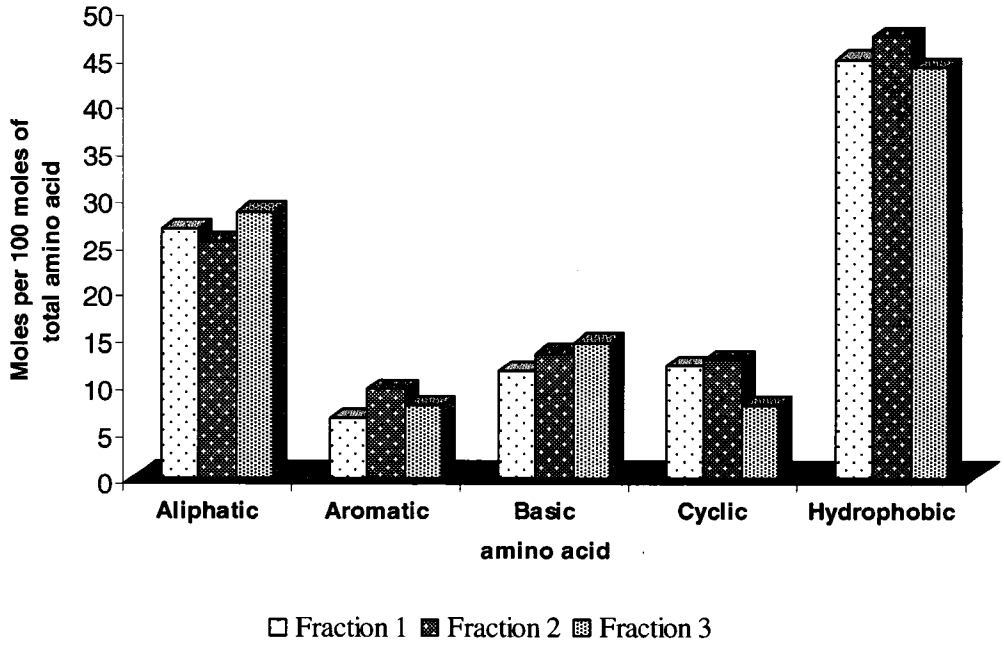

Figure 5.2: Comparison of amino acid contents among fractions from Bio-Gel P2.

Amino acids were categorized according to their side chains.

Aliphatic amino acid: Ala, Val, Met, Leu, and Ile
Aromatic amino acid: Phe, Trp and Tyr
Basic amino acid: Arg, Lys and His
Cyclic amino acid: Pro
Hydrophobic amino acid: Ala, Val, Met, Leu, Ile, Phe, Trp, Tyr and Pro

Fraction 1 Fraction 2 Fraction 3

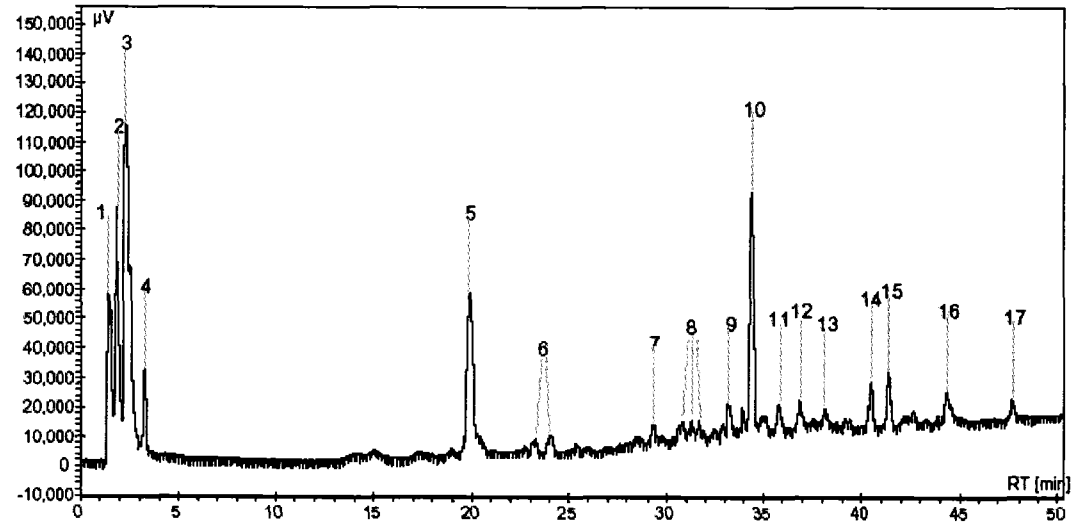

Figure 5.3: RP-HPLC chromatogram of a peptide fraction from Bio-Gel P2. A portion (80 µl) of peptide sample [(0.0052 g of fraction II/ml of 0.1% TFA in water (solvent A)] was injected to the HPLC system. The peptides were eluted at a flow rate of 1.0 ml/min starting with 0.1% TFA in acetonitrile (solvent B) for 10 min and a linear gradient from 0 to 70% of solvent B for 60 min.
See methods for other details.

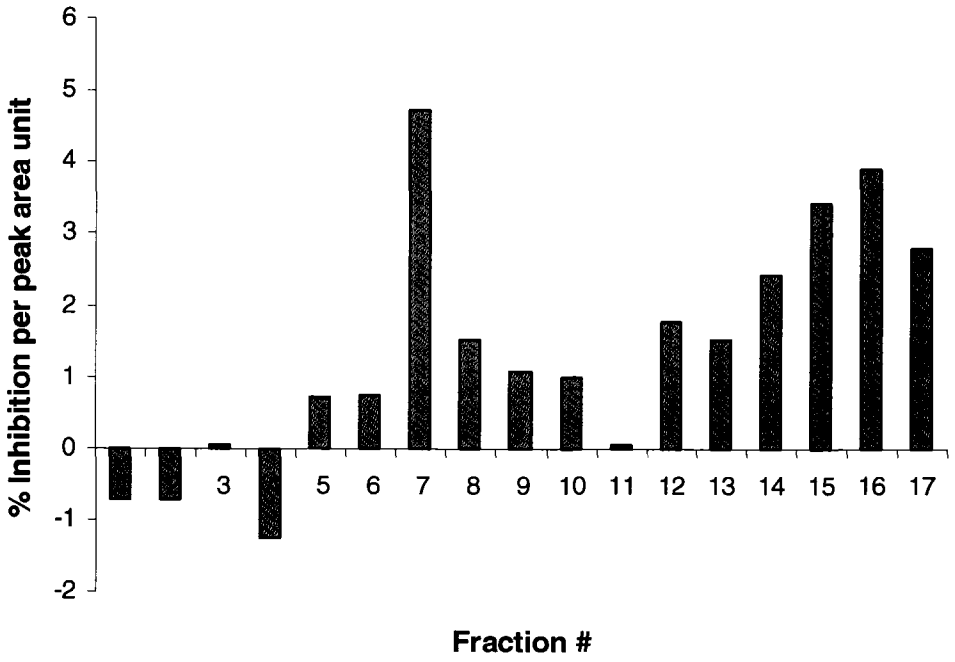

Figure 5.4: ACE inhibitory activity determined in fractions obtained by RP-HPLC. Fractions from one run of RP-HPLC were dried and added with 80 µl of 0.1 M sodium borate buffer, pH 8.3 containing 0.3 M NaCl. Then the solutions obtained were used in the ACE inhibitory activity assay.

Table 5.1: ACE inhibitory activity of fractions of the 03 FM permeate obtained from Bio-Gel P2 gel filtration column.

Fraction	IC_{50} (μg peptide/ml)	Yield[1]
I	38.8 ± 4.3	7.4 ± 2.8
II	12.2 ± 0.3	5.7 ± 2.3
III	448.2 ± 10.5	8.1 ± 3.2
IV	Not detected	34.8 ± 4.5

[1] Percentage of dry weight of the permeate of 03FM used for purification expressed as mean ± SD.

Table 5.2: Amino acid composition of fractions obtained by gel filtration chromatography of 03FM permeate on Bio-Gel P2.

Amino acid	Fraction		
	I	II	III
	Moles/ 100 moles of total amino acids		
Asparagine and aspartic acid	7.1	4.4	7.5
Threonine	5.7	4.9	4.5
Serine	6.5	8.9	7.0
Glutamine and glutamic acid	21.4	17.3	15.5
Glycine	3.3	4.4	7.5
Alanine	4.4	6.4	10.2
Valine	6.0	4.2	5.1
Methionine	2. 5	2.4	1.0
Isoleucine	4.3	3.6	3.3
Leucine	9.2	8.6	8.7
Tyrosine	2.8	4.5	4.1
Phenylalanine	3.4	4.9	3.6
Histidine	2.5	3.2	2.4
Lysine	6.5	7.5	7.3
Tryptophan	0.0	0.0	0.0
Arginine	2.4	2.4	4.6
Proline	11.9	12.4	7.7

Cysteine was not analyzed

Table 5.3: Peptides identified by LC/MS/MS in the fractions from RP-HPLC.

No.	Fraction[a]	Observed mass	Calculated mass[b]	Protein fragment[c]	Sequence	ACE inhibitory peptide[d]	IC_{50}[e] (μM)	Blood pressure reduction (mm Hg) at {mg/kg body weight}	Ref
1	7	510.33	509.32	β-CN f(82-86)	VVVPP	VPP	9	29 {0.6}	[7], [8]
2		524.31	523.30	β-CN f(150-154)	PLPPT	LPP	9.6		[16]
3		649.32	648.31	β-CN f(192-196)	LYQEP				
4		652.37	651.36	β-CN f(149-154)	QPLPPT	LPP	9.6		[16]
5		366.20	365.20	α_{S1}-CN f(142-144)	LAY	LAYFYP	65		[17]
6		481.22	480.22	α_{S1}-CN f(93-96)	GYLE				
7		609.28	608.28	α_{S1}-CN f(93-97)	GYLEQ				
8		651.33	650.30	α_{S1}-CN f(87-91)	PSERY	VPSERYL	232.8		[18]
9		654.31	653.30	α_{S1}-CN f(9-14)	pQGLPQE				
10		722.33	721.33	α_{S1}-CN f(156-162)	LDAYPSG				
11		375.69	749.37	α_{S1}-CN f(86-91)	VPSERY	VPSERYL	232.8		[18]
12		577.30	576.29	α_{S2}-CN f(99-103)	LYQGP				
13		627.37	626.36	α_{S2}-CN f(117-122)	VPITPT				
14		502.25	1002.48	α_{S2}-CN f(26-34)	MAINPSKEN	NMAINPSK	60		[19]
15		498.76	995.51	κ-CN f(42-49)	YYQQKPVA				
16	16	513.28	512.27	β-CN f(49-52)	IHPF	DKIHPF	257		[20]
17		552.37	551.37	β-CN f(135-139)	LPLPL				
18		568.34	567.34	β-CN f(172-176)	PVPQK	SKVLPVPQKV	39		[21]
						LPVPQ	5	32 {1}	[22]
19		671.42	670.41	β-CN f(83-88)	VVPPFL	VPP	9	29{0.6}	[7], [8]

No.	Fraction[a]	Observed mass	Calculated mass[b]	Protein fragment[c]	Sequence	ACE inhibitory peptide[d]	IC_{50}[e] (μM)	Blood pressure reduction (mm Hg) at {mg/kg body weight}	Ref
20		735.40	734.40	β-CN f(58-63)	LVYPFP	VYP	288	21{8}	[23]
						VYPFPG	221	22 {8}	
						FP	315	27 {8}	
21		388.19	774.37	β-CN f(179-184)	PYPQRD	PYP	220		[24]
22		609.30	608.29	κ-CN f(33-37)	SRYPS				
23		687.38	686.38	κ-CN f(73-77)	ILQWQ				
24		612.32	1222.63	κ-CN f(40-49)	LNYYQQKPVA				
25		485.79	969.56	α_{S1}-CN f(15-22)	VLNENLLR				
26		779.37	778.35	α_{S2}-CN f(18-23)	pETYKQE				
27	15	513.28	512.27	β-CN f(49-52)	IHPF	DKIHPF	257		[20]
28		568.34	567.34	β-CN f(172-176)	PVPQK	SKVLPVPQ	39		[21]
						KVLPVPQ	5	32 {1}	[22]
29		646.36	645.35	β-CN f(139-143)	LLQSW	LQSW	500	2 {1}	[22]
30		706.37	705.35	β-CN f(185-190)	MPIQAF				
31		388.18	774.37	β-CN f(179-184)	PYPQRD	PYP	220		[24]
32		488.75	975.51	β-CN f(111-118)	FPKYPVEP	FP	315	27 {8}	[23]
33		668.33	667.32	α_{S1}-CN f(145-149)	FYPEL				
34		806.41	805.40	α_{S1}-CN f(24-30)	FVAPFPE	FP	315	27 {8}	[23]
						PFPE	>1000,		[26]
						FPE	n.d.		[27]
						VAP	2		[24]
						FVAP	10		[24]
35		485.78	969.56	α_{S1}-CN f(15-22)	VLNENLLR				
36		609.30	608.29	κ-CN f(33-37)	SRYPS				

No.	Fraction[a]	Observed mass	Calculated mass[b]	Protein fragment[c]	Sequence	ACE inhibitory peptide[d]	IC_{50}[e] (μM)	Blood pressure reduction (mm Hg) at {mg/kg body weight}	Ref
37		636.34	635.33	κ-CN f(55-59)	FLPYP	PYP	220		[24]
38		687.39	686.38	κ-CN f(73-77)	ILQWQ				
39		612.32	1222.63	κ-CN f(40-49)	LNYYQQKPVA				
40	17	513.28	512.27	β-CN f(49-52)	IHPF	DKIHPF	257		[20]
41		568.34	567.34	β-CN f(172-176)	PVPQK	SKVLPVPQ	39		[21]
						KVLPVPQ	5	32 {1}	[22]
42		742.45	741.44	β-CN f(203-209)	GPFPIIV	FP,	315	27 {8}	[23]
						LLYQEPVLGP -VRGPFPIIV	21		[21]
43		770.48	769.47	β-CN f(82-88)	VVVPPFL	VPP	9	29 {0.6}	[7], [8]
44		388.18	774.37	β-CN f(179-184)	PYPQRD	PYP	220		[24]
45		488.75	975.51	β-CN f(111-118)	FPKYPVEP	FP	315	27 {8}	[23]
46		499.31	996.61	β-CN f(201-209)	VRGPFPIIV	FP	315	27 {8}	[23]
						VRGPFP	592		[18]
						LLYQEPVLGP -VRGPFPIIV	21		[21]
47		587.29	1172.56	β-CN f(177-186)	AVPYPQRDMP	AVPYPQR	15		[28]
						AVP	340		[24]
						PYP	220		[24]
						AVPYP	80		[24]
48		609.30	608.29	κ-CN f(33-37)	SRYPS				
49		527.27	1052.53	κ-CN f(40-47)	LNYYQQKP				
50		612.32	1222.63	κ-CN f(40-49)	LNYYQQKPVA				

No.	Fraction[a]	Observed mass	Calculated mass[b]	Protein fragment[c]	Sequence	ACE inhibitory peptide[d]	IC_{50}[e] (μM)	Blood pressure reduction (mm Hg) at {mg/kg body weight}	Ref
51	14	568.34	567.34	β-CN f(172-176)	PVPQK	SKVLPVPQ	39, 5		[21]
						KVLPVPQ		32 {1}	[22]
52		622.32	621.32	β-CN f(59-63)	VYPFP	VYP	288	21 {8}	[23]
						VYPFPG	221	22 {8}	
						FP	315	27 {8}	
53		646.36	645.35	β-CN f(139-143)	LLQSW	LQSW	500	2 {1}	[22]
54		706.34	705.35	β-CN f(185-190)	MPIQAF				
55		388.19	774.37	β-CN f(179-184)	PYPQRD	PYP	220		[24]
56		488.76	975.50	β-CN f(111-118)	FPKYPVEP	FP	315	27 {8}	[23]
57		636.34	635.33	κ-CN f(55-59)	FLPYP	PYP	220		[24]
58		687.39	686.38	κ-CN f(73-77)	ILQWQ				
59		612.32	1222.63	κ-CN f(40-49)	LNYYQQKPVA				
60		668.33	667.32	κ-CN f(145-149)	FYPEL				
61		485.78	969.56	κ-CN f(15-22)	VLNENLLR				
62	12	439.29	438.28	β-CN f(135-138)	LPLP				
63		439.29	438.28	β-CN f(74-77)	IPPL	IPP	5	24 {0.3}	[7], [8]
64		491.29	490.28	β-CN f(58-61)	LVYP	VYP	288	21 {8}	[23]
65		513.28	512.27	β-CN f(49-52)	IHPF	DKIHPF	257		[20]
66		533.27	532.26	β-CN f(140-143)	LQSW	LQSW	500	2 {1}	[22]
67		568.34	567.34	β-CN f(172-176)	PVPQK	SKVLPVPQ	39	32 {1}	[21]
						KVLPVPQ	5		[22]
68		388.18	774.37	β-CN f(179-184)	PYPQRD	PYP	220		[24]
69		805.39	804.38	β-CN f(155-161)	VMFPPQS	FP	315	27 {8}	[23]

No.	Fraction[a]	Observed mass	Calculated mass[b]	Protein fragment[c]	Sequence	ACE inhibitory peptide[d]	IC_{50}[e] (μM)	Blood pressure reduction (mm Hg) at {mg/kg body weight}	Ref
70		828.42	827.42	β-CN f(62-69)	FPGPIPNS	FP	315	27 {8}	[23]
						YPFPGPI	500		[25]
						YPFPGPIPN	15	7 {7.5}	[29]
71		587.29	1172.56	β-CN f(177-186)	AVPYPQRDMP	AVPYPQR	15		[28]
						AVP	340		[24]
						PYP	220		[24]
						AVPYP	80		[24]
72		848.42	847.41	β-Lg f(151-157)	FNPTQLE				
73		633.36	632.35	κ-CN f(26-30)	IPIQY				
74	8	510.33	509.32	β-CN f(82-86)	VVVPP	VPP	9	29 {0.6}	[7], [8]
75		559.29	558.28	β-CN f(185-189)	MPIQA				
76		568.34	567.34	β-CN f(172-176)	PVPQK	SKVLPVPQ	39	32 {1}	[21]
						KVLPVPQ	5		
77		575.28	574.28	β-CN f(157-161)	FPPQS	FP	315	27 {8}	[23]
78		635.34	634.33	β-CN f(149-154)	pQPLPPT	LPP	9.6		[16]
79		643.28	642.27	β-CN f(89-94)	pQPEVMG				
80		388.19	774.37	β-CN f(179-184)	PYPQRD	PYP	220		[24]
81		421.20	420.20	α_{S1}-CN f(176-179)	APSF				
82		545.27	544.25	α_{S1}-CN f(167-171)	SDIPN				
83		557.32	556.32	κ-CN f(119-123)	IPTIN				
84		804.45	803.44	κ-CN f(162-169)	VQVTSTAV				
85	13	439.29	438.28	β-CN f(74-77)	IPPL	IPP	5	24 {0.3}	[7], [8]
86		491.29	490.28	β-CN f(58-61)	LVYP	VYP	288	21 {8}	[23]

No.	Fraction[a]	Observed mass	Calculated mass[b]	Protein fragment[c]	Sequence	ACE inhibitory peptide[d]	IC_{50}[e] (μM)	Blood pressure reduction (mm Hg) at {mg/kg body weight}	Ref
86		568.34	567.34	β-CN f(172-176)	PVPQK	SKVLPVPQ	39		[21]
						KVLPVPQ	5	32 {1}	
88		741.40	740.39	β-CN f(62-68)	FPGPIPN	FP	315	27 {8}	[23]
						YPFPGPI	500		[25]
						YPFPGPIPN	15	7 {7.5}	[29]
89		805.39	804.38	β-CN f(155-161)	VMFPPQS	FP	315	27 {8}	[23]
90		828.43	827.42	β-CN f(62-69)	FPGPIPNS	FP	315	27{8}	[23]
						YPFPGPI	500		[25]
						YPFPGPIPN	15	7 {7.5}	[29]
91		485.78	969.56	α_{S1}-CN f(15-22)	VLNENLLR				
92		541.31	540.29	BSA f(409-413)	pQNALI				
93		497.24	496.24	κ-CN f(103-106)	LSFM				
94		687.38	686.38	κ-CN f(73-77)	ILQWQ				
95		719.37	718.36	β-Lg f(151-156)	FNPTQL				
96	9	478.26	477.26	β-CN f(187-190)	IQAF				
97		491.28	490.28	β-CN f(58-61)	LVYP	VYP	288	21 {8}	[23]
98		510.33	509.32	β-CN f(82-86)	VVVPP	VPP	9	29 {0.6}	[7], [8]
99		568.35	567.34	β-CN f(172-176)	PVPQK	SKVLPVPQ	39		[21]
						KVLPVPQ	5	32 {1}	[22]
100		639.38	638.38	β-CN f(172-177)	PVPQKA	SKVLPVPQ	39		[21]
						KVLPVPQ	5	32 {1}	[22]
101		388.19	774.37	β-CN f(179-184)	PYPQRD	PYP	220		[24]
102		658.34	657.37	κ-CN f(120-125)	PTINTI				
103		658.38	657.37	κ-CN f(119-124)	IPTINT				

No.	Fraction[a]	Observed mass	Calculated mass[b]	Protein fragment[c]	Sequence	ACE inhibitory peptide[d]	IC_{50}[e] (μM)	Blood pressure reduction (mm Hg) at {mg/kg body weight}	Ref
104		586.28	585.28	α_{S2}-CN f(97-100)	QYLY				
105		701.38	700.38	α_{S1}-CN f(15-20)	VLNENL				
106		777.41	776.41	α_{S1}-CN f(167-173)	VPLGTQY				
107	10	491.26	490.28	β-CN f(58-61)	LVYP	VYP	288	21 {8}	[23]
108		510.33	509.32	β-CN f(82-86)	VVVPP	VPP	9	29 {0.6}	[7], [8]
109		554.32	553.29	β-CN f(111-114)	FPKY	FP	315	27 {8}	[23]
110		568.35	567.34	β-CN f(172-176)	PVPQK	SKVLPVPQ	39		[21]
						KVLPVPQ	5	32 {1}	[22]
111		388.19	774.37	β-CN f(179-184)	PYPQRD	PYP	220		[24]
112		805.39	804.38	β-CN f(155-161)	VMFPPQS	FP	315	27 {8}	[23]
113		436.26	870.52	β-CN f(165-172)	LSQSKVLP				
114		518.77	1035.53	β-CN f(64-73)	GPIPNSLPQN	LPQ			[29]
						YPFPGPIPN	14	7 {7.5}	
115		594.31	593.31	α_{S1}-CN f(92-96)	LGYLE	LGY	104		[16]
116		659.34	658.33	α_{S1}-CN f(25-30)	VAPFPE	FP	315	27 {8}	[23]
						PFPE	>1000		[7],[8]
						FPE	n.d.		[27]
						VAP	2		[24]
117		349.18	348.16	κ-CN f(19-21)	SDK				
118		658.38	657.37	κ-CN f(119-124)	IPTINT				
119	6	486.24	485.25	α_{S1}-CN f(11-14)	LPQE				
120		565.26	564.25	α_{S1}-CN f(158-163)	AYPSGA				
121		460.25	459.27	β-CN f(139-142)	LLQS				
122		559.31	558.30	β-CN f(77-81)	LTQTP				

No.	Fraction[a]	Observed mass	Calculated mass[b]	Protein fragment[c]	Sequence	ACE inhibitory peptide[d]	IC_{50}[e] (μM)	Blood pressure reduction (mm Hg) at {mg/kg body weight}	Ref
123		681.32	680.31	β-CN f(52-57)	FAQTQS				
124	5	576.28	575.27	β-CN f(54-58)	QTQSL				
125		594.28	593.28	β-CN f(52-56)	FAQTQ				
126		681.31	680.31	β-CN f(52-57)	FAQTQS				
127		601.32	600.32	κ-CN f(50-54)	LINNQ				

n.d., not determined

[a] Fraction present in a decreasing order of the ACE inhibitory activity as shown in Figure 3.3.

[b] Calculated monoisotopic mass.

[c] Protein sequence was obtained from one of the Mascot search engines (Matrix Science Inc., Boston, MA).

[d] Previously reported ACE inhibitory peptides.

[e] Peptide concentration needed to inhibit 50% ACE activity.

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CHAPTER 6

FINAL REMARKS

6.1 FUNCTIONAL FOODS IN THE TREATMENT AND PREVENTION OF CARDIOVASCULAR DISEASE

Cardiovascular disease (CVD) is a leading cause of global morbidity and mortality [1], responsible for one third of all deaths worldwide. CVD is a disease affecting the heart or blood vessels, including arteriosclerosis, coronary artery disease, heart valve disease, arrhythmia, heart failure, endocarditis, diseases of the aorta and its branches, disorders of the peripheral vascular system, and congenital heart disease. Hypertension is a highly prevalent risk factor for CVD [1]. Angiotensin converting enzyme (ACE) inhibitor is one of the recommended remedies for hypertension [2]. Synthetic ACE inhibitors including captopril, enalapril, and ramipril, have been prescribed to patients with hypertension and shown effective reduction and control in blood pressure. However, patients may suffer from their adverse effects such as dry cough, hyperkalemia, hypotension, dizziness and drowsiness [3]. Modifications in lifestyles including exercise, no smoking and diet control can assist in the effective management of hypertension. Dietary Approaches to Stop Hypertension (DASH), which is a diet rich in fruits, vegetables, and low-fat dairy products, has significant correlation with a decrease in blood pressure [4]. Research has shown that food containing ACE inhibitory peptides had effectively reduced blood pressure in hypertensive subjects [5], [6], [7]. No adverse effect was observed in the tested subjects. This indicates that ACE inhibitory peptides may play an important role in prevention and treatment of hypertension. As consumer awareness of nutrition and health relationship increases, foods containing ACE inhibitory peptides may have a good opportunity in functional food market. At present, there are some functional foods containing ACE inhibitors already available in the market as listed below.

- Calpis™ sour milk – a milk beverage containing VPP and IPP peptides – from The Calpis Food Industry Co., Ltd., Japan
- Katsuobushi Oligopeptide – a protease digest of Katsuobushi (dried bonito) containing peptide LKPNM – from Nippon Supplement, Inc., Japan
- BioZate® – a hydrolyzed whey protein isolate supplement – from Davisco Foods International, Inc., USA
- Evolus® – a milk beverage containing VPP and IPP peptides – from Valio Ltd., Finland

Information from our study can facilitate the production of functional food containing ACE inhibitory peptides. The research demonstrated the formation of ACE inhibitory peptides in casein pancreatin hydrolysate (CPH) and milk fermented with *Lactobacillus casei* ADA03 (03FM). This indicates that both CPH and 03FM could be classified as functional food containing ACE inhibitors, however, the efficacy of the product needs to be validated. Furthermore, the isolated fractions of CPH and 03FM could be used as a functional food ingredient, which exhibits ACE inhibitory activity.

Lactobacillus casei ADA03 is classified and proved as probiotic, therefore, 03FM can be suggested to be a dairy product that contains probiotic bacteria and exhibit ACE inhibitory activity. Besides ACE inhibitory activity, *Lactobacillus casei* ADA03 has shown to exhibit other physiological effects including antimicrobial activity against *Helicobacter pylori* [8], and immunostimulatory [9], cytotoxic [10], and apoptotic activities [10]. This reflects multifunctional effects that can be delivered by 03FM.

6.2 SUMMARY OF THE PRESENT RESEARCH

Our research has focused on the optimization for the production condition of ACE inhibitory peptide as well as the isolation and the identification of such peptides (Fig. 6.1). The present study demonstrated the formation of ACE inhibitory peptides by means of enzymatic hydrolysis and fermentation by lactic acid bacteria. The optimized condition of the production of ACE inhibitory peptides was investigated by comparing incubation times in the hydrolysis or by comparing lactic

acid bacteria strains and incubation times in the fermentation. Our study found the highest ACE inhibitory activity in the casein pancreatin hydrolysate (CPH) incubated for 8 h at 37 °C and in the milk fermented with *Lactobacillus casei* ADA03 (03FM) incubated for 24 h at 37 °C.

Methods of isolation (membrane and chromatographic techniques) of ACE inhibitory peptides were also explored. High ACE inhibitory activity was found in the permeate fractions obtained by ultrafiltration and fractions obtained by gel filtration chromatography on Bio-Gel P2, indicating that these techniques can be employed to enrich ACE inhibitory activity in both the hydrolysate and fermented milk fractions. Furthermore, the application of ultrafiltration followed by Bio-Gel P2 decreased the purification time of ACE inhibitory peptides compared to a few-step column chromatographic methods traditionally used for protein purification. Ultrafiltration or Bio-Gel P2 increased ACE inhibitory activity of 03FM more effectively than CPH (Table 6.1). However, more CPH fractions were recovered than 03FM fractions by either technique (Table 6.1).

Amino acid analysis of active fractions of CPH and 03FM indicated that hydrophobic amino acids play a prominent role in the inhibition of ACE. Characterization of peptides confirmed the significance of hydrophobic amino acids at the C-terminal end. Only few peptides identified in the present study have been previously described. This indicates the discovery of novel ACE inhibitory peptides in the present study.

6.3 RECOMMENDATIONS FOR FUTURE RESEARCH

- *In vivo* study of physiological activity of active ingredient is essential to the scientific evidence. Therefore, the verification of antihypertensive property of CPH and its fraction, and 03FM and its fraction in animal model and in clinical study is needed for the efficacy of the active ingredient.

- Synthesis of the ACE inhibitory peptide identified in the present study may be needed to verify its efficacy and validate the dose of a product in which it is present.

• Design of functional food product has to be developed to deliver the active ingredient. Furthermore, the dose of the product has to be validated.

• Purification and characterization of proteinases produced by *Lactobacillus casei* ADA03 should facilitate further studies to understand the role of enzymes involved in the formation of ACE inhibitory peptides.

• A new protein source for the ACE inhibitory peptides of interest may be investigated by using the understanding of the structure – inhibition relationships of ACE inhibitor and the specificity of the enzyme.

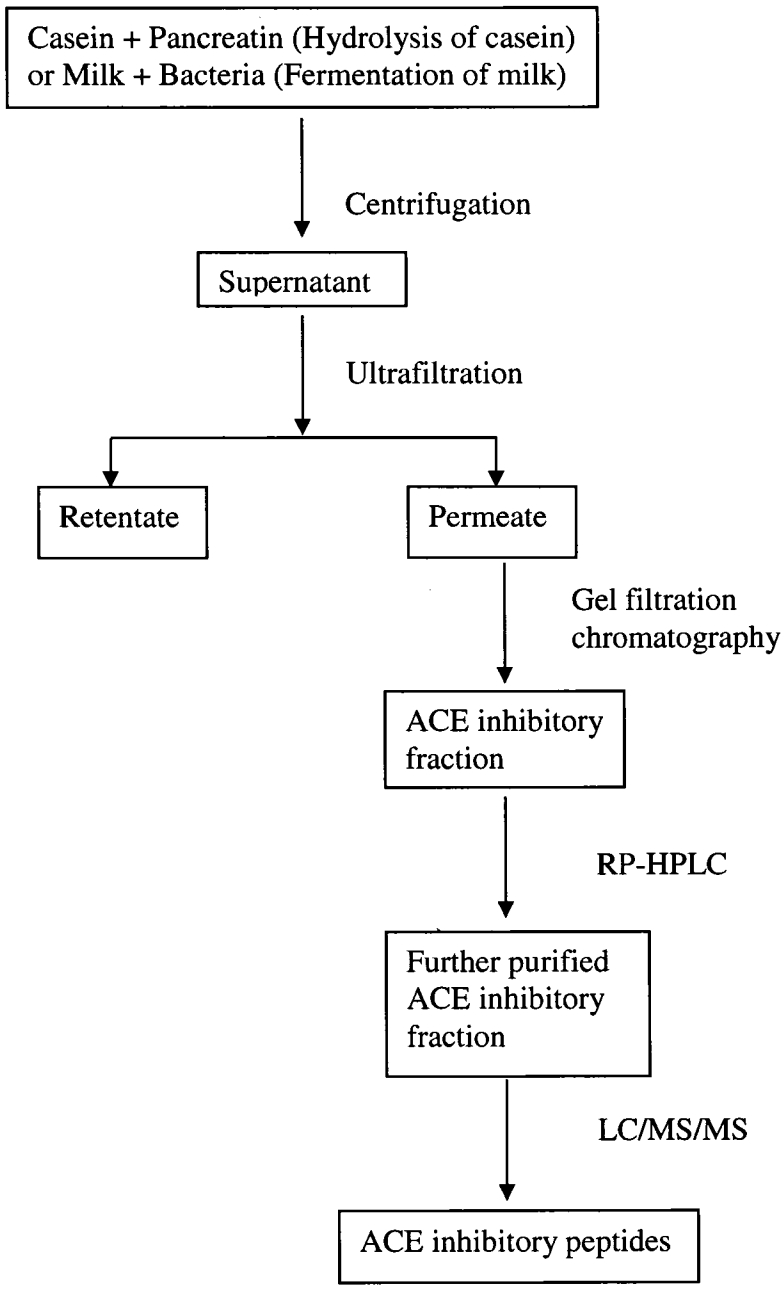

Figure 6.1: Schematic overview of the present study

Table 6.1: ACE inhibitory activities and yields of fractions of casein pancreatin hydrolysate (CPH) and milk fermented with *Lactobacillus* casei ADA03 (03FM)

	IC_{50} (μg peptide/ml)	Yield [1] (%)
CPH		
Supernatant	803	82
Permeate	464	47
Bio-Gel P2 fraction	319	9.4
03FM		
Supernatant	1118	66
Permeate	252	14
Bio-Gel P2 fraction	12	0.8

[1] Percentage of dry weight of casein used in the hydrolysis or skim milk used in the fermentation

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Appendix A

Statistical Analysis results of CPH and its fractions

Table A1: ANOVA for the yield of CPH at different hydrolysis time

Tests of Between-Subjects Effects

Dependent Variable: yield_CPH

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	3.619[a]	6	.603	.045	.999
Intercept	202861.714	1	202861.714	15214.629	.000
time	3.619	6	.603	.045	.999
Error	186.667	14	13.333		
Total	203052.000	21			
Corrected Total	190.286	20			

a. R Squared = .019 (Adjusted R Squared = -.401)

Table A2: ANOVA for the yield of permeate at different hydrolysis time

Tests of Between-Subjects Effects

Dependent Variable: yield_permeate

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	311.072[a]	6	51.845	2.830	.051
Intercept	43566.298	1	43566.298	2378.258	.000
time	311.072	6	51.845	2.830	.051
Error	256.460	14	18.319		
Total	44133.830	21			
Corrected Total	567.532	20			

a. R Squared = .548 (Adjusted R Squared = .354)

Table A3: ANOVA and Tukey's test for the yield of supernatant at different hydrolysis time

Tests of Between-Subjects Effects

Dependent Variable: yield_supernatant

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	78.464[a]	6	13.077	4.119	.014
Intercept	142048.035	1	142048.035	44742.521	.000
time	78.464	6	13.077	4.119	.014
Error	44.447	14	3.175		
Total	142170.946	21			
Corrected Total	122.911	20			

a. R Squared = .638 (Adjusted R Squared = .483)

yield_supernatant

Tukey HSD[a,b]

time	N	Subset 1	Subset 2
2	3	79.1407	
4	3	80.4185	
8	3	82.1556	82.1556
6	3	82.4167	82.4167
12	3	82.4981	82.4981
10	3	83.4167	83.4167
18	3		85.6667
Sig.		.114	.262

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = 3.175.

a. Uses Harmonic Mean Sample Size = 3.000.

b. Alpha = .05.

Multiple Comparisons

Dependent Variable: yield_supernatant

Tukey HSD

(I) time	(J) time	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
2	4	-1.2778	1.45483	.970	-6.2454	3.6899
	6	-3.2759	1.45483	.330	-8.2436	1.6917
	8	-3.0148	1.45483	.417	-7.9825	1.9528
	10	-4.2759	1.45483	.114	-9.2436	.6917
	12	-3.3574	1.45483	.305	-8.3250	1.6102
	18	-6.5259*	1.45483	.007	-11.4936	-1.5583
4	2	1.2778	1.45483	.970	-3.6899	6.2454
	6	-1.9981	1.45483	.807	-6.9658	2.9695
	8	-1.7370	1.45483	.885	-6.7047	3.2306
	10	-2.9981	1.45483	.423	-7.9658	1.9695
	12	-2.0796	1.45483	.778	-7.0473	2.8880
	18	-5.2481*	1.45483	.035	-10.2158	-.2805
6	2	3.2759	1.45483	.330	-1.6917	8.2436
	4	1.9981	1.45483	.807	-2.9695	6.9658
	8	.2611	1.45483	1.000	-4.7065	5.2287
	10	-1.0000	1.45483	.991	-5.9676	3.9676
	12	-.0815	1.45483	1.000	-5.0491	4.8862
	18	-3.2500	1.45483	.338	-8.2176	1.7176
8	2	3.0148	1.45483	.417	-1.9528	7.9825
	4	1.7370	1.45483	.885	-3.2306	6.7047
	6	-.2611	1.45483	1.000	-5.2287	4.7065
	10	-1.2611	1.45483	.972	-6.2287	3.7065
	12	-.3426	1.45483	1.000	-5.3102	4.6250
	18	-3.5111	1.45483	.262	-8.4787	1.4565
10	2	4.2759	1.45483	.114	-.6917	9.2436
	4	2.9981	1.45483	.423	-1.9695	7.9658
	6	1.0000	1.45483	.991	-3.9676	5.9676
	8	1.2611	1.45483	.972	-3.7065	6.2287
	12	.9185	1.45483	.994	-4.0491	5.8862
	18	-2.2500	1.45483	.715	-7.2176	2.7176
12	2	3.3574	1.45483	.305	-1.6102	8.3250
	4	2.0796	1.45483	.778	-2.8880	7.0473
	6	.0815	1.45483	1.000	-4.8862	5.0491
	8	.3426	1.45483	1.000	-4.6250	5.3102
	10	-.9185	1.45483	.994	-5.8862	4.0491
	18	-3.1685	1.45483	.364	-8.1362	1.7991
18	2	6.5259*	1.45483	.007	1.5583	11.4936
	4	5.2481*	1.45483	.035	.2805	10.2158
	6	3.2500	1.45483	.338	-1.7176	8.2176
	8	3.5111	1.45483	.262	-1.4565	8.4787
	10	2.2500	1.45483	.715	-2.7176	7.2176
	12	3.1685	1.45483	.364	-1.7991	8.1362

Based on observed means.

*. The mean difference is significant at the .05 level.

Table A4: ANCOVA and LSD test for the ACE inhibitory activity of different fractions at the hydrolysis time of 2 h

Tests of Between-Subjects Effects

Dependent Variable: Inhibition

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	28847.096[a]	3	9615.699	319.435	.000
Intercept	2369.293	1	2369.293	78.708	.000
Fraction	4662.106	2	2331.053	77.438	.000
Peptide_Conc	25666.076	1	25666.076	852.630	.000
Error	2287.770	76	30.102		
Total	101287.470	80			
Corrected Total	31134.865	79			

a. R Squared = .927 (Adjusted R Squared = .924)

Estimates

Dependent Variable: Inhibition

Fraction	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
Per	39.016[a]	1.077	36.870	41.162
Ret	20.211[a]	1.058	18.105	22.317
Sup	29.959[a]	1.056	27.856	32.062

a. Covariates appearing in the model are evaluated at the following values: Peptide_Conc = 567.7726.

Pairwise Comparisons

Dependent Variable: Inhibition

(I) Fraction	(J) Fraction	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a]	
					Lower Bound	Upper Bound
Per	Ret	18.805*	1.512	.000	15.794	21.816
	Sup	9.057*	1.508	.000	6.053	12.061
Ret	Per	-18.805*	1.512	.000	-21.816	-15.794
	Sup	-9.748*	1.495	.000	-12.725	-6.771
Sup	Per	-9.057*	1.508	.000	-12.061	-6.053
	Ret	9.748*	1.495	.000	6.771	12.725

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

a. Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).

Table A5: ANCOVA and LSD test for the ACE inhibitory activity of different fractions at the hydrolysis time of 4 h

Tests of Between-Subjects Effects

Dependent Variable: Inhibition

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	32059.944[a]	3	10686.648	197.571	.000
Intercept	1961.568	1	1961.568	36.265	.000
Fraction	4407.648	2	2203.824	40.744	.000
Peptide_Conc	30358.124	1	30358.124	561.250	.000
Error	3894.492	72	54.090		
Total	121517.673	76			
Corrected Total	35954.437	75			

a. R Squared = .892 (Adjusted R Squared = .887)

Estimates

Dependent Variable: Inhibition

Fraction	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
Per	41.773[a]	1.422	38.937	44.608
Ret	23.055[a]	1.510	20.044	26.066
Sup	34.755[a]	1.471	31.823	37.687

a. Covariates appearing in the model are evaluated at the following values: Peptide_Conc = 607.8473.

Pairwise Comparisons

Dependent Variable: Inhibition

(I) Fraction	(J) Fraction	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a]	
					Lower Bound	Upper Bound
Per	Ret	18.717*	2.086	.000	14.559	22.876
	Sup	7.018*	2.046	.001	2.940	11.096
Ret	Per	-18.717*	2.086	.000	-22.876	-14.559
	Sup	-11.699*	2.109	.000	-15.904	-7.495
Sup	Per	-7.018*	2.046	.001	-11.096	-2.940
	Ret	11.699*	2.109	.000	7.495	15.904

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

a. Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).

Table A6: ANCOVA and LSD test for the ACE inhibitory activity of different fractions at the hydrolysis time of 6 h

Tests of Between-Subjects Effects

Dependent Variable: Inhibition

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	29260.462[a]	3	9753.487	154.105	.000
Intercept	1595.331	1	1595.331	25.206	.000
Fraction	3835.622	2	1917.811	30.301	.000
Peptide_Conc	27211.685	1	27211.685	429.944	.000
Error	4367.092	69	63.291		
Total	108936.106	73			
Corrected Total	33627.554	72			

a. R Squared = .870 (Adjusted R Squared = .864)

Estimates

Dependent Variable: Inhibition

Fraction	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
Per	40.883[a]	1.628	37.634	44.131
Ret	22.925[a]	1.628	19.676	26.173
Sup	32.532[a]	1.591	29.358	35.707

a. Covariates appearing in the model are evaluated at the following values: Peptide_Conc = 629.9620.

Pairwise Comparisons

Dependent Variable: Inhibition

(I) Fraction	(J) Fraction	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a]	
					Lower Bound	Upper Bound
Per	Ret	17.958*	2.309	.000	13.352	22.564
	Sup	8.350*	2.277	.000	3.808	12.892
Ret	Per	-17.958*	2.309	.000	-22.564	-13.352
	Sup	-9.608*	2.276	.000	-14.149	-5.067
Sup	Per	-8.350*	2.277	.000	-12.892	-3.808
	Ret	9.608*	2.276	.000	5.067	14.149

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

a. Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).

Table A7: ANCOVA and LSD test for the ACE inhibitory activity of different fractions at the hydrolysis time of 8 h

Tests of Between-Subjects Effects

Dependent Variable: Inhibition

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	31560.825[a]	3	10520.275	214.761	.000
Intercept	4529.099	1	4529.099	92.457	.000
Fraction	5195.671	2	2597.835	53.032	.000
Peptide_Conc	28491.007	1	28491.007	581.615	.000
Error	3673.954	75	48.986		
Total	130596.079	79			
Corrected Total	35234.778	78			

a. R Squared = .896 (Adjusted R Squared = .892)

Estimates

Dependent Variable: Inhibition

Fraction	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
Per	44.843[a]	1.329	42.195	47.491
Ret	25.238[a]	1.374	22.501	27.975
Sup	33.318[a]	1.403	30.523	36.112

a. Covariates appearing in the model are evaluated at the following values: Peptide_Conc = 553.1360.

Pairwise Comparisons

Dependent Variable: Inhibition

(I) Fraction	(J) Fraction	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a]	
					Lower Bound	Upper Bound
Per	Ret	19.605*	1.916	.000	15.789	23.421
	Sup	11.525*	1.939	.000	7.663	15.387
Ret	Per	-19.605*	1.916	.000	-23.421	-15.789
	Sup	-8.080*	1.961	.000	-11.986	-4.173
Sup	Per	-11.525*	1.939	.000	-15.387	-7.663
	Ret	8.080*	1.961	.000	4.173	11.986

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

a. Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).

Table A8: ANCOVA and LSD test for the ACE inhibitory activity of different fractions at the hydrolysis time of 10 h

Tests of Between-Subjects Effects

Dependent Variable: Inhibition

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	29287.576[a]	3	9762.525	171.429	.000
Intercept	2880.099	1	2880.099	50.574	.000
Fraction	2795.104	2	1397.552	24.541	.000
Peptide_Conc	26084.082	1	26084.082	458.033	.000
Error	3872.468	68	56.948		
Total	123109.535	72			
Corrected Total	33160.044	71			

a. R Squared = .883 (Adjusted R Squared = .878)

Estimates

Dependent Variable: Inhibition

Fraction	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
Per	42.244[a]	1.574	39.104	45.384
Ret	27.283[a]	1.510	24.270	30.295
Sup	37.133[a]	1.540	34.059	40.207

a. Covariates appearing in the model are evaluated at the following values: Peptide_Conc = 626.3975.

Pairwise Comparisons

Dependent Variable: Inhibition

(I) Fraction	(J) Fraction	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a]	
					Lower Bound	Upper Bound
Per	Ret	14.962*	2.181	.000	10.610	19.313
	Sup	5.111*	2.202	.023	.717	9.505
Ret	Per	-14.962*	2.181	.000	-19.313	-10.610
	Sup	-9.850*	2.157	.000	-14.155	-5.546
Sup	Per	-5.111*	2.202	.023	-9.505	-.717
	Ret	9.850*	2.157	.000	5.546	14.155

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

a. Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).

Table A9: ANCOVA and LSD test for the ACE inhibitory activity of different fractions at the hydrolysis time of 12 h

Tests of Between-Subjects Effects

Dependent Variable: Inhibition

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	26251.972[a]	3	8750.657	139.471	.000
Intercept	1646.400	1	1646.400	26.241	.000
Fraction	1749.690	2	874.845	13.944	.000
Peptide_Conc	25465.737	1	25465.737	405.881	.000
Error	4329.190	69	62.742		
Total	103600.600	73			
Corrected Total	30581.163	72			

a. R Squared = .858 (Adjusted R Squared = .852)

Estimates

Dependent Variable: Inhibition

Fraction	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
Per	37.076[a]	1.588	33.907	40.244
Ret	25.152[a]	1.620	21.921	28.384
Sup	32.426[a]	1.617	29.200	35.652

a. Covariates appearing in the model are evaluated at the following values: Peptide_Conc = 635.9687.

Pairwise Comparisons

Dependent Variable: Inhibition

(I) Fraction	(J) Fraction	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a]	
					Lower Bound	Upper Bound
Per	Ret	11.923*	2.273	.000	7.389	16.458
	Sup	4.650*	2.267	.044	.126	9.173
Ret	Per	-11.923*	2.273	.000	-16.458	-7.389
	Sup	-7.274*	2.288	.002	-11.838	-2.709
Sup	Per	-4.650*	2.267	.044	-9.173	-.126
	Ret	7.274*	2.288	.002	2.709	11.838

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

a. Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).

Table A10: ANCOVA and LSD test for the ACE inhibitory activity of different fractions at the hydrolysis time of 18 h

Tests of Between-Subjects Effects

Dependent Variable: Inhibition

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	29522.189[a]	3	9840.730	240.458	.000
Intercept	5665.921	1	5665.921	138.447	.000
Fraction	5374.610	2	2687.305	65.664	.000
Peptide_Conc	26577.861	1	26577.861	649.430	.000
Error	2701.044	66	40.925		
Total	147775.766	70			
Corrected Total	32223.233	69			

a. R Squared = .916 (Adjusted R Squared = .912)

Estimates

Dependent Variable: Inhibition

Fraction	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
Per	49.171[a]	1.339	46.497	51.846
Ret	28.349[a]	1.339	25.675	31.023
Sup	44.212[a]	1.306	41.605	46.819

a. Covariates appearing in the model are evaluated at the following values: Peptide_Conc = 714.4306.

Pairwise Comparisons

Dependent Variable: Inhibition

(I) Fraction	(J) Fraction	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a]	
					Lower Bound	Upper Bound
Per	Ret	20.822*	1.902	.000	17.025	24.620
	Sup	4.959*	1.871	.010	1.224	8.694
Ret	Per	-20.822*	1.902	.000	-24.620	-17.025
	Sup	-15.863*	1.871	.000	-19.598	-12.129
Sup	Per	-4.959*	1.871	.010	-8.694	-1.224
	Ret	15.863*	1.871	.000	12.129	19.598

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

a. Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).

Table A11: ANCOVA and LSD test for the ACE inhibitory activity of permeate fractions at different hydrolysis times

Tests of Between-Subjects Effects

Dependent Variable: Inhibition

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	48500.317[a]	7	6928.617	115.254	.000
Intercept	11803.508	1	11803.508	196.345	.000
Time	1504.973	6	250.829	4.172	.001
Peptide_Conc	46505.267	1	46505.267	773.591	.000
Error	6672.889	111	60.116		
Total	247174.960	119			
Corrected Total	55173.206	118			

a. R Squared = .879 (Adjusted R Squared = .871)

Estimates

Dependent Variable: Inhibition

Time	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
2	37.804[a]	1.829	34.180	41.427
4	37.841[a]	1.828	34.219	41.463
6	40.430[a]	1.938	36.589	44.271
8	46.493[a]	1.780	42.966	50.020
10	38.383[a]	2.007	34.407	42.359
12	36.242[a]	1.828	32.621	39.864
18	44.002[a]	2.004	40.031	47.973

a. Covariates appearing in the model are evaluated at the following values: Peptide_Conc = 552.8894.

Pairwise Comparisons

Dependent Variable: Inhibition

(I) Time	(J) Time	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a]	
					Lower Bound	Upper Bound
2	4	-.037	2.585	.989	-5.159	5.085
	6	-2.626	2.665	.327	-7.906	2.654
	8	-8.689*	2.550	.001	-13.743	-3.636
	10	-.580	2.718	.832	-5.965	4.806
	12	1.561	2.585	.547	-3.561	6.683
	18	-6.198*	2.715	.024	-11.579	-.818
4	2	.037	2.585	.989	-5.085	5.159
	6	-2.589	2.664	.333	-7.868	2.691
	8	-8.652*	2.550	.001	-13.706	-3.599
	10	-.543	2.716	.842	-5.925	4.840
	12	1.598	2.585	.538	-3.523	6.720
	18	-6.161*	2.714	.025	-11.539	-.784
6	2	2.626	2.665	.327	-2.654	7.906
	4	2.589	2.664	.333	-2.691	7.868
	8	-6.063*	2.631	.023	-11.278	-.849
	10	2.046	2.790	.465	-3.483	7.575
	12	4.187	2.664	.119	-1.092	9.466
	18	-3.572	2.788	.203	-9.098	1.953
8	2	8.689*	2.550	.001	3.636	13.743
	4	8.652*	2.550	.001	3.599	13.706
	6	6.063*	2.631	.023	.849	11.278
	10	8.110*	2.685	.003	2.789	13.431
	12	10.251*	2.550	.000	5.197	15.305
	18	2.491	2.682	.355	-2.824	7.806
10	2	.580	2.718	.832	-4.806	5.965
	4	.543	2.716	.842	-4.840	5.925
	6	-2.046	2.790	.465	-7.575	3.483
	8	-8.110*	2.685	.003	-13.431	-2.789
	12	2.141	2.715	.432	-3.240	7.522
	18	-5.619*	2.831	.050	-11.230	-.008
12	2	-1.561	2.585	.547	-6.683	3.561
	4	-1.598	2.585	.538	-6.720	3.523
	6	-4.187	2.664	.119	-9.466	1.092
	8	-10.251*	2.550	.000	-15.305	-5.197
	10	-2.141	2.715	.432	-7.522	3.240
	18	-7.760*	2.713	.005	-13.136	-2.383
18	2	6.198*	2.715	.024	.818	11.579
	4	6.161*	2.714	.025	.784	11.539
	6	3.572	2.788	.203	-1.953	9.098
	8	-2.491	2.682	.355	-7.806	2.824
	10	5.619*	2.831	.050	.008	11.230
	12	7.760*	2.713	.005	2.383	13.136

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

a. Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).

Table A12: ANOVA and Tukey's test for aromatic amino acid content of casein and permeate fractions at different hydrolysis times

Tests of Between-Subjects Effects

Dependent Variable: Aromatic

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	17.929[a]	7	2.561	111.725	.000
Intercept	1620.063	1	1620.063	70667.939	.000
Trt	17.929	7	2.561	111.725	.000
Error	.183	8	.023		
Total	1638.175	16			
Corrected Total	18.113	15			

a. R Squared = .990 (Adjusted R Squared = .981)

Aromatic

Tukey HSD[a,b]

Trt	N	Subset 1	2	3	4	5
1.00	2	7.9000				
2.00	2		8.8500			
6.00	2			10.0350		
12.00	2			10.3350	10.3350	
10.00	2			10.5000	10.5000	
18.00	2				10.6600	10.6600
4.00	2					11.1050
8.00	2					11.1150
Sig.		1.000	1.000	.155	.460	.168

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = .023.

a. Uses Harmonic Mean Sample Size = 2.000.

b. Alpha = .05.

Multiple Comparisons

Dependent Variable: Aromatic

Tukey HSD

(I) Trt	(J) Trt	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.00	2.00	-.9500*	.15141	.003	-1.5491	-.3509
	4.00	-3.2050*	.15141	.000	-3.8041	-2.6059
	6.00	-2.1350*	.15141	.000	-2.7341	-1.5359
	8.00	-3.2150*	.15141	.000	-3.8141	-2.6159
	10.00	-2.6000*	.15141	.000	-3.1991	-2.0009
	12.00	-2.4350*	.15141	.000	-3.0341	-1.8359
	18.00	-2.7600*	.15141	.000	-3.3591	-2.1609
2.00	1.00	.9500*	.15141	.003	.3509	1.5491
	4.00	-2.2550*	.15141	.000	-2.8541	-1.6559
	6.00	-1.1850*	.15141	.001	-1.7841	-.5859
	8.00	-2.2650*	.15141	.000	-2.8641	-1.6659
	10.00	-1.6500*	.15141	.000	-2.2491	-1.0509
	12.00	-1.4850*	.15141	.000	-2.0841	-.8859
	18.00	-1.8100*	.15141	.000	-2.4091	-1.2109
4.00	1.00	3.2050*	.15141	.000	2.6059	3.8041
	2.00	2.2550*	.15141	.000	1.6559	2.8541
	6.00	1.0700*	.15141	.002	.4709	1.6691
	8.00	-.0100	.15141	1.000	-.6091	.5891
	10.00	.6050*	.15141	.048	.0059	1.2041
	12.00	.7700*	.15141	.013	.1709	1.3691
	18.00	.4450	.15141	.183	-.1541	1.0441
6.00	1.00	2.1350*	.15141	.000	1.5359	2.7341
	2.00	1.1850*	.15141	.001	.5859	1.7841
	4.00	-1.0700*	.15141	.002	-1.6691	-.4709
	8.00	-1.0800*	.15141	.001	-1.6791	-.4809
	10.00	-.4650	.15141	.155	-1.0641	.1341
	12.00	-.3000	.15141	.542	-.8991	.2991
	18.00	-.6250*	.15141	.040	-1.2241	-.0259
8.00	1.00	3.2150*	.15141	.000	2.6159	3.8141
	2.00	2.2650*	.15141	.000	1.6659	2.8641
	4.00	.0100	.15141	1.000	-.5891	.6091
	6.00	1.0800*	.15141	.001	.4809	1.6791
	10.00	.6150*	.15141	.044	.0159	1.2141
	12.00	.7800*	.15141	.012	.1809	1.3791
	18.00	.4550	.15141	.168	-.1441	1.0541
10.00	1.00	2.6000*	.15141	.000	2.0009	3.1991
	2.00	1.6500*	.15141	.000	1.0509	2.2491
	4.00	-.6050*	.15141	.048	-1.2041	-.0059
	6.00	.4650	.15141	.155	-.1341	1.0641
	8.00	-.6150*	.15141	.044	-1.2141	-.0159
	12.00	.1650	.15141	.942	-.4341	.7641
	18.00	-.1600	.15141	.950	-.7591	.4391
12.00	1.00	2.4350*	.15141	.000	1.8359	3.0341
	2.00	1.4850*	.15141	.000	.8859	2.0841
	4.00	-.7700*	.15141	.013	-1.3691	-.1709
	6.00	.3000	.15141	.542	-.2991	.8991
	8.00	-.7800*	.15141	.012	-1.3791	-.1809
	10.00	-.1650	.15141	.942	-.7641	.4341
	18.00	-.3250	.15141	.460	-.9241	.2741
18.00	1.00	2.7600*	.15141	.000	2.1609	3.3591
	2.00	1.8100*	.15141	.000	1.2109	2.4091
	4.00	-.4450	.15141	.183	-1.0441	.1541
	6.00	.6250*	.15141	.040	.0259	1.2241
	8.00	-.4550	.15141	.168	-1.0541	.1441
	10.00	.1600	.15141	.950	-.4391	.7591
	12.00	.3250	.15141	.460	-.2741	.9241

Based on observed means.

*. The mean difference is significant at the .05 level.

Table A13: ANOVA and Tukey's test for basic amino acid content of casein and permeate fractions at different hydrolysis times

Tests of Between-Subjects Effects

Dependent Variable: Basic

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	18.540[a]	7	2.649	98.806	.000
Intercept	3128.445	1	3128.445	116705.8	.000
Trt	18.540	7	2.649	98.806	.000
Error	.214	8	.027		
Total	3147.199	16			
Corrected Total	18.755	15			

a. R Squared = .989 (Adjusted R Squared = .979)

Basic

Tukey HSD[a,b]

Trt	N	Subset 1	Subset 2	Subset 3
1.00	2	11.5850		
10.00	2		13.7150	
12.00	2		13.7850	
6.00	2		13.9400	
18.00	2		14.0450	
8.00	2		14.2650	
4.00	2			15.1300
2.00	2			15.4000
Sig.		1.000	.107	.716

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = .027.

a. Uses Harmonic Mean Sample Size = 2.000.

b. Alpha = .05.

Multiple Comparisons

Dependent Variable: Basic

Tukey HSD

(I) Trt	(J) Trt	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.00	2.00	-3.8150*	.16373	.000	-4.4629	-3.1671
	4.00	-3.5450*	.16373	.000	-4.1929	-2.8971
	6.00	-2.3550*	.16373	.000	-3.0029	-1.7071
	8.00	-2.6800*	.16373	.000	-3.3279	-2.0321
	10.00	-2.1300*	.16373	.000	-2.7779	-1.4821
	12.00	-2.2000*	.16373	.000	-2.8479	-1.5521
	18.00	-2.4600*	.16373	.000	-3.1079	-1.8121
2.00	1.00	3.8150*	.16373	.000	3.1671	4.4629
	4.00	.2700	.16373	.716	-.3779	.9179
	6.00	1.4600*	.16373	.000	.8121	2.1079
	8.00	1.1350*	.16373	.002	.4871	1.7829
	10.00	1.6850*	.16373	.000	1.0371	2.3329
	12.00	1.6150*	.16373	.000	.9671	2.2629
	18.00	1.3550*	.16373	.001	.7071	2.0029
4.00	1.00	3.5450*	.16373	.000	2.8971	4.1929
	2.00	-.2700	.16373	.716	-.9179	.3779
	6.00	1.1900*	.16373	.001	.5421	1.8379
	8.00	.8650*	.16373	.010	.2171	1.5129
	10.00	1.4150*	.16373	.000	.7671	2.0629
	12.00	1.3450*	.16373	.001	.6971	1.9929
	18.00	1.0850*	.16373	.002	.4371	1.7329
6.00	1.00	2.3550*	.16373	.000	1.7071	3.0029
	2.00	-1.4600*	.16373	.000	-2.1079	-.8121
	4.00	-1.1900*	.16373	.001	-1.8379	-.5421
	8.00	-.3250	.16373	.540	-.9729	.3229
	10.00	.2250	.16373	.847	-.4229	.8729
	12.00	.1550	.16373	.971	-.4929	.8029
	18.00	-.1050	.16373	.997	-.7529	.5429
8.00	1.00	2.6800*	.16373	.000	2.0321	3.3279
	2.00	-1.1350*	.16373	.002	-1.7829	-.4871
	4.00	-.8650*	.16373	.010	-1.5129	-.2171
	6.00	.3250	.16373	.540	-.3229	.9729
	10.00	.5500	.16373	.107	-.0979	1.1979
	12.00	.4800	.16373	.185	-.1679	1.1279
	18.00	.2200	.16373	.859	-.4279	.8679
10.00	1.00	2.1300*	.16373	.000	1.4821	2.7779
	2.00	-1.6850*	.16373	.000	-2.3329	-1.0371
	4.00	-1.4150*	.16373	.000	-2.0629	-.7671
	6.00	-.2250	.16373	.847	-.8729	.4229
	8.00	-.5500	.16373	.107	-1.1979	.0979
	12.00	-.0700	.16373	1.000	-.7179	.5779
	18.00	-.3300	.16373	.524	-.9779	.3179
12.00	1.00	2.2000*	.16373	.000	1.5521	2.8479
	2.00	-1.6150*	.16373	.000	-2.2629	-.9671
	4.00	-1.3450*	.16373	.001	-1.9929	-.6971
	6.00	-.1550	.16373	.971	-.8029	.4929
	8.00	-.4800	.16373	.185	-1.1279	.1679
	10.00	.0700	.16373	1.000	-.5779	.7179
	18.00	-.2600	.16373	.747	-.9079	.3879
18.00	1.00	2.4600*	.16373	.000	1.8121	3.1079
	2.00	-1.3550*	.16373	.001	-2.0029	-.7071
	4.00	-1.0850*	.16373	.002	-1.7329	-.4371
	6.00	.1050	.16373	.997	-.5429	.7529
	8.00	-.2200	.16373	.859	-.8679	.4279
	10.00	.3300	.16373	.524	-.3179	.9779
	12.00	.2600	.16373	.747	-.3879	.9079

Based on observed means.

*. The mean difference is significant at the .05 level.

Table A14: ANOVA and Tukey's test for cyclic amino acid content of casein and permeate fractions at different hydrolysis times

Tests of Between-Subjects Effects

Dependent Variable: Cyclic

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	5.356[a]	7	.765	42.645	.000
Intercept	1834.623	1	1834.623	102243.0	.000
Trt	5.356	7	.765	42.645	.000
Error	.144	8	.018		
Total	1840.123	16			
Corrected Total	5.500	15			

a. R Squared = .974 (Adjusted R Squared = .951)

Cyclic

Tukey HSD[a,b]

Trt	N	Subset 1	2	3	4	5
4.00	2	9.7200				
2.00	2	10.1900	10.1900			
8.00	2		10.4700	10.4700		
6.00	2		10.5900	10.5900		
12.00	2			10.8550	10.8550	
10.00	2			10.9550	10.9550	
18.00	2				11.1250	
1.00	2					11.7600
Sig.		.089	.172	.077	.524	1.000

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = .018.

a. Uses Harmonic Mean Sample Size = 2.000.

b. Alpha = .05.

Multiple Comparisons

Dependent Variable: Cyclic
Tukey HSD

(I) Trt	(J) Trt	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.00	2.00	1.5700*	.13395	.000	1.0399	2.1001
	4.00	2.0400*	.13395	.000	1.5099	2.5701
	6.00	1.1700*	.13395	.000	.6399	1.7001
	8.00	1.2900*	.13395	.000	.7599	1.8201
	10.00	.8050*	.13395	.004	.2749	1.3351
	12.00	.9050*	.13395	.002	.3749	1.4351
	18.00	.6350*	.13395	.019	.1049	1.1651
2.00	1.00	-1.5700*	.13395	.000	-2.1001	-1.0399
	4.00	.4700	.13395	.089	-.0601	1.0001
	6.00	-.4000	.13395	.172	-.9301	.1301
	8.00	-.2800	.13395	.487	-.8101	.2501
	10.00	-.7650*	.13395	.006	-1.2951	-.2349
	12.00	-.6650*	.13395	.015	-1.1951	-.1349
	18.00	-.9350*	.13395	.002	-1.4651	-.4049
4.00	1.00	-2.0400*	.13395	.000	-2.5701	-1.5099
	2.00	-.4700	.13395	.089	-1.0001	.0601
	6.00	-.8700*	.13395	.003	-1.4001	-.3399
	8.00	-.7500*	.13395	.007	-1.2801	-.2199
	10.00	-1.2350*	.13395	.000	-1.7651	-.7049
	12.00	-1.1350*	.13395	.000	-1.6651	-.6049
	18.00	-1.4050*	.13395	.000	-1.9351	-.8749
6.00	1.00	-1.1700*	.13395	.000	-1.7001	-.6399
	2.00	.4000	.13395	.172	-.1301	.9301
	4.00	.8700*	.13395	.003	.3399	1.4001
	8.00	.1200	.13395	.978	-.4101	.6501
	10.00	-.3650	.13395	.238	-.8951	.1651
	12.00	-.2650	.13395	.543	-.7951	.2651
	18.00	-.5350*	.13395	.048	-1.0651	-.0049
8.00	1.00	-1.2900*	.13395	.000	-1.8201	-.7599
	2.00	.2800	.13395	.487	-.2501	.8101
	4.00	.7500*	.13395	.007	.2199	1.2801
	6.00	-.1200	.13395	.978	-.6501	.4101
	10.00	-.4850	.13395	.077	-1.0151	.0451
	12.00	-.3850	.13395	.198	-.9151	.1451
	18.00	-.6550*	.13395	.016	-1.1851	-.1249
10.00	1.00	-.8050*	.13395	.004	-1.3351	-.2749
	2.00	.7650*	.13395	.006	.2349	1.2951
	4.00	1.2350*	.13395	.000	.7049	1.7651
	6.00	.3650	.13395	.238	-.1651	.8951
	8.00	.4850	.13395	.077	-.0451	1.0151
	12.00	.1000	.13395	.992	-.4301	.6301
	18.00	-.1700	.13395	.888	-.7001	.3601
12.00	1.00	-.9050*	.13395	.002	-1.4351	-.3749
	2.00	.6650*	.13395	.015	.1349	1.1951
	4.00	1.1350*	.13395	.000	.6049	1.6651
	6.00	.2650	.13395	.543	-.2651	.7951
	8.00	.3850	.13395	.198	-.1451	.9151
	10.00	-.1000	.13395	.992	-.6301	.4301
	18.00	-.2700	.13395	.524	-.8001	.2601
18.00	1.00	-.6350*	.13395	.019	-1.1651	-.1049
	2.00	.9350*	.13395	.002	.4049	1.4651
	4.00	1.4050*	.13395	.000	.8749	1.9351
	6.00	.5350*	.13395	.048	.0049	1.0651
	8.00	.6550*	.13395	.016	.1249	1.1851
	10.00	.1700	.13395	.888	-.3601	.7001
	12.00	.2700	.13395	.524	-.2601	.8001

Based on observed means.

*. The mean difference is significant at the .05 level.

Table A15: ANOVA and Tukey's test for aliphatic amino acid content of casein and permeate fractions at different hydrolysis times

Tests of Between-Subjects Effects

Dependent Variable: Aliphatic

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	3.043[a]	7	.435	16.770	.000
Intercept	11751.102	1	11751.102	453382.3	.000
Trt	3.043	7	.435	16.770	.000
Error	.207	8	.026		
Total	11754.352	16			
Corrected Total	3.250	15			

a. R Squared = .936 (Adjusted R Squared = .880)

Aliphatic

Tukey HSD[a,b]

Trt	N	Subset 1	2	3	4
2.00	2	26.4900			
1.00	2	26.6500			
6.00	2	26.7300	26.7300		
4.00	2	26.9450	26.9450	26.9450	
10.00	2		27.2950	27.2950	27.2950
8.00	2		27.3400	27.3400	27.3400
12.00	2			27.5700	27.5700
18.00	2				27.7850
Sig.		.210	.062	.055	.160

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = .026.

a. Uses Harmonic Mean Sample Size = 2.000.

b. Alpha = .05.

Multiple Comparisons

Dependent Variable: Aliphatic

Tukey HSD

(I) Trt	(J) Trt	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.00	2.00	.1600	.16099	.963	-.4771	.7971
	4.00	-.2950	.16099	.620	-.9321	.3421
	6.00	-.0800	.16099	.999	-.7171	.5571
	8.00	-.6900*	.16099	.033	-1.3271	-.0529
	10.00	-.6450*	.16099	.047	-1.2821	-.0079
	12.00	-.9200*	.16099	.006	-1.5571	-.2829
	18.00	-1.1350*	.16099	.002	-1.7721	-.4979
2.00	1.00	-.1600	.16099	.963	-.7971	.4771
	4.00	-.4550	.16099	.210	-1.0921	.1821
	6.00	-.2400	.16099	.794	-.8771	.3971
	8.00	-.8500*	.16099	.010	-1.4871	-.2129
	10.00	-.8050*	.16099	.014	-1.4421	-.1679
	12.00	-1.0800*	.16099	.002	-1.7171	-.4429
	18.00	-1.2950*	.16099	.001	-1.9321	-.6579
4.00	1.00	.2950	.16099	.620	-.3421	.9321
	2.00	.4550	.16099	.210	-.1821	1.0921
	6.00	.2150	.16099	.863	-.4221	.8521
	8.00	-.3950	.16099	.329	-1.0321	.2421
	10.00	-.3500	.16099	.447	-.9871	.2871
	12.00	-.6250	.16099	.055	-1.2621	.0121
	18.00	-.8400*	.16099	.011	-1.4771	-.2029
6.00	1.00	.0800	.16099	.999	-.5571	.7171
	2.00	.2400	.16099	.794	-.3971	.8771
	4.00	-.2150	.16099	.863	-.8521	.4221
	8.00	-.6100	.16099	.062	-1.2471	.0271
	10.00	-.5650	.16099	.089	-1.2021	.0721
	12.00	-.8400*	.16099	.011	-1.4771	-.2029
	18.00	-1.0550*	.16099	.003	-1.6921	-.4179
8.00	1.00	.6900*	.16099	.033	.0529	1.3271
	2.00	.8500*	.16099	.010	.2129	1.4871
	4.00	.3950	.16099	.329	-.2421	1.0321
	6.00	.6100	.16099	.062	-.0271	1.2471
	10.00	.0450	.16099	1.000	-.5921	.6821
	12.00	-.2300	.16099	.823	-.8671	.4071
	18.00	-.4450	.16099	.227	-1.0821	.1921
10.00	1.00	.6450*	.16099	.047	.0079	1.2821
	2.00	.8050*	.16099	.014	.1679	1.4421
	4.00	.3500	.16099	.447	-.2871	.9871
	6.00	.5650	.16099	.089	-.0721	1.2021
	8.00	-.0450	.16099	1.000	-.6821	.5921
	12.00	-.2750	.16099	.685	-.9121	.3621
	18.00	-.4900	.16099	.160	-1.1271	.1471
12.00	1.00	.9200*	.16099	.006	.2829	1.5571
	2.00	1.0800*	.16099	.002	.4429	1.7171
	4.00	.6250	.16099	.055	-.0121	1.2621
	6.00	.8400*	.16099	.011	.2029	1.4771
	8.00	.2300	.16099	.823	-.4071	.8671
	10.00	.2750	.16099	.685	-.3621	.9121
	18.00	-.2150	.16099	.863	-.8521	.4221
18.00	1.00	1.1350*	.16099	.002	.4979	1.7721
	2.00	1.2950*	.16099	.001	.6579	1.9321
	4.00	.8400*	.16099	.011	.2029	1.4771
	6.00	1.0550*	.16099	.003	.4179	1.6921
	8.00	.4450	.16099	.227	-.1921	1.0821
	10.00	.4900	.16099	.160	-.1471	1.1271
	12.00	.2150	.16099	.863	-.4221	.8521

Based on observed means.

*. The mean difference is significant at the .05 level.

Appendix B

Statistical Analysis results of milk fermented with probiotics

Table B1: ANOVA and Tukey's test for the viable plate count of ADA03 at different incubation time

Tests of Between-Subjects Effects

Dependent Variable: Count

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2.427E+018[a]	2	1.213E+018	11.870	.008
Intercept	3.364E+019	1	3.364E+019	329.087	.000
Time	2.427E+018	2	1.213E+018	11.870	.008
Error	6.133E+017	6	1.022E+017		
Total	3.668E+019	9			
Corrected Total	3.040E+018	8			

a. R Squared = .798 (Adjusted R Squared = .731)

Count

Tukey B[a,b]

Time	N	Subset 1	Subset 2
48	3	1E+009	
18	3		2E+009
24	3		3E+009

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = 102222222222222200.000.

a. Uses Harmonic Mean Sample Size = 3.000.

b. Alpha = .05.

Table B2: ANOVA and Tukey's test for the viable plate count of ADA05 at different incubation time

Tests of Between-Subjects Effects

Dependent Variable: Count

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2.167E+016[a]	2	1.083E+016	.051	.951
Intercept	2.484E+019	1	2.484E+019	117.355	.000
Time	2.167E+016	2	1.083E+016	.051	.951
Error	1.058E+018	5	2.117E+017		
Total	2.700E+019	8			
Corrected Total	1.080E+018	7			

a. R Squared = .020 (Adjusted R Squared = -.372)

Count

Tukey HSD[a,b,c]

Time	N	Subset 1
18	2	2E+009
48	3	2E+009
24	3	2E+009
Sig.		.956

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = 211666666666666600.000.

a. Uses Harmonic Mean Sample Size = 2.571.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

c. Alpha = .05.

Table B3: ANOVA and Tukey's test for the viable plate count of MR100 at different incubation time

Tests of Between-Subjects Effects

Dependent Variable: Count

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2.342E+016[a]	2	1.171E+016	1.956	.236
Intercept	4.456E+018	1	4.456E+018	744.283	.000
Time	2.342E+016	2	1.171E+016	1.956	.236
Error	2.993E+016	5	5.987E+015		
Total	4.766E+018	8			
Corrected Total	5.335E+016	7			

a. R Squared = .439 (Adjusted R Squared = .214)

Count

Tukey HSD[a,b,c]

Time	N	Subset 1
18	2	7E+008
24	3	7E+008
48	3	8E+008
Sig.		.219

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = 5986666666666660.000.

a. Uses Harmonic Mean Sample Size = 2.571.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

c. Alpha = .05.

Table B4: ANOVA and Tukey's test for the viable plate count of MR110 at different incubation time

Tests of Between-Subjects Effects

Dependent Variable: Count

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	3.427E+017[a]	2	1.713E+017	50.000	.000
Intercept	6.816E+018	1	6.816E+018	1989.205	.000
Time	3.427E+017	2	1.713E+017	50.000	.000
Error	1.713E+016	5	3.427E+015		
Total	7.131E+018	8			
Corrected Total	3.598E+017	7			

a. R Squared = .952 (Adjusted R Squared = .933)

Count

Tukey HSD[a,b,c]

Time	N	Subset 1	Subset 2
48	3	7E+008	
24	3		1E+009
18	2		1E+009
Sig.		1.000	.803

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = 3426666666666666.000.

a. Uses Harmonic Mean Sample Size = 2.571.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

c. Alpha = .05.

Table B5: ANOVA and Tukey's test for the viable plate count of probiotics at 18 h

Tests of Between-Subjects Effects

Dependent Variable: Count

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2.457E+018[a]	3	8.191E+017	32.763	.001
Intercept	1.680E+019	1	1.680E+019	672.055	.000
Bacteria	2.457E+018	3	8.191E+017	32.763	.001
Error	1.250E+017	5	2.500E+016		
Total	2.165E+019	9			
Corrected Total	2.582E+018	8			

a. R Squared = .952 (Adjusted R Squared = .923)

Count

Tukey B[a,b,c]

Bacteria	N	Subset 1	Subset 2
100	2	7E+008	
110	2	1E+009	
005	2		2E+009
003	3		2E+009

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = 25000000000000000.000.

a. Uses Harmonic Mean Sample Size = 2.182.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

c. Alpha = .05.

Table B6: ANOVA and Tukey's test for the viable plate count of probiotics at 24 h

Tests of Between-Subjects Effects

Dependent Variable: Count

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	5.838E+018[a]	3	1.946E+018	18.345	.001
Intercept	2.895E+019	1	2.895E+019	272.938	.000
Bacteria	5.838E+018	3	1.946E+018	18.345	.001
Error	8.487E+017	8	1.061E+017		
Total	3.564E+019	12			
Corrected Total	6.687E+018	11			

a. R Squared = .873 (Adjusted R Squared = .825)

Count

Tukey HSD[a,b]

Bacteria	N	Subset 1	Subset 2	Subset 3
100	3	7E+008		
110	3	1E+009	1E+009	
005	3		2E+009	2E+009
003	3			3E+009
Sig.		.642	.066	.133

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = 106083333333333300.000.

a. Uses Harmonic Mean Sample Size = 3.000.

b. Alpha = .05.

Table B7: ANOVA and Tukey's test for the viable plate count of probiotics at 48 h

Tests of Between-Subjects Effects

Dependent Variable: Count

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2.218E+018[a]	3	7.392E+017	7.937	.009
Intercept	1.532E+019	1	1.532E+019	164.525	.000
Bacteria	2.218E+018	3	7.392E+017	7.937	.009
Error	7.451E+017	8	9.313E+016		
Total	1.829E+019	12			
Corrected Total	2.963E+018	11			

a. R Squared = .749 (Adjusted R Squared = .654)

Count

Tukey B[a,b]

Bacteria	N	Subset 1	Subset 2
110	3	7E+008	
100	3	8E+008	
003	3	1E+009	1E+009
005	3		2E+009

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = 93133333333333300.000.

a. Uses Harmonic Mean Sample Size = 3.000.

b. Alpha = .05.

Table B8: ANOVA and Tukey's test for the pH of ADA03 at different incubation time

Tests of Between-Subjects Effects

Dependent Variable: pH

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.851[a]	2	.426	51.218	.000
Intercept	145.042	1	145.042	17451.563	.000
Time	.851	2	.426	51.218	.000
Error	.050	6	.008		
Total	145.943	9			
Corrected Total	.901	8			

a. R Squared = .945 (Adjusted R Squared = .926)

pH

Tukey B[a,b]

Time	N	Subset 1	Subset 2	Subset 3
48	3	3.6600		
24	3		3.9733	
18	3			4.4100

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = .008.

a. Uses Harmonic Mean Sample Size = 3.000.

b. Alpha = .05.

Table B9: ANOVA and Tukey's test for the pH of ADA05 at different incubation time

Tests of Between-Subjects Effects

Dependent Variable: pH

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1.508[a]	2	.754	87.337	.000
Intercept	157.670	1	157.670	18262.920	.000
Time	1.508	2	.754	87.337	.000
Error	.052	6	.009		
Total	159.230	9			
Corrected Total	1.560	8			

a. R Squared = .967 (Adjusted R Squared = .956)

pH

Tukey HSD[a,b]

Time	N	Subset 1	Subset 2	Subset 3
48	3	3.7067		
24	3		4.1433	
18	3			4.7067
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = .009.

a. Uses Harmonic Mean Sample Size = 3.000.

b. Alpha = .05.

Table B10: ANOVA and Tukey's test for the pH of MR100 at different incubation time

Tests of Between-Subjects Effects

Dependent Variable: pH

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.124[a]	2	.062	1120.200	.000
Intercept	134.328	1	134.328	2417906	.000
Time	.124	2	.062	1120.200	.000
Error	.000	6	5.56E-005		
Total	134.453	9			
Corrected Total	.125	8			

a. R Squared = .997 (Adjusted R Squared = .996)

pH

Tukey HSD[a,b]

Time	N	Subset 1	Subset 2	Subset 3
48	3	3.7067		
24	3		3.8933	
18	3			3.9900
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = 5.56E-005.

a. Uses Harmonic Mean Sample Size = 3.000.

b. Alpha = .05.

Table B11: ANOVA and Tukey's test for the pH of MR110 at different incubation time

Tests of Between-Subjects Effects

Dependent Variable: pH

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.064[a]	2	.032	2869.000	.000
Intercept	113.068	1	113.068	1E+007	.000
Time	.064	2	.032	2869.000	.000
Error	6.67E-005	6	1.11E-005		
Total	113.132	9			
Corrected Total	.064	8			

a. R Squared = .999 (Adjusted R Squared = .999)

pH

Tukey HSD[a,b]

Time	N	Subset 1	Subset 2	Subset 3
48	3	3.4300		
24	3		3.5733	
18	3			3.6300
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = 1.11E-005.

a. Uses Harmonic Mean Sample Size = 3.000.

b. Alpha = .05.

Table B12: ANOVA and Tukey's test for the pH of probiotics at 18 h

Tests of Between-Subjects Effects

Dependent Variable: pH

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2.006[a]	3	.669	231.957	.000
Intercept	210.087	1	210.087	72862.546	.000
Bacteria	2.006	3	.669	231.957	.000
Error	.023	8	.003		
Total	212.117	12			
Corrected Total	2.029	11			

a. R Squared = .989 (Adjusted R Squared = .984)

pH

Tukey B[a,b]

Bacteria	N	Subset			
		1	2	3	4
110	3	3.6300			
100	3		3.9900		
003	3			4.4100	
005	3				4.7067

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = .003.

a. Uses Harmonic Mean Sample Size = 3.000.

b. Alpha = .05.

Table B13: ANOVA and Tukey's test for the pH of probiotics at 24 h

Tests of Between-Subjects Effects

Dependent Variable: pH

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.514[a]	3	.171	20.309	.000
Intercept	182.130	1	182.130	21596.467	.000
Bacteria	.514	3	.171	20.309	.000
Error	.067	8	.008		
Total	182.712	12			
Corrected Total	.581	11			

a. R Squared = .884 (Adjusted R Squared = .840)

pH

Tukey HSD[a,b]

Bacteria	N	Subset 1	Subset 2	Subset 3
110	3	3.5733		
100	3		3.8933	
003	3		3.9733	3.9733
005	3			4.1433
Sig.		1.000	.718	.185

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = .008.

a. Uses Harmonic Mean Sample Size = 3.000.

b. Alpha = .05.

Table B14: ANOVA and Tukey's test for the pH of probiotics at 48 h

Tests of Between-Subjects Effects

Dependent Variable: pH

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.158[a]	3	.053	36.476	.000
Intercept	157.760	1	157.760	109428.9	.000
Bacteria	.158	3	.053	36.476	.000
Error	.012	8	.001		
Total	157.929	12			
Corrected Total	.169	11			

a. R Squared = .932 (Adjusted R Squared = .906)

pH

Tukey B[a,b]

Bacteria	N	Subset 1	Subset 2
110	3	3.4300	
003	3		3.6600
005	3		3.7067
100	3		3.7067

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = .001.

a. Uses Harmonic Mean Sample Size = 3.000.

b. Alpha = .05.

Table B15: ANOVA and Tukey's test for the titrable acidity of ADA03 at different incubation time

Tests of Between-Subjects Effects

Dependent Variable: Acidity_wv

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1.005[a]	2	.502	42.027	.000
Intercept	15.682	1	15.682	1312.048	.000
Time	1.005	2	.502	42.027	.000
Error	.072	6	.012		
Total	16.758	9			
Corrected Total	1.076	8			

a. R Squared = .933 (Adjusted R Squared = .911)

Acidity_wv

Tukey HSD[a,b]

Time	N	Subset 1	Subset 2	Subset 3
18	3	.9300		
24	3		1.2840	
48	3			1.7460
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = .012.

a. Uses Harmonic Mean Sample Size = 3.000.

b. Alpha = .05.

Table B16: ANOVA and Tukey's test for the titrable acidity of ADA05 at different incubation time

Tests of Between-Subjects Effects

Dependent Variable: Acidity_wv

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.891[a]	2	.445	26.831	.001
Intercept	11.209	1	11.209	675.410	.000
Time	.891	2	.445	26.831	.001
Error	.100	6	.017		
Total	12.199	9			
Corrected Total	.990	8			

a. R Squared = .899 (Adjusted R Squared = .866)

Acidity_wv

Tukey HSD[a,b]

Time	N	Subset 1	Subset 2	Subset 3
18	3	.7500		
24	3		1.0800	
48	3			1.5180
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = .017.

a. Uses Harmonic Mean Sample Size = 3.000.

b. Alpha = .05.

Table B17: ANOVA and Tukey's test for the titrable acidity of MR100 at different incubation time

Tests of Between-Subjects Effects

Dependent Variable: Acidity_wv

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.152[a]	2	.076	15.158	.005
Intercept	16.794	1	16.794	3356.036	.000
Time	.152	2	.076	15.158	.005
Error	.030	6	.005		
Total	16.975	9			
Corrected Total	.182	8			

a. R Squared = .835 (Adjusted R Squared = .780)

Acidity_wv

Tukey HSD[a,b]

Time	N	Subset 1	Subset 2
18	3	1.2060	
24	3	1.3680	1.3680
48	3		1.5240
Sig.		.069	.079

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = .005.

a. Uses Harmonic Mean Sample Size = 3.000.

b. Alpha = .05.

Table B18: ANOVA and Tukey's test for the titrable acidity of MR110 at different incubation time

Tests of Between-Subjects Effects

Dependent Variable: Acidity

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.001[a]	2	.000	9.484	.014
Intercept	.382	1	.382	7920.083	.000
Time	.001	2	.000	9.484	.014
Error	.000	6	4.82E-005		
Total	.383	9			
Corrected Total	.001	8			

a. R Squared = .760 (Adjusted R Squared = .680)

Acidity

Tukey HSD[a,b]

Time	N	Subset 1	Subset 2
18	3	.19333	
24	3	.20667	.20667
48	3		.21800
Sig.		.123	.193

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = 4.82E-005.

a. Uses Harmonic Mean Sample Size = 3.000.

b. Alpha = .05.

Table B19: ANOVA and Tukey's test for the titrable acidity of probiotics at 18 h

Tests of Between-Subjects Effects

Dependent Variable: Acidity_wv

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2.107[a]	3	.702	1300.467	.000
Intercept	16.851	1	16.851	31205.000	.000
Bacteria	2.107	3	.702	1300.467	.000
Error	.004	8	.001		
Total	18.962	12			
Corrected Total	2.111	11			

a. R Squared = .998 (Adjusted R Squared = .997)

Acidity_wv

Tukey B[a,b]

Bacteria	N	Subset 1	Subset 2	Subset 3	Subset 4
005	3	.7500			
003	3		.9300		
100	3			1.2060	
110	3				1.8540

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = .001.

a. Uses Harmonic Mean Sample Size = 3.000.

b. Alpha = .05.

Table B20: ANOVA and Tukey's test for the titrable acidity of probiotics at 24 h

Tests of Between-Subjects Effects

Dependent Variable: Acidity_wv

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1.391[a]	3	.464	55.198	.000
Intercept	24.573	1	24.573	2926.418	.000
Bacteria	1.391	3	.464	55.198	.000
Error	.067	8	.008		
Total	26.031	12			
Corrected Total	1.458	11			

a. R Squared = .954 (Adjusted R Squared = .937)

Acidity_wv

Tukey HSD[a,b]

Bacteria	N	Subset 1	Subset 2	Subset 3
005	3	1.0800		
003	3	1.2840	1.2840	
100	3		1.3680	
110	3			1.9920
Sig.		.098	.687	1.000

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = .008.

a. Uses Harmonic Mean Sample Size = 3.000.

b. Alpha = .05.

Table B21: ANOVA and Tukey's test for the titrable acidity of probiotics at 48 h

Tests of Between-Subjects Effects

Dependent Variable: Acidity_wv

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.673[a]	3	.224	11.489	.003
Intercept	35.583	1	35.583	1822.827	.000
Bacteria	.673	3	.224	11.489	.003
Error	.156	8	.020		
Total	36.412	12			
Corrected Total	.829	11			

a. R Squared = .812 (Adjusted R Squared = .741)

Acidity_wv

Tukey B[a,b]

Bacteria	N	Subset 1	Subset 2
005	3	1.5180	
100	3	1.5240	
003	3	1.7460	
110	3		2.1000

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = .020.

a. Uses Harmonic Mean Sample Size = 3.000.

b. Alpha = .05.

Table B22: ANOVA and Tukey's test for the proteolytic activity of ADA03 at different incubation time

Tests of Between-Subjects Effects

Dependent Variable: Dff_SerineNH2

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.091[a]	2	.046	29.875	.004
Intercept	.355	1	.355	232.383	.000
time	.091	2	.046	29.875	.004
Error	.006	4	.002		
Total	.540	7			
Corrected Total	.097	6			

a. R Squared = .937 (Adjusted R Squared = .906)

Dff_SerineNH2

Tukey HSD[a,b,c]

time	N	Subset 1	Subset 2
18	2	.1500	
48	2	.1550	
24	3		.3833
Sig.		.990	1.000

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = .002.

a. Uses Harmonic Mean Sample Size = 2.250.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

c. Alpha = .05.

Table B23: ANOVA and Tukey's test for the proteolytic activity of ADA05 at different incubation time

Tests of Between-Subjects Effects

Dependent Variable: Dff_SerineNH2

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.054[a]	2	.027	63.176	.001
Intercept	.814	1	.814	1896.238	.000
time	.054	2	.027	63.176	.001
Error	.002	4	.000		
Total	.879	7			
Corrected Total	.056	6			

a. R Squared = .969 (Adjusted R Squared = .954)

Dff_SerineNH2

Tukey B[a,b,c]

time	N	Subset 1	Subset 2	Subset 3
48	2	.2500		
24	3		.3167	
18	2			.4750

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = .000.

a. Uses Harmonic Mean Sample Size = 2.250.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

c. Alpha = .05.

Table B24: ANOVA for the proteolytic activity of MR100 at different incubation time

Tests of Between-Subjects Effects

Dependent Variable: Dff_SerineNH2

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.012[a]	2	.006	1.929	.239
Intercept	3.321	1	3.321	1070.094	.000
time	.012	2	.006	1.929	.239
Error	.016	5	.003		
Total	3.395	8			
Corrected Total	.027	7			

a. R Squared = .436 (Adjusted R Squared = .210)

Table B25: ANOVA for the proteolytic activity of MR110 at different incubation time

Tests of Between-Subjects Effects

Dependent Variable: Dff_SerineNH2

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.004[a]	2	.002	.536	.615
Intercept	4.371	1	4.371	1276.968	.000
time	.004	2	.002	.536	.615
Error	.017	5	.003		
Total	4.596	8			
Corrected Total	.021	7			

a. R Squared = .177 (Adjusted R Squared = -.153)

Table B26: ANOVA and Tukey's test for the proteolytic activity of probiotics at 18 h

Tests of Between-Subjects Effects

Dependent Variable: Dff_SerineNH2

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.511[a]	3	.170	62.608	.000
Intercept	2.493	1	2.493	916.688	.000
Probiotic	.511	3	.170	62.608	.000
Error	.016	6	.003		
Total	3.541	10			
Corrected Total	.527	9			

a. R Squared = .969 (Adjusted R Squared = .954)

Dff_SerineNH2

Tukey B[a,b,c]

Probiotic	N	Subset 1	2	3	4
003	2	.1500			
005	2		.4750		
100	3			.6333	
110	3				.7800

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = .003.

a. Uses Harmonic Mean Sample Size = 2.400.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

c. Alpha = .05.

Table B27: ANOVA and Tukey's test for the proteolytic activity of probiotics at 24 h

Tests of Between-Subjects Effects

Dependent Variable: Dff_SerineNH2

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.373[a]	3	.124	77.785	.000
Intercept	3.224	1	3.224	2015.021	.000
Probiotic	.373	3	.124	77.785	.000
Error	.013	8	.002		
Total	3.610	12			
Corrected Total	.386	11			

a. R Squared = .967 (Adjusted R Squared = .954)

Dff_SerineNH2

Tukey B[a,b]

Probiotic	N	Subset 1	Subset 2	Subset 3
005	3	.3167		
003	3	.3833		
100	3		.6200	
110	3			.7533

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = .002.

a. Uses Harmonic Mean Sample Size = 3.000.

b. Alpha = .05.

Table B28: ANOVA and Tukey's test for the proteolytic activity of probiotics at 48 h

Tests of Between-Subjects Effects

Dependent Variable: Dff_SerineNH2

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.545[a]	3	.182	63.993	.001
Intercept	1.702	1	1.702	599.828	.000
Probiotic	.545	3	.182	63.993	.001
Error	.011	4	.003		
Total	2.258	8			
Corrected Total	.556	7			

a. R Squared = .980 (Adjusted R Squared = .964)

Dff_SerineNH2

Tukey B[a,b]

Probiotic	N	Subset 1	Subset 2
003	2	.1550	
005	2	.2500	
100	2		.7150
110	2		.7250

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = .003.

a. Uses Harmonic Mean Sample Size = 2.000.

b. Alpha = .05.