PRODUCTION AND CHARACTERIZATION OF ANGIOTENSIN I-CONVERTINE ENZYME INHIBITORY PEPTIDES FROM WHEY FERMENTATION WITH LACTIC ACID BACTERIA

By

Jae-Eun Ahn

Department of Food Science and Agricultural Chemistry
Macdonald Campus of McGill University
Montreal, Quebec, Canada

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PRODUCTION OF ACE INHIBITORY PEPTIDES FROM FERMENTED WHEY

Dedication

To my parents Sa-Sup Ahn and Jeong-Ja Kang.

ABSTRACT

Whey media, containing 2% (w/v) whey powder, 1% (w/v) glucose, and 0.5% (w/v) yeast extract, were fermented with nine *Lactobacillus* strains to produce angiotensin I-converting enzyme (ACE) inhibitory peptides. *Lb. brevis*, *Lb. helveticus*, and *Lb. paracasei* were most effective in producing whey hydrolysates that contained potent ACE inhibitors, with the inhibition rate ranging from 93.3 ± 0.3 to 100%. The hydrolysates of three *Lactobacillus* strains were partially purified by dialysis (6,000-8,000 Da cut-off) to remove larger molecules, and subsequently subjected to RP-HPLC, equipped with a Delta Pak C_{18} column. Each chromatogram displayed at least three distinct peaks at the hydrophobic region of the elution profile. Altogether fourteen peaks were purified and assayed for ACE inhibitory activity. All peaks except one exhibited ACE inhibitory activities, with IC_{50} ranging from 5.3 ± 0.1 to 2637.8 ± 366.9μg/ml. Three of these peaks contained pentapeptides, which consisted of mostly hydrophobic or aromatic amino acids at the C-terminal.

RÉSUMÉ

Le milieu, composé à 2% (p/v) de poudre de lactosérum, 1% (p/v) de glucose, et 0,5% (p/v) d'extrait de levure a été fermenté avec neuf souches de *Lactobacillus* pour produire des peptides inhibiteurs de l'enzyme de conversion de l'angiotensine-I (ACE). *Lb. brevis, Lb. helveticus, et Lb. paracasei* ont été les plus efficaces pour produire l'hydrolysat de lactosérum contenant l'inhibiteur de l'ACE actif, à un taux d'inhibition variant entre 93,3 ± 0.3 et 100%. Les hydrolysats de trois souches de *Lactobacillus* ont été partiellement purifiés pour séparer les molécules de 6 000 à 8 000 Da de celles qui sont plus grandes, suivi de l'analyse sur RP-HPLC équipé d'une colonne Delta-Pak C_{18}. Chaque chromatogramme a démontré au moins trois pics distincts dans la région hydrophobique. Dans l'ensemble, quatorze pics ont été purifiés et vérifiés pour de l'activité d'inhibition de l'ACE; tous sauf un ont démontré une activité d'inhibition de l'ACE (IC_{50} = 5,3 ± 0,1 à 2637,8 ± 366,9 µg/ml). Trois de ces pics contenaient des pentapeptides composés surtout des acides aminés hydrophobiques ou aromatiques au C-terminal.

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LIST OF ABBREVIATIONS

ACE	Angiotensin I-converting enzyme
BW	Body weight
CFU	Colony forming unit
DBP	Diastolic blood pressure
DW	Distilled water
FD	Freeze-dried
HPLC	High performance liquid chromatography
KKS	Kallikrein kinin system
LAB	Lactic acid bacteria
ODS	Octadecylsilica
OPA	*o*-phthaldialdehyde
RAS	Renin-angiotensin system
RP-HPLC	Reversed-phase high performance liquid chromatography
SBP	Systolic blood pressure
SHR	Spontaneously hypertensive rats
TFA	Trifluoroacetic acid
WP	Whey powder
YE	Yeast extract

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FOREWORD

This thesis is submitted in the form of original papers suitable for journal publication. The first chapter is a general introduction and literature review presenting the theory and background information on this topic. The next two chapters represent the body of the thesis, each being a complete manuscript. The last section is a summary of the major conclusions. The format of this thesis has been approved by the Faculty of Graduate Studies and Research at McGill University, and follows the conditions outlined in the Guidelines concerning Thesis Presentation, which are as follows:

"Candidates have the option of including, as part of the thesis, the text of a paper(s) submitted or to be submitted for publication, or the clearly-duplicated text of a published paper(s). These texts must be bound as an integral part of the thesis.

If this option is chosen, **connecting texts that provide logical bridges between the different papers are mandatory**. The thesis must still conform to all other requirements of the "Guidelines for Thesis Preparation". **The thesis must include**: A Table of Contents, an abstract in English and French, an introduction which clearly states the rationale and objectives of the study, a comprehensive review of the literature, a final conclusion and summary, and a thorough bibliography or reference list.

Additional material must be provided where appropriate (e.g. in appendices) and in sufficient detail to allow a clear and precise judgment to be made of the importance and originality of the research reported in the thesis.

CONTRIBUTION OF CO-AUTHOR TO MANUSCRIPTS FOR PUBLICATIONS

Dr. Byong H. Lee, my supervisor, is co-author on all publications presented in this thesis and contributed in a supervisory role. Dr. Lee fully reviewed the manuscripts. This project was supported the grant awarded to Dr. Lee by Dairy Farmers of Canada.

Dr. Marcos DiFalco carried out the amino acid sequencing of the bioactive peptides on Edman micro-sequencer (492 Procise®).

A. Part of this work has been prepared for publications

Ahn, Jae-Eun and Lee, Byong H. (2001). Angiotensin I-converting enzyme inhibitory peptides derived from food proteins. *CRC Rev. Food Sci. Technol.*

B. Part of this work has been presented at a scientific meeting

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GENERAL INTRODUCTION

According to the definition of 'Hypertension' by the World Health Organization (WHO), 25% of the population in industrialized countries suffer from hypertension, which contributes to various diseases of the circulatory system, including stroke, dementia associated with stroke, heart attack, arteriosclerosis, as well as other cardiovascular diseases (Sugai, 1998). For years physicians have been prescribing angiotensin I-converting enzyme (ACE) inhibitors, one of the many types of synthetic antihypertensive drugs that are available on the market, to the hypertensive patients.

However, due to the growth of health conscious consumers demanding natural products rather than chemically synthesized ones, nutraceuticals and functional foods, have been gaining popularity since the mid 90s. In fact, the market for nutraceuticals and functional foods is growing quickly worldwide; it is estimated that $250 billion may be attributable to the use of this type of product if taken at its broadest definition, and demand from the larger European population would bring that figure up to $500 billion (Anonymous, 1994).

Even before the terms "nutraceuticals" and "functional foods" were coined, ACE inhibitors derived from food proteins have been studied continuously since the late 70s, following the discovery of ACE inhibitory peptides from snake venom. Some of the food sources that have been under investigation include bovine casein (Maruyama and Suzuki, 1982; Maruyama et al., 1985, 1987a,b; Karaki et al., 1990; Nakamura et al., 1995a,b;

Pihlanto-Leppälä, 1998; Sugai, 1998), zein (Maruyama et al., 1989; Miyoshi et al., 1991a,b), gelatin (Oshima et al., 1979), soy sauce (Kinoshita et al., 1993), and fish products, such as tuna muscle (Kohama et al., 1988, 1991; Yokoyama et al., 1992) and bonito (Matsumura et al., 1993a,b). Among these, bovine caseins have been most extensively studied for the isolation of ACE inhibitory peptides from enzymatic hydrolysis of the proteins or from fermentation with lactic acid bacteria. As a matter of fact, the two food-derived peptides with the highest ACE inhibitory activity reported so far were isolated from fermenting bovine caseins with a starter culture containing two strains of lactic acid bacteria (Nakamura et al., 1995a).

In contrast to caseins, ACE inhibitory peptides from whey proteins are less studied and characterized (Chiba and Yoshikawa, 1991; Mullally et al., 1996, 1997a,b; Pihlanto-Leppälä, 1998). Whey, which is the serum liquid portion remaining after removal of fat and casein from milk during manufacture of cheeses and caseins, was traditionally regarded as an undesirable by-product in the dairy industry. However, due to the recognition that whey is a potentially valuable source of nutrients, such as proteins, lactose, and minerals, it is gradually transforming to a highly functional and nutritious co-product. Whey proteins represent about 20% of total milk proteins. This, along with its lactose content of 40-50 gL^{-1}, makes whey cheap and readily available source for use as fermentation media. However, fermenting whey has not been successful in isolating ACE inhibitory peptides with high activity even though some studies have been done (Pihlanto-Leppälä et al., 1998; Belem et al, 1999; Haileselassie et al., 1999)

The objectives of this study were:

i) to optimize the growth conditions of lactic acid bacteria for the production of potential ACE inhibitory peptides from whey fermentation

ii) to determine if whey hydrolysates produced from the fermentation possess ACE inhibitory activity and select the strains that produce the most potent ACE inhibitory peptides

iii) to purify and identify the potential ACE inhibitory peptides produced from the selected lactic acid bacteria

CHAPTER 1

LITERATURE REVIEW

ANGIOTENSIN I-CONVERTING ENZYME INHIBITORY PEPTIDES DERIVED FROM FOOD PROTEINS

This chapter was summarized as a publication entitled "Angiotensin I-converting enzyme inhibitory peptides derived from food proteins", which was co-authored by Jae-Eun Ahn and Byong H. Lee. This paper was written by Jae-Eun Ahn and supervised by Dr. Byong H. Lee, who acted in an editorial capacity, evaluating the manuscript. This chapter serves as an introduction to the thesis, showing the background for the research that was done and the significance of this project. One manuscript will be submitted to *CRC Rev. Food Sci. Technol.*

1.1 ABSTRACT

Angiotensin I-converting enzyme (ACE) inhibitors have been developed as antihypertensive agents. The first competitive ACE inhibitors were discovered from naturally occurring peptides in snake venom, and subsequently the first commercial ACE inhibitor was developed as an analogue to one of the snake venom derived ACE inhibitory peptides. For the past two decades, a number of ACE inhibitory peptides have also been isolated from various food proteins. Among these, bovine milk proteins, especially caseins, have been most extensively studied for the production of ACE inhibitors by enzymatic digestion as well as fermentation with lactic acid bacteria (LAB). Some of the peptides were active *in vitro* as well as *in vivo*. This chapter reviews the definition of ACE, origin of ACE inhibitors, and some of the properties of food-derived ACE inhibitors.

Key words: ACE inhibitors, antihypertensive agents, enzymatic digestion, fermentation, lactic acid bacteria

1.2 INTRODUCTION

Angiotensin I-converting enzyme (ACE, EC 3.4.15.1) is a zinc metallopeptidase that acts mainly as a dipeptidyl carboxypeptidase. It is an acidic glycoprotein composed of a single, large polypeptide chain and 1 mol/mol zinc (Soffer, 1976, 1981; Ondetti and Cushman, 1982; Ehlers and Riordan, 1989) and present as a membrane-bound form in endothelial cells, in epithelial or neuroepithelial cells, and in the brain, and as a soluble form in blood and numerous body fluids (Skidgel and Erdös, 1993). In most mammalian species, the predominant form, i.e. a somatic form, with a molecular weight of 140-160 kDa occurs in most tissues, although in humans primarily in kidney and lung. A lower molecular weight form (90-110 kDa) is found exclusively in germinal cells (El-Dorry et al., 1982; Lanzillo et al., 1985). As shown in Figure 1.1, ACE has been classically associated with the renin-angiotensin system (RAS) and kallikrein kinin system (KKS), both of which play important roles in peripheral blood pressure regulation in addition to water and salt metabolism (Corvol et al., 1995; Sugai, 1998).

In RAS, reduced sodium delivery at the macula densa, decreased renal perfusion pressure, and sympathetic activation all stimulate renin secretion by the juxtaglomerular cell (Sealey and Laragh, 1990). Subsequently, renin cleaves the inactive decapeptide angiotensin I from the prohormone angiotensinogen, a non-inhibiting member of the serpin superfamily of serine protease inhibitors (Doolittle, 1983). Finally, ACE raises blood pressure by cleaving the C-terminal His-Leu from angiotensin I and produces angiotensin II (an octapeptide), which is a potent vasoconstrictor that directly acts on

vascular smooth muscle cells (Folkow et al., 1961). On the other hand, in KKS, ACE acts as kininase II and catalyzes the degradation of the nonapeptide bradykinin by successively removing two C-terminal dipeptides (Yang and Erdös, 1967; Yang et al., 1970). Bradykinin promotes vasodilation by stimulating the production of arachidonic acid metabolites, nitric oxide, and endothelium-derived hyperpolarizing a factor in vascular endothelium (Vanhoutte, 1989). Consequently, inhibition of ACE can lower blood pressure (Meisel, 1993) by reducing the vasoconstricting activity of angiotensin II and by augmenting the vasodilating activity of bradykinin (Sugai, 1998).

1.3 ACE INHIBITORS

1.3.1 ACE inhibitors derived from snake venom

The first competitive inhibitors to ACE were reported from naturally occurring peptides in venom of a Brazillian snake, *Bothrops jararaca* (Ferreira et al., 1970; Ondetti et al., 1971). Ferreira et al. (1970) isolated nine peptides from alcoholic extracts of the snake venom that were originally reported to contain bradykinin-potentiating factor (Ferreira, 1965) and subsequently shown to inhibit the activity of ACE in lung extracts (Bakhle, 1968), perfused lung (Bakhle et al., 1969), and intact lung *in vivo* (Ng and Vane, 1970). The peptides ranged in size from pentapeptide to tridecapeptide with a high proline content as well as presence of glutamic acid, arginine, and tryptophan in most of the peptides. Among them, only the sequence of pentapeptide was determined (Table 1.1) (Ferreira et al., 1970). Ondetti et al. (1971) isolated and characterized six similar

peptides with inhibitory activity against ACE from the venom of *Bothrops jararaca* (Table 1.1). These peptides contained nine to thirteen amino acids per molecule and had a sequence of pro-pro at the C-terminal in addition to high proline content within the peptides. Similar features were also observed from three of five undecapeptides with ACE inhibitory isolated from the venom of a Japanese snake, *Agkistrodon halys blomhoffii* (Table 1.1) (Kato and Suzuki, 1969, 1970, 1971). Several investigators reported that C-terminal amino acids of inhibitory peptides are important to bind with the active site of ACE, and favourable amino acids are the aromatic (Trp, Tyr, and Phe) and imino acid (Pro) (Cheung et al., 1980; Saito et al., 1994b).

1.3.2 Commercial ACE inhibitors as antihypertensive drugs

Currently, ACE inhibitors are widely used for the treatment of hypertensive patients. These agents inhibit the generation of angiotensin II and thereby make it possible to chronically block the renin-angiotensin system. The first ACE inhibitor to be used in humans was teprotide (Ondetti et al., 1971), a nonapeptide that is effective only when administered parenterally. The first orally active ACE inhibitor was captopril (D-3-mercapto-2-methyl-propanoyl-L-proline) (Cushman et al., 1977), which is analogous to C-terminal dipeptide of ACE inhibitory peptides derived from snake venoms. Captopril contains a sulfhydryl group that strongly binds to the zinc ion on the active site of ACE (Figure 1.2) (Cushman et al., 1977). Unlike captopril, fosinopril contains phosphinyl group as its reactive moiety, but the majority of other ACE inhibitors contain a carboxyl moiety as shown in Table 1.2 (Brown and Vaughan, 1998). Most commercial ACE

inhibitors are administered as an ester that is metabolized in the liver to form the active drug (Brunner et al., 1985; Kelly and O'Malley, 1990). Given alone or in combination with other antihypertensive drugs, these compounds are very effective in lowering blood pressure of most hypertensive patients. However, administration of these ACE inhibitors requires a careful monitoring since it may cause a number of side effects, including hypotension, hyperkalemia, renal impairment, dry cough, angioneurotic edema, taste disturbance, and rash (Waeber et al., 1995).

1.4 ACE INHIBITORS DERIVED FROM FOOD PROTEINS

Following the discovery of ACE inhibitory peptides in snake venom, numerous trials have demonstrated the presence of ACE inhibitory substances in various foods. Food derived ACE inhibitors were first reported by Oshima et al. (1979); they found peptide inhibitors of ACE in digests of gelatin with bacterial collagenase. Thereafter, many other ACE inhibitors have been discovered from enzymatic hydrolysates or the related synthetic peptides of bovine caseins (Maruyama and Suzuki, 1982; Maruyama et al., 1985, 1987a,b; Karaki et al., 1990; Yamamoto et al., 1994a,b, 1999; Nakamura et al., 1995a,b), whey proteins (Abubakar et al., 1996; Mullally et al., 1996, 1997a,b; Pihlanto-Leppälä et al., 2000), human casein (Kohmura et al., 1989, 1990a), zein (Maruyama et al., 1989; Miyoshi et al., 1991a,b), soy sauce (Kinoshita et al., 1993), sake and sake lees (Saito et al., 1992, 1994a,b), and other food proteins. Many studies have also been performed on fish proteins, such as tuna (Kohama et al., 1988, 1991), bonito (Yokoyama et al., 1992; Matsumura et al., 1993a,b), and sardine (Ukeda et al., 1992; Matsui et al.,

1993) muscles. The most active ACE inhibitory peptides found in these studies are summarized in Table 1.3 and Table 1.4. These peptides contain 2-12 amino acid residues, several of them with proline in the C-terminus. In addition to showing potent ACE inhibitory activity *in vitro*, these peptides have also demonstrated antihypertensive effects in spontaneously hypertensive rats (SHR) by oral administration (Table 1.5).

1.4.1 ACE inhibitors derived from gelatin

Food derived ACE inhibitory peptides were first reported by Oshima et al (1979). These peptides were produced by digesting gelatin, which is rich in proline forming imino bonds (Eastoe, 1967), with *Clostridium histolyticum* derived collagenase (clostridiopeptidase A, EC 3.4.24.3), which specifically hydrolyses the X-Gly peptide bond of the sequence Pro-X-Gly-Pro. Altogether nine ACE-inhibitory peptides, with 3 to 12 amino acid residues and a N-terminal Gly-Pro sequence, were isolated from the digests, which underwent alcohol fractionation followed by a series of chromatography treatment. Among these, only six peptides having C-terminal Ala-Hyp significantly inhibited ACE activity (IC_{50} = 8.3-37 μM) (Table 1.3), and their inhibitory potency was in the same order as the inhibitory peptides isolated from the snake venom (Cheung and Cushman, 1973). In addition to the exogenous bacterial collagenase, Oshima et al. (1979) also used endogenous mammalian proteinases, namely trypsin (EC 3.4.21.4) and α-chymotrypsin (EC 3.4.21.1), to produce ACE inhibitory peptides from gelatin.

1.4.2 ACE inhibitors derived from α-zein

ACE inhibitory peptides were produced by enzymatic hydrolysis of α- and γ-zeins, two major components of maize (*Zea mays*) endosperm protein (Maruyama et al., 1989; Miyoshi et al., 1991a,b). Maruyama et al. (1989) isolated a hexapeptide (Val-His-Leu-Pro-Pro-Pro) with ACE inhibitory effect from a thermolysin hydrolysate of γ-zein, which is rich in proline residues (Esen, 1987). However, its potency (IC_{50} = 200 μM) was much lower than the related synthetic pentapeptide (Val-His-Leu-Pro-Pro, IC_{50} = 18 μM) and tripeptide (Leu-Pro-Pro, IC_{50} = 9.6 μM); fragments with these sequences were not easily produced from γ-zein by enzymatic hydrolysis (Maruyama et al., 1989). Miyoshi et al. (1991a) isolated much stronger ACE inhibitors from thermolysin hydrolysate of α-zein. Most of these peptides were tripeptides having four different C-terminal amino acid residues (Pro, Tyr, Ala, and Gln). Leu-Arg-Pro was the most potent inhibitor, and it was also effective in blood pressure reduction of spontaneously hypertensive rats (SHR) after an intravenous injection. Furthermore, when the unpurified thermolysin hydrolysate of α-zein was orally administered to spontaneously hypertensive rats, their blood pressure was reduced (Miyoshi et al., 1991b).

1.4.3 ACE inhibitors derived from fish

An ACE inhibitory octapeptide was discovered and isolated from acid extract of tuna muscle by a combination of column chromatography techniques (Kohama et al., 1988). The peptide had an amino acid sequence of Pro-Thr-His-Ile-Lys-Trp-Gly-Asp,

and it showed IC_{50} values of 1 and 2 μM for ACEs from bovine and rabbit lungs, respectively. Unlike the ACE inhibitors from snake venom that had a C-terminal Pro-Pro or Ala-Pro sequence (Cheung and Cushman, 1973), the octapeptide had a C-terminal Gly-Asp sequence. The structure-activity relationship of the venom peptides and their synthetic analogues indicated that the ACE had little affinity for substrates or competitive inhibitors with C-terminal dicarboxylic acids, but an antepenultimate aromatic amino acid residue appeared to enhance binding (Cushman et al., 1973; Cheung et al., 1980). Even though the antepenultimate amino acid of the octapeptide was aromatic (Trp), it had dicarboxylic acid (Asp) at the C-terminal, indicating that its inhibition mechanism to ACE was different from that of venom peptides. It was later suggested that the octapeptide inhibited ACE non-competitively and localized its binding affinity to ACE in parts other than its C-terminal dipeptide (Kohama et al., 1991).

In addition to tuna, bonito was also under investigation for the presence of ACE inhibitors (Yokoyama et al., 1992; Matsumura et al., 1993a,b). Yokoyama et al. (1992) isolated eight ACE inhibitory peptides (Table 1.3) from thermolysin digest of dried bonito, a Japanese traditional seasoning made of bonito muscle, using HPLC. Four of them (Ile-Val-Gly-Arg-Pro-Arg-His-Gln-Gly, Ile-Trp-His-His-Thr, Ala-leu-Pro-His-Ala, Phe-Gln-Pro) were found in the primary structure of actin. When fragments of the active peptides were obtained by chemical synthesis or further thermolysin hydrolysis, some of the di- and tripeptides showed higher inhibitory activity than their parental peptides. In other study, six ACE inhibitory peptides were isolated from a bonito bowels autolysate that was treated with ultrafiltration and column chromatographies (Matsumura et al.,

1993a,b). Four of these peptides were tripeptides, and they were composed of a N-terminal hydrophobic amino acid residue, a C-terminal proline, and a basic amino acid residue in the middle (Table 1.3). Among these tripeptides, Val-Arg-Pro and Leu-Arg-Pro were also discovered in β-caseins (Kohmura et al., 1990b) and α-zein (Miyoshi et al., 1991a), suggesting that many proteins might contain these sequences.

1.4.4 ACE inhibitors derived from sake and sake lees

Saito et al. (1992, 1994a,b) isolated nine peptides that inhibit ACE from sake and its by product, sake lees. While the ACE inhibitors in sake existed as free peptides, sake lees had to be treated with protease to produce these peptides. They were short peptides with 5 or fewer amino acid residues, but unlike the inhibitory peptides produced from gelatin and zeins, most of these peptides did not contain proline residue. Instead, many of them had a tryptophan or tyrosine residue at the C-terminus. The only proline-containing ACE inhibitor was the pentapeptide (Ile-Tyr-Pro-Arg-Tyr) from sake lees, but its potency was slightly lower than a dipeptide (Val-Trp), which was also isolated from sake lees (Table 1.3). When fragments of the pentapeptide were orally administered to SHR, the systolic blood pressure (SBP) was reduced (Saito et al., 1994b).

1.4.5 ACE inhibitors derived from chicken muscle and ovalbumin

Recently, ACE inhibitors were produced from the thermolysin digest of chicken muscle and the peptic digest of ovalbumin, a typical egg protein (Fujita et al., 2000).

Seven potent ACE inhibitory peptides with 3-9 amino acid residues were isolated from the chicken muscle digest that was fractionated on an octadecylsilica (ODS) column and purified on phenyl and cyanopropyl columns. Among them, the nonapeptide (Ile-Val-Gly-Arg-Pro-Arg-His-Gln-Gly) had exactly the same amino acid sequence as an ACE inhibitory peptide that was isolated from the thermolysin digest of dried bonito (Yokoyama et al., 1992), and the tripeptide (Ile-Lys-Trp) showed the most potent ACE inhibitory activity (IC_{50} = 0.21 μM). On the other hand, six peptides with ACE inhibitory effect were isolated from the peptic digest of ovalbumin that underwent same column chromatography treatments. Among the di- to octapeptides, the most potent ACE inhibitor was the octapeptide (Glu-Arg-Lys-Ile-Lys-Val-Tyr-Leu, IC_{50} = 1.2 μM). In addition to exerting ACE inhibitory activity, some of the peptides isolated from both digests showed antihypertensive activity after intravenous administration at a dose of 10 mg/kg in SHR as well as oral administration at a dose of 60 mg/kg in SHR. Generally, dipeptides and tripeptides were more effective in lowering blood pressure of SHR than larger peptides, such as octapeptides and nonapeptides.

1.4.6 ACE inhibitors derived from porcine skeletal muscle

Arihara et al. (2001) isolated two ACE inhibitory peptides from thermolysin digest of porcine skeletal muscle myosin. Both peptides were pentapeptides with amino acid sequence of Met-Asn-Pro-Pro-Lys (IC_{50} = 945.5 μM) and Ile-Thr-Thr-Asn-Pro (IC_{50} = 549.0 μM), which were found in the primary structure of the porcine myosin heavy chain at 79-83 and 306-310, respectively. The ACE inhibitory activity of these

pentapeptides was relatively low compared with other food protein derived ACE inhibitory peptides. On the other hand, three synthetic tripeptides (Met-Asn-Pro, Asn-Pro-Pro, and Thr-Asn-Pro) that corresponded to sequences within the pentapeptides exhibited higher inhibitory activity than the original peptides (Table 1.3). All three tripeptides had proline residue at the C-terminal.

1.4.7 ACE inhibitors derived from milk caseins

Casein represents approximately 80% of total proteins contained in milk. Milk casein is composed of α-, β-, and γ-casein. α-Casein is further divided into α_s-casein, which is precipitated by Ca^{2+}, and κ-casein, which is not precipitated by Ca^{2+} and plays a role in making casein micelle in milk. Casein is hydrolysed to peptides by trypsin in the intestinal tract.

The first casein-derived ACE inhibitor was a dodecapeptide (Phe-Phe-Val-Ala-Pro-Phe-Pro-Glu-Val-Phe-Gly-Lys, IC_{50} = 77 μM) that was obtained from a tryptic hydrolysate (Maruyama and Suzuki, 1982). The peptide corresponded to f23-34 of α_{s1}-casein B variant (Grosclaude et al., 1970) except that the glutamine residue had changed to glutamic acid; it was speculated that termination of the trypsin hydrolysis with concentrated HCl resulted in the conversion. The same workers isolated a pentapeptide (Phe-Phe-Val-Ala-Pro) from the dodecapeptide by hydrolysing it with a proline-specific endopeptidase (Maruyama et al., 1985). This peptide had a C-terminal Ala-Pro sequence, and it was 13 times more potent than the dodecapeptide *in vitro* (Table 1.4). In addition

to the two peptides derived from α_{s1}-casein, a heptapeptide, which corresponded to f177-183 of β-casein, was isolated (Maruyama et al., 1985). The ACE inhibitory activity of the heptapeptide was higher than the dodecapeptide but lower than the pentapeptide. When peptides corresponding to fragments of the ACE inhibitors isolated previously were synthesized, only Val-Ala-Pro, which corresponded to the C-terminal tripeptide of pentapeptide exhibited higher inhibitory activity (IC_{50} = 2.0 μM) than the original peptide (IC_{50} = 6.0 μM) (Maruyama et al., 1985). However, D-Val-Ala-Pro showed lower inhibitory activity (IC_{50} = 550 μM), confirming a report, which suggested that ACE showed high stereospecificity for an amino acid residue at position 3 from the C-terminal, but showed little stereospecificity at position 4 (Oshima and Nagasawa, 1979). Maruyama et al. (1987a) also investigated the antihypertensive activity of the dodecapeptide and pentapeptide. When administered intravenously to anesthetized rats, the dodecapeptide antagonized their pressor response to angiotensin I at a dose of 14.2 mg/kg, but not the pentapeptide. The antihypertensive effect of tryptic hydrolysate of casein was also verified when decreased blood pressure in SHR was resulted from a single intraperitoneal or oral administration (Karaki et al., 1990).

Lactic acid bacteria (LAB) possess extracellular proteinases, which hydrolyze milk protein restrictively and liberate peptides in culture medium (Monnet et al., 1987; Zevaco and Gripon, 1988). *Lactobacillus helveticus* has especially strong proteolytic activity. Yamamoto et al. (1993) hydrolyzed α_{s1}-casein and β-casein with an extracellular proteinase, which was purified from a *Lactobacillus helveticus* CP790, and identified 25 main peptides from the hydrolysates. Among these peptides, β-casein derived peptide

with 27 amino acid residues showed the highest ACE inhibitory activity with IC_{50} value of 4 μM (Yamamoto et al., 1994a). The hydrolysates also demonstrated antihypertensive effect in SHR by oral administration. The same group of workers purified and identified ten more peptides from a casein hydrolysate, produced by an extracellular proteinase from *Lactobacillus helveticus* CP790 (Maeno et al., 1996). Subsequently, synthetic peptides corresponding to the identified peptide were examined for antihypertensive activity in SHR. Among these peptides, a heptapeptide (Lys-Val-Leu-Pro-Val-Pro-Gln), found in β-casein, showed strong antihypertensive effect. However, the antihypertensive peptide did not have strong ACE inhibitory activity. When a hexapeptide with C-terminal proline was liberated from the heptapeptide by pancreatic digestion, the hexapeptide showed stronger ACE inhibitory activity. On the other hand, two peptides (Thr-Lys-Val-Leu-Pro-Val-Pro, Ala-Tyr-Phe-Tyr-Pro) with proline residue at the C-terminal did not show strong inhibitory activity, thus suggesting that both proline residue in the C-terminal and the amino acid sequence were important for ACE inhibition (Maeno et al., 1996).

In addition to enzymatic hydrolysis, a low heat skim milk medium was fermented with *Lactobacillus helveticus* CP790 and *Lactobacillus helveticus* CP791, which was a variant defective in proteinase activity, and tested for antihypertensive activity (Yamamoto et al., 1994a). The milk fermented with *Lactobacillus helveticus* CP790 showed antihypertensive activity on SBP in SHR rats; however, the milk fermented with *Lactobacillus helveticus* CP791 did not. This result indicated that proteinases in the LAB liberated active peptides during fermentation.

For the first time, Nakamura et al. (1995a) isolated and identified two ACE inhibitory peptides from Calpis (Calpis Food Industry Co., Ltd., Tokyo, Japan) sour milk, which is a popular Japanese soft drink prepared by fermenting skim milk with a starter containing *Lactobacillus helveticus* and *Saccharomyces cerevisiae*. The two tripeptides purified from the fermented sour milk by a four-step HPLC, were Val-Pro-Pro and Ile-Pro-Pro, which occur at three positions in bovine caseins (β-casein, f74-76; β-casein, f84-86, and k-casein, f108-110). Ile-Pro-Pro was also present in the C-terminal sequence of some bradykinin potentiating peptides that also had ACE inhibitory activity (Ferreira et al., 1970; Kato and Suzuki, 1971; Fisher et al., 1979). Both peptides exhibited potent ACE inhibitory activity (IC_{50} = 9 and 5 μM, respectively) as well as antihypertensive activity after a single oral administration (0.6 and 0.3 mg/kg of body weight, respectively) to SHR. The amount of the peptides required to show antihypertensive effect was relatively small compared with other ACE inhibitory peptides reported so far (Kohmura et al., 1989; Karaki et al., 1990). It was also found that long-term feeding of the sour milk in SHR significantly lowered the SBP and ACE activity in the abdominal aorta of the rats (Nakamura et al., 1996). Traces of the tripeptides (Val-Pro-Pro and Ile-Pro-Pro) were detected in the abdominal aorta after oral administration of the sour milk to SHR; however, these peptides were not detected in the aorta of SHR that were given saline (Masuda et al., 1996). This result suggested that the small peptides were absorbed directly in the intestine without being decomposed by digestive enzymes, reached the abdominal aorta, inhibited the ACE, and consequently showed antihypertensive effects. Antihypertensive effects of the sour milk were also studied in hypertensive patients, who were taking antihypertensive medication (Hata et al., 1996). The SBP and DBP of a

group ingesting daily dose of sour milk (2 ml/kg of BW) decreased significantly after 8 weeks, but no significant changes in blood pressure were observed in the placebo group ingesting the same amount of artificially acidified milk.

In another study, a yogurt-like product that was produced by fermenting skim milk with *Lactobacillus helveticus* CPN4 demonstrated strong antihypertensive activity in SHR (Yamamoto et al., 1996). From this product, a dipeptide with ACE inhibitory activity was isolated by Sep-pak C-18 cartridge followed by two-step HPLC (Yamamoto et al., 1999). The amino acid sequence of the dipeptide (Tyr-Pro) was mostly found at C-terminal regions of bovine casiens, i.e., α_{s1}-casein, f146-147; α_{s1}-casein, f159-160; β-casein, f114-115; and κ-casein f58-59. The same amino acid sequence was also found in an active pentapeptide isolated from sake lees (Saito et al., 1994b). Although the antihypertensive activity of the dipeptide was in the same order as the tripeptides isolated from sour milk, its ACE inhibitory activity was much lower (IC_{50} = 720 μM) (Table 1.4).

Pihlanto-Leppälä et al. (1998) fermented skim milk with five different lactic acid starters, followed by enzymatic digestion with a combination of pepsin and trypsin; they reported that fermentation alone could not produce ACE inhibitory activity but enzymatic digestion was required to produce inhibitory activity. The hydrolysates were fractionated by size exclusion and reversed phase chromatography, and several peptides with ACE inhibitory activity were purified and identified. The size of these peptides ranged from six to eight amino acid residues and they were originated from α_{s1}- and β-caseins. Among these, a α_{s1}-casein derived hexapeptide (Thr-Thr-Met-Pro-Leu-Trp) showed the

highest inhibitory activity (IC_{50} = 51 μM), but it was much weaker than the tripeptides isolated from Calpis sour milk.

1.4.8 ACE inhibitors derived from whey proteins

In contrast to caseins, ACE inhibitory peptides from whey proteins are less studied and characterized. Chiba and Yoshikawa (1991) reported that albutensin A, derived from bovine serum albumin f208-216, exhibited ACE inhibitory activity in addition to ileum contracting and relaxing activities. Mullally et al. (1996) detected ACE inhibitory activity in synthetic tetrapeptides, i.e., β-lactorphin (Tyr-Leu-Leu-Phe), β-lactotensin (His-Ile-Arg-Leu), and α-lactorphin (Tyr-Gly-Leu-Phe), corresponding to sequences of β-lactoglobulin f102-105 and f146-148, and α-lactalbumin f50-53, respectively. Among these, β-lactorphin was the most potent ACE inhibitor (IC_{50} = 171.8 μM). The most potent whey protein-derived ACE inhibitor reported so far is a synthetic peptide (Ala-Leu-Pro-Met-His-Ile-Arg) corresponding to a tryptic fragment of β-lactoglobulin (f142-148) with IC_{50} of 42.6 μM (Mullally et al., 1997a). Attempts were made to purify and identify a number of ACE inhibitory peptides from whey proteins by enzymatic digestion with a group of enzymes (Pihlanto-Leppälä et al., 2000) or by fermentation with various lactic acid starters in combination with enzymatic hydrolysis (Pihlanto-Leppälä et al., 1998). However, these peptides were not as potent as the synthetic heptapeptide identified by Mullally et al. (1997a).

1.5 CONCLUSION

A number of ACE inhibitory peptides have been identified in enzymatic digest of fermented food proteins. Most of these peptides are small in length, i.e., less than ten amino acid residues, and have C-terminal amino acid residues that are aromatic (Trp, Tyr, and Phe) or with imino group (Pro). Therefore, both peptide size and the amino acid sequence of the peptides contribute to inhibiting ACE. In addition, due to the specificity of ACE, it was determined that C-terminal dipeptides are generally the more potent fragment of ACE inhibitory peptides (Cheung et al., 1980). It was noted that food-derived ACE inhibitors do not have the potency of antihypertensive drugs, such as captopril, but food-derived peptides with potency within the IC_{50} 100-500 μM range are considered to be of nutritive/physiological importance in that they could be active following oral administration (Sekiya et al., 1992). Therefore, ACE inhibitory peptides derived from food proteins represent potential nutraceutical or functional food ingredients for the prevention of hypertension.

Table 1.1 ACE inhibitory peptides from alcoholic extracts of venom of *Bothrops jararaca* and *Agkistrodon halys blomhoffii*

Peptides	Snake species	IC_{50} (μM)[a]	Reference
Pyr[b]-Lys-Trp-Ala-Pro	*Bothrops jararaca*	0.07	Ferreira et al., 1970
Pyr-Trp-Pro-Arg-Pro-Thr-Pro-Gln-Ile-Pro-Pro	*Bothrops jararaca*	2	Ondettie et al., 1971
Pyr-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro	*Bothrops jararaca*	3	Ondettie et al., 1971
Pyr-Asn-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro	*Bothrops jararaca*	3	Ondettie et al., 1971
Pyr-Asn-Trp-Pro-His-Pro-Gln-Ile-Pro-Pro	*Bothrops jararaca*	9	Ondettie et al., 1971
Pyr-Ser-Trp-Pro-Gly-Pro-Ans-Ile-Pro-Pro	*Bothrops jararaca*	39	Ondettie et al., 1971
Pyr-Gly-Gly-Trp-Pro-Arg-Pro-Gly-Pro-Glu-Ile-Pro-Pro	*Bothrops jararaca*	13	Ondettie et al., 1971
Pyr-Gly-Leu-Pro-Pro-Gly-Pro-Pro-Ile-Pro-Pro	*Agkistrodon halys blomhoffii*	Not available	Kato and Suzuki, 1970
Pyr-Lys-Trp-Asp-Pro-Pro-Pro-Val-Ser-Pro-Pro	*Agkistrodon halys blomhoffii*	Not available	Kato and Suzuki, 1971
Pyr-Gly-Leu-Pro-Pro-Arg-Pro-Lys-Ile-Pro-Pro	*Agkistrodon halys blomhoffii*	Not available	Kato and Suzuki, 1971

[a] The concentration of an ACE inhibitor required to inhibit 50% of ACE activity.
[b] L-Pyroglutamic acid (L-2-pyrrolidone-5-carboxylic acid)

Table 1.2 Pharmacology of ACE inhibitors approved in the United States.

	Captopril	Enalapril	Lisinopril	Benazepril	Quinapril	Ramipril	Trandolapril	Moexipril	Fosinopril
Zinc ligand	Sulfhydryl	Carboxyl	Carboxyl	Carboxyl	Carboxyl	Carboxyl	Carboxyl	Carboxyl	Phosphinyl
Prodrug	No	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes
t_{max} active drug, h	0.7 – 0.9	2– 8	6 – 8	1 – 2	2	3	4 – 10	1.5	3
$t_{1/2}$ active drug, h	1.7	11	12	10 – 11	1.9 – 2.5, 25 terminal	Triphasic 4, 9 – 18, >50	15 – 24 terminal	2 – 9	12
Route of elimination	Kidney	Kidney	Kidney	Kidney	Kidney	Kidney	Kidney, liver	Kidney	Liver, kidney
Dosage range, mg	6.25 –300	2.5 – 40	5 – 40	5 – 80	5 – 80	1.25 – 20	1 – 8	7.5 – 30	10 – 80
F [a], %	75 – 91	60	6 – 60	>37	>60	50 – 60	70	13	36

[a] F indicates bioavailability
(Adapted from Brown and Vaughan, 1998)

Table1.3 ACE inhibitory peptides derived from food proteins.

Peptides	Source	Preparation	IC_{50} (μM)[a]	Reference
GPHypGTDGAHyp[b]	Gelatin	Collagenase	10.5	Oshima et al., 1979
GPHypGA(P)[c]Hyp	Gelatin	Collagenase	11.3[d]	Oshima et al., 1979
GPAGAHyp	Gelatin	Collagenase	8.3	Oshima et al., 1979
GPPGAHyp	Gelatin	Collagenase	8.6	Oshima et al., 1979
GPIGSVGAHyp	Gelatin	Collagenase	31.8	Oshima et al., 1979
GPAGAPGAA	Gelatin	Collagenase	37.0	Oshima et al., 1979
VHLPPP	γ-Zein	Thermolysin	200	Maruyama et al., 1989
VHLPP	γ-Zein	Synthesis	18	Maruyama et al., 1989
LPP	γ-Zein	Synthesis	9.6	Maruyama et al., 1989
LRP	α-Zein	Thermolysin	0.27	Miyoshi et al., 1991a
LSP	α-Zein	Thermolysin	1.7	Miyoshi et al., 1991a
LQP	α-Zein	Thermolysin	1.9	Miyoshi et al., 1991a
PTHIKWGD	Tuna	Acid extraction	1-2	Kohama et al., 1988
IKPLNY	Bonito muscle	Thermolysin	43	Yokoyama et al., 1992
IVGRPRHQG	Bonito muscle	Thermolysin	6.2	Yokoyama et al., 1992
IWHHT	Bonito muscle	Thermolysin	5.1	Yokoyama et al., 1992
ALPHA	Bonito muscle	Thermolysin	10	Yokoyama et al., 1992
LKPNM	Bonito muscle	Thermolysin	17	Yokoyama et al., 1992
IY	Bonito muscle	Thermolysin	3.7	Yokoyama et al., 1992
FQP	Bonito muscle	Thermolysin	12	Yokoyama et al., 1992
DYGLYP	Bonito muscle	Thermolysin	62	Yokoyama et al., 1992
VRP	Bonito bowels	Autolysis	2.2	Matsumura et al., 1993a
IKP	Bonito bowels	Autolysis	2.5	Matsumura et al., 1993a
LRP	Bonito bowels	Autolysis	1.0	Matsumura et al., 1993a
IRP	Bonito bowels	Autolysis	1.8	Matsumura et al., 1993a

[a] The concentration of an ACE inhibitor required to inhibit 50% of ACE activity
[b] Hydroxyproline
[c] Ratio of A : P = 44.4 : 55.6
[d] Value based on average molecular weight

Table1.3 ACE inhibitory peptides derived from food proteins (Continued)

Peptides	Source	Preparation	IC_{50} (μM)[a]	Reference
MNPPK	Porcine myosin	Thermolysin	945.5	Arihara et al., 2001
ITTNP	Porcine myosin	Thermolysin	549.0	Arihara et al., 2001
MNP	Porcine myosin	Synthesis	66.6	Arihara et al., 2001
NPP	Porcine myosin	Synthesis	290.5	Arihara et al., 2001
TNP	Porcine myosin	Synthesis	207.4	Arihara et al., 2001
VY	Sake	Brewing	7.1	Saito et al., 1992
YGGY	Sake	Brewing	16.2	Saito et al., 1992
IYPRY	Sake lees	Protease	4.1	Saito et al., 1992
VW	Sake lees	Protease	1.4	Saito et al., 1994b
VWY	Sake lees	Protease	9.4	Saito et al., 1994b
YW	Sake lees	Protease	10.5	Saito et al., 1994b
LKA	Chicken muscle	Thermolysin	8.5	Fujita et al., 2000
LKP	Chicken muscle	Thermolysin	0.32	Fujita et al., 2000
LAP	Chicken muscle	Thermolysin	3.5	Fujita et al., 2000
FQKPKR	Chicken muscle	Thermolysin	14	Fujita et al., 2000
IVGRPRHQG	Chicken muscle	Thermolysin	2.4	Fujita et al., 2000
FKGRYYP	Chicken muscle	Thermolysin	0.55	Fujita et al., 2000
IKW	Chicken muscle	Thermolysin	0.21	Fujita et al., 2000
FGRCVSP	Ovalbumin	Pepsin	6.2	Fujita et al., 2000
ERKIKVYL	Ovalbumin	Pepsin	1.2	Fujita et al., 2000
FFGRCVSP	Ovalbumin	Pepsin	0.4	Fujita et al., 2000
LW	Ovalbumin	Pepsin	6.8	Fujita et al., 2000
FCF	Ovalbumin	Pepsin	11	Fujita et al., 2000
NIFYCP	Ovalbumin	Pepsin	15	Fujita et al., 2000

[a] The concentration of an ACE inhibitor required to inhibit 50% of ACE activity

Table 1.4 ACE inhibitory peptides derived from milk proteins.

Peptides	Source	Preparation	IC_{50} (μM)[a]	Reference
FFVAPFPEVFGK	α_{s1}-Casein	Trypsin	77	Maruyama and Suzuki, 1982
FFVAP	α_{s1}-Casein	Peptidase	6.0	Maruyama et al., 1985
AVPYPQR	β-Casein	Trypsin	15	Maruyama et al., 1985
DELQDKIHPFAQTQSLVYPFPGPIPNS	β-Casein	Extracellular proteinase	4	Yamamoto et al., 1994a
VPP	β-, κ-Caseins	Fermentation	9	Nakamura et al., 1995a
IPP	β-, κ-Caseins	Fermentation	5	Nakamura et al., 1995a
YP	α_{s1}-, β-, κ-Caseins	Fermentation	720	Yamamoto et al., 1999
YLLF	β-Lactoglobulin	Synthesis	171.8	Mullally et al., 1996
HIRL	β-Lactoglobulin	Synthesis	1153.2	Mullally et al., 1996
YGLF	α-Lactalbumin	Synthesis	733.3	Mullally et al., 1996
ALPMHIR	β-Lactoglobulin	Synthesis	42.6	Mullally et al., 1997a

[a] The concentration of an ACE inhibitor required to inhibit 50% of ACE activity

Table 1.5 Antihypertensive peptides derived from bovine caseins.

Peptide	Source	Preparation	IC_{50} [a] (μM)	Dose (mg/kg)	SBP[b] (mm Hg)	Reference
FFVAPFPEVFGK	α_{s1}-Casein	Trypsin	77	100	-13.0[c]	Maruyama et al., 1987a
TTMPLW	α_{s1}-Casein	Trypsin	16	100	-13.6[c]	Kohmura et al., 1989
AVPYPQR	β-Casein	Trypsin	15	100	-10.2[c]	Kohmura et al., 1989
KVLPVPQ	β-Casein	Proteinase	>1000	1	-24.1[d]	Yamamoto et al., 1999
KVLPVP	β-Casein	Digestive enzyme	5	1	-32.2[d]	Yamamoto et al., 1999
YKVPQL	α_{s1}-Casein	Proteinase	22	1	-12.5[d]	Yamamoto et al., 1999
IPP	β-, κ-Caseins	Fermentation	5	1	-15.1[d]	Nakamura et al., 1995b
VPP	β-, κ-Caseins	Fermentation	9	1.4	-20.0[d]	Nakamura et al., 1995b
YP	α_{s1}-, β-, κ-Caseins	Fermentation	720	1	-27.4[d]	Yamamoto et al., 1996

[a] The concentration of an ACE inhibitor required to inhibit 50% of ACE activity.
[b] Systolic blood pressure.
[c] Changes of systolic blood pressure in SHR at 3h after oral administration.
[d] Changes of systolic blood pressure in SHR at 6h after oral administration.

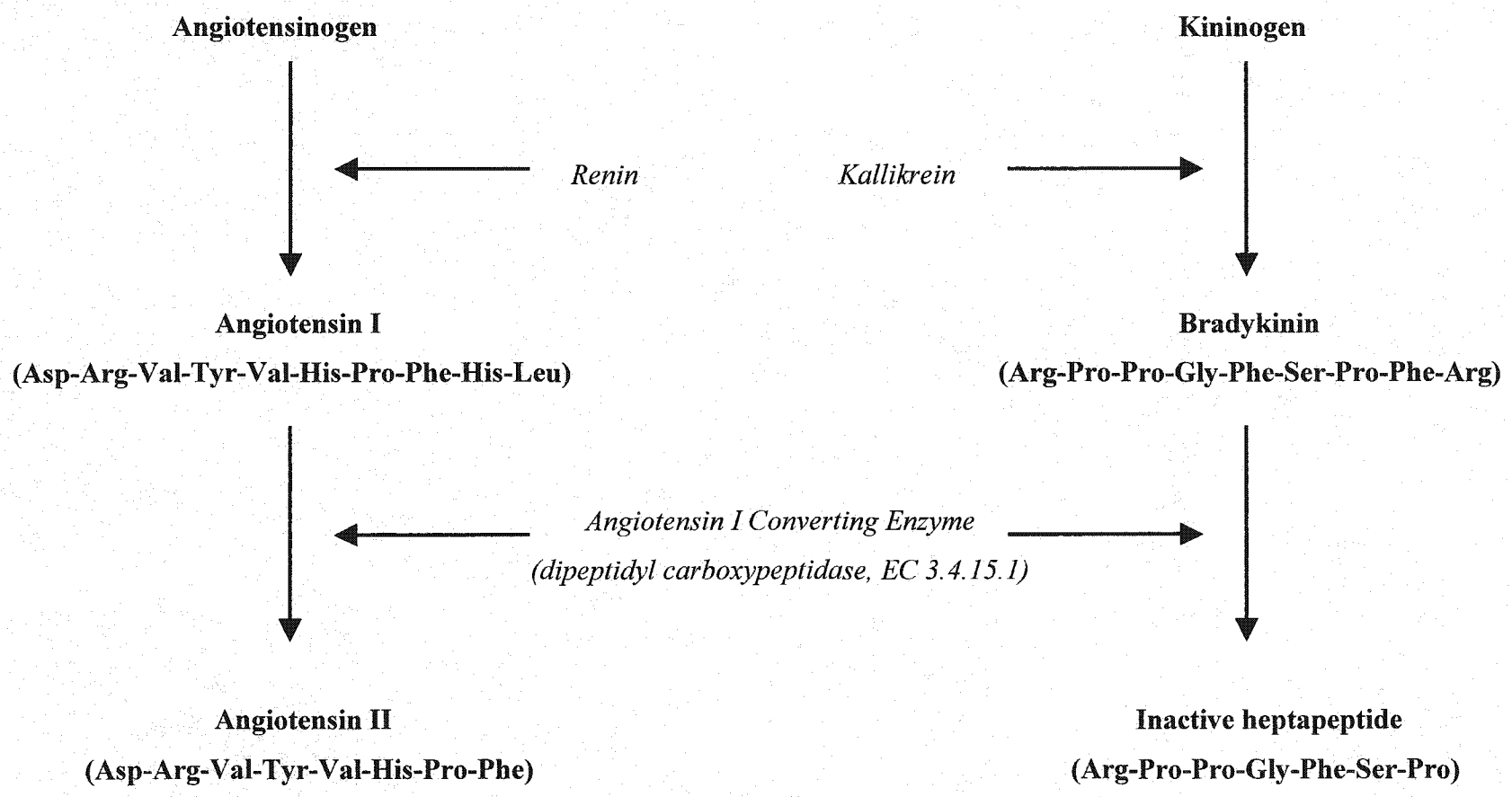

Figure 1.1 Renin-angiotensin system (RAS) and kallikrein-kinin system (KKS).

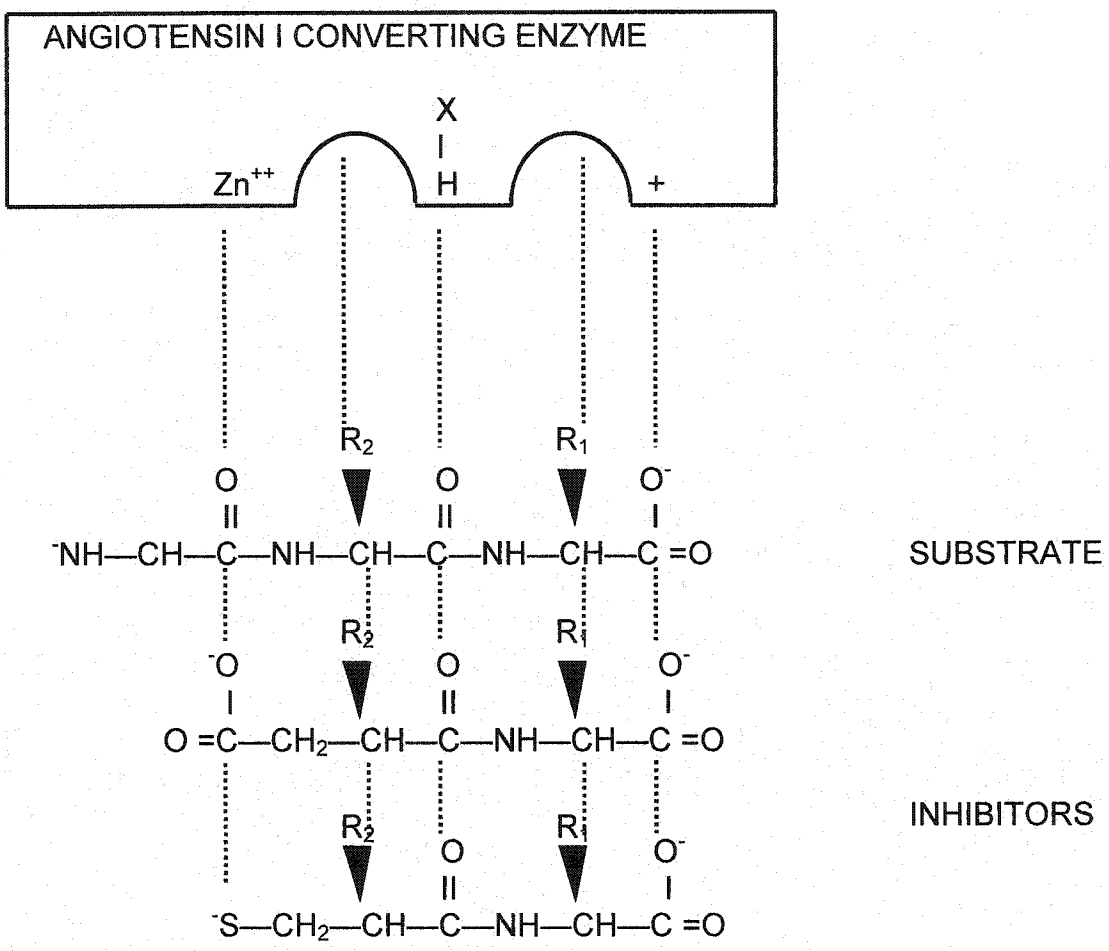

Figure 1.2 Diagrammatic model of a hypothetical active site of angiotensin I converting enzyme. By analogy to bovine pancreatic carboxypeptidase A, the active site of ACE was proposed to contain three important groups that participate in binding of peptide substrates or competitive inhibitors: a carboxyl-binding group, a group with affinity for the C-terminal peptide bond, and a tightly bound zinc ion that could coordinate with the carbonyl of the penultimate (scissile) peptide bond. The circular clefts on the active site represents portions of the active site that interact with substituents R_1 and R_2 of substrates or competitive inhibitors by an undetermined mechanism; X-H represents a hydrogen bonding residue at the active site of ACE (Adapted from Cushman et al., 1977).

CHAPTER 2

PRODUCTION OF ANGIOTENSIN I-CONVERTING ENZYME INHIBITORY PEPTIDES FROM WHEY FERMENTATION WITH LACTIC ACID BACTERIA

In order to verify if angiotensin I-converting enzyme inhibitory peptides could be derived from whey fermentation, whey media were fermented with various lactic acid bacteria. Optimum conditions for the fermentation were established to achieve higher biomass that would induce higher proteolytic activity. These conditions were used to prepare whey hydrolysates for further studies.

The major results of this study were summarized as a manuscript suitable for journal publication. The manuscript entitled "Production of angiotensin I-converting enzyme inhibitory peptides from whey fermentation with lactic acid bacteria" was co-authored by Jae-Eun Ahn and Byong H. Lee. The project was supervised by Dr. Byong H. Lee, while the actual experimental work and the manuscript writing were done by Jae-Eun Ahn.

2.1 ABSTRACT

Nine strains of *Lactobacillus* were respectively used to ferment whey media, containing different concentration of whey powder, glucose, and yeast extract. The fermentation was carried out at 30 or 37 °C, according to the optimum growth temperature of each strain. The strains showed the optimal growth in whey medium containing 2% (w/v) whey powder. Whey media supplemented with glucose and yeast extract increased the growth rate, and the optimal growth was achieved at 1% (w/v) glucose and 0.5% (w/v) yeast extract. However, glucose effect was not as significant as the yeast extract supplementation in improving the growth of the *Lactobacillus* species. Subsequently, whey medium, containing 2% (w/v) whey powder, 1% glucose, and 0.5% yeast extract, was fermented with each *Lactobacillus* strain. Whey hydrolysates from the fermentation were assayed for ACE inhibitory activity. All hydrolysates exhibited the inhibitory activity, but *Lb. brevis*, *Lb. helveticus*, and *Lb. paracasei* showed the highest activities with inhibition rate ranging from 93.1 ± 0.3 and 100 %.

Key words: *Lactobacillus* strains, fermentation, whey, supplementation, ACE inhibitory activity

2.2 INTRODUCTION

Angiotensin-I-converting enzyme (ACE) is a dipeptide-liberating exopeptidase, which plays an important role in peripheral blood pressure regulation. It raises blood pressure by converting the inactive decapeptide angiotensin I to the potent vasoconstrictor octapeptide angiotensin II, as well as inactivating the vasodilating nonapeptide, bradykinin. Inhibition of ACE may exert an antihypertensive effect as a consequence of the decrease of angiotensin II as well as increase of bradykinin.

Peptides that inhibit ACE have been found in many different food proteins (Ariyoshi, 1993; Okamoto et al., 1995; Yamamoto, 1997), most extensively in milk proteins (Pihlanto-Leppälä, 1998; Takano, 1998, 2000; Yamamoto and Takano, 1999; Nurminen, 2000). Several casein-derived ACE inhibitors have been reported (Maruyama and Suzuki, 1982; Maruyama et al., 1985, 1987a,b; Karaki et al., 1990; Nakamura et al., 1995a; Maeno et al., 1996; Sugai, 1998). However, only limited studies have been carried out on whey protein-derived ACE inhibitors. Albutensin A, a peptide derived from serum albumin, was shown to inhibit ACE (Chiba and Yoshikawa, 1991). Synthetic di- and tetra-peptides corresponding to α-lactalbumin (α-la) and β-lactoglobulin (β-lg) sequences were shown to inhibit ACE (Mullally et al., 1996, 1997a,b).

Whey proteins represent about 20% of total milk proteins and are well known for their high nutritional value and versatile functional properties in food products. These proteins can also provide elemental nitrogen for the growth of lactic acid bacteria (LAB),

which possess proteinases that catalyze the hydrolysis of native or denatured protein molecules, and peptidases that catalyze the degradation of the smaller peptides produced by proteinases action (Law and Kolstad, 1983; Christensen et al., 1999; Siezen, 1999). Thus, the proteolytic system of lactic acid bacteria can convert whey proteins into smaller peptides, which may exhibit ACE inhibitory activity.

In this study, optimizing culture medium for the growth of lactic acid bacteria (LAB) during whey fermentation was investigated in order to produce whey hydrolysates that contain potential ACE inhibitory peptides. Subsequently, ACE inhibitory activities of these hydrolysates were determined.

2.3 MATERIALS AND METHODS

2.3.1 Chemicals

Hydrochloric acid (HCl), sodium borate ($Na_2B_4O_7 \cdot 10H_2O$), Tween 80 ($C_{64}H_{124}O_{26}$), and glycerol ($HOCH_2CH(OH)CH_2OH$) were purchased from Fisher Scientific Company (Fair Lawn, NJ, USA), while glucose ($C_6H_{12}O_6$), sodium chloride (NaCl), ethyl acetate ($C_4H_8O_2$), Hip-His-Leu, and ACE from rabbit lung were obtained from Sigma Chemicals Co. (St. Louis, MO, USA). Whey powder, yeast extract, and MRS were obtained from Pascobel Inc. (Longueuil, QC, Canada), Becton Dickinson Co. (Cockeysville, MD, USA), and Institut Rosell Inc. (Montreal, QC, Canada), respectively.

2.3.2 Microorganisms

The lactic acid bacteria (LAB) used in this study are commonly used for the fermentation of dairy and food products. Altogether 9 different *Lactobacillus* species (Table 2.1) that were available in our laboratory were used. Stock cultures were maintained at –80°C in MRS broth, containing 50% (v/v) glycerol. As required, these cultures were thawed and reactivated by two transfers in MRS broth.

2.3.3 Inoculum preparation

10 µl of each *Lactobacillus* from the frozen stock was inoculated to 10 ml of MRS broth, containing 1% glucose and 0.1% Tween 80, and grown at 30 or 37 °C, depending on the optimum incubation temperature for each strain (Table 2.1) for 15-18 h. A portion (50 µl) of this culture was transferred to 50 ml MRS broth in a 125 ml Erlenmeyer flask and incubated at 30 or 37 °C for 15-18 h on a rotatory shaker (Forma Scientific Inc., Marietta, OH, USA) at 150 rpm. Prior to inoculations, MRS broth was autoclaved at 121 °C for 15 min.

2.3.4 Growth optimum and effect of whey media compositions

Whey media, containing different concentrations of whey powder (12 % protein), glucose, and yeast extract (Table 2.2), were prepared for fermentation with different strains of *Lactobacillus*. Each medium, prepared at a volume of 200 ml in 500 ml

Erlenmeyer flask and autoclaved at 121 °C for 15 min, was inoculated with a seed culture at a rate of 0.1% (v/v) and subsequently incubated at 30 or 37 °C for 24 h on a rotatory shaker at 150 rpm. The viable cell count was achieved at 6 h intervals by serial dilutions of each sample in 0.85% saline solution followed by pour-plating on MRS agar.

2.3.5 Whey fermentation for the production of ACE inhibitory peptides

The procedure for whey fermentation followed by whey hydrolysates preparation is represented in Figure 2.1. For whey fermentation, 0.1% (v/v) of each *Lactobacillus* strain, which was precultured for 15-18h in MRS broth, was inoculated in 500 ml Erlenmeyer flask containing 200 ml of sterile whey medium (pH 6). The whey medium used in this study was composed of 2% (w/v) whey powder, 1% (w/v) glucose, and 0.5% (w/v) yeast extract. The fermentation was carried out at 30 or 37 °C, according to their optimal incubation temperature, for 48 h with mild agitation (150 rpm). At 12h intervals, viable cell count and pH of each whey medium were monitored. When the fermentation ceased, each sample was centrifuged (10,000×*g*, 10 min) to remove cell mass and insoluble denatured proteins and other components. The supernatant was collected, frozen (48h), and freeze-dried (7 days) using Flexi-Dry MP-Microprocessor controlled bench top lyophilizer (FTS Systems, Inc., Stone Ridge, NY), which is equipped with a condenser and a vacuum pump. The freeze-dried hydrolysates were stored at –4 °C for further analysis.

2.3.6 *In vitro* assay for ACE inhibitory activity

The inhibitory activity against ACE was measured *in vitro* by the method of Cushman and Cheung (1971), modified by Nakamura et al. (1995a). This method is based on the liberation of hippuric acid from a synthetic peptide substrate hippuryl-L-histidyl-L-leucine (Hip-His-Leu), catalyzed by ACE. The procedure for the assay is represented in Figure 2.2. After the Hip-His-Leu was dissolved in 0.1 M sodium borate buffer (pH 8.3) containing 0.3 M NaCl, 200 μl of 5 mM Hip-His-Leu solution was mixed with 80 μl of the freeze-dried whey hydrolysate, dissolved in distilled water at a concentration of 200 mg/ml (adjusted to pH 8.3), and then preincubated at 37 °C for 5 min. The reaction was initiated by the addition of 20 μl of ACE from rabbit lung dissolved in distilled water (0.1 U/ml), and the mixture was incubated for 30 min at 37 °C. The reaction was stopped by addition of 100 μl of 1 M HCl. The hippuric acid liberated by ACE was extracted from the acidified solution into 1.2 ml of ethyl acetate by vortex mixing. After a brief centrifugation, a portion (1.0 ml) of ethyl acetate layer was transferred to a clean tube. The ethyl acetate layer was evaporated and the hippuric acid was dissolved in 1.0 ml distilled water and the amount formed was measured spectrophotometrically at 228 nm. The amount of hippuric acid liberated from Hip-His-Leu under test conditions – but in the absence of an inhibitor – is defined as 100% ACE activity, and the extent of inhibition was calculated as follows:

$$(B - A)/(B - C) \times 100\%$$

where

A = the optical density in the presence of ACE and ACE inhibitory component,

B = the optical density without ACE inhibitory component, and

C = the optical density without ACE.

2.4 RESULTS AND DISCUSSION

2.4.1 Growth optimum and effect of whey media compositions

The major components of whey powder are lactose (74% w/w) and protein (12% w/w), which can provide carbon and nitrogen sources, respectively, necessary for promoting growth of lactic acid bacteria during fermentation. In order to determine the optimum whey powder concentration needed for the growth of LAB, four different culture media, containing 1, 2, 5, and 10% (w/v) whey powder, were fermented with *Lb. brevis*. Figure 2.3 shows the kinetics of microbial growth at different whey powder concentrations. The growth profiles obtained from all fermentations were very similar except for the medium containing 1% whey powder, which showed a brief stationary phase followed by death phase. In other media, the stationary phase was much longer, extending to 18h of fermentation. Only a slight increase of biomass was observed with an increase in whey powder concentration up to 5%. Above this level there was no significant increase in growth. For economical reason, culture medium containing 2% whey powder appeared to be optimal in this study as only a slight increase in biomass was achieved by increasing whey substrate by more than two fold.

The effect of yeast extract on the growth rate of *Lb. brevis* in whey fermentation was observed by fermenting three whey media (2% w/v whey powder) supplemented with different yeast extract concentration at 0, 0.5, and 1% (w/v), and monitoring the viable cell count at 6 h intervals. Figure 2.4 shows that growth of *Lb. brevis* was very low in the absence of yeast extract, but the yield increased more than 10 fold when yeast extract was added at a concentration of 0.5%. On the other hand, only a slight difference on the growth rate was observed between 0.5% supplementation and 1% supplementation. This observation was consistent with the result obtained from a study carried out by Arasaratnam et al. (1996), who reported that growth of *Lb. delbrueckii* in whey fermentation increased with an increase in yeast extract supplementation up to 10 g/L and did not observe any significant increase in growth above this level. Arasaratnam et al. (1996) suggested that this could be resulted from carbon source limitation.

It is well known that lactic acid bacteria have complex nutritional requirements for growth (Cox et al., 1977). Because whey is relatively low in essential amino acids in free form needed for the growth of these bacteria, most whey fermentations use supplements, especially yeast extract, to achieve good growth and productivity (Aeschlimann and von Stockar, 1990; Arasarathnam et al., 1996; Amrane and Prigent, 1997). In addition to nitrogen in the form of amino acids and peptides, yeast extract also supplies growth factors, such as vitamins of the B group and several organic acids including pyruvic and glyceric acid (Taso and Hanson, 1975; Cox et al., 1977).

Whey medium composed of 2% (w/v) whey powder alone can supply carbon source for proliferation of LAB, as it contains approximately 15 g/L lactose. However, lactose is not a fermentable or preferred carbon source for many microorganisms (Yang and Silva, 1995). The effect of glucose supplementation on the growth rate of *Lb. brevis* was observed by preparing three whey media, containing equal amount of whey powder (2% w/v) and yeast extract (0.5% w/v), but different concentrations of glucose (0, 1, and 2% w/v). In Figure 2.5, the growth of *Lb. brevis* slightly increased with glucose supplementation. However, the growth rates at 1% and 2% supplementations were almost identical. This could be due to inhibitory effect exerted by higher sugar concentration as suggested by Goncalves et al. (1991).

2.4.2 Whey fermentation for the production of ACE inhibitory peptides

Whey medium was fermented with selected *Lactobacillus* species for the purpose of producing potentially active peptides that inhibit ACE activity. This was based on the fact that LAB possess a number of proteinases and peptidases, which hydrolyze milk proteins to small peptides and free amino acids required for cell growth during fermentation (Law and Kolstad, 1983; Christensen et al., 1999; Siezen, 1999). Whey medium subjected to fermentation was composed of 2% (w/v) whey powder, 1% (w/v) glucose, and 0.5% (w/v) yeast extract.

Figure 2.6 shows the kinetics of microbial growth and pH change during fermentation with *Lb. brevis*. The maximal growth (about 2.0×10^8 CFU/ml) was reached

at 15 h of fermentation. However, the fermentation was continued for another 36 h to give ample time for the culture, which generally exhibits relatively weak proteolytic activity, to hydrolyze whey proteins that are globular molecules with a high degree of secondary and tertiary structure, thus making them less susceptible to proteolysis in contrast to caseins, having an open and largely random structure (Thomas and Pritchard, 1987). During fermentation, the pH decreased from about 6 to 4.1 due to lactic acid production. However, the pH decreased steadily even after the maximal microbial growth was reached. In fact, *Lb. brevis* maintained a very slow death phase. It was assumed that cell proliferation and cell lysis were undergoing simultaneously, suggesting that proteolytic activity also continued.

2.4.3 ACE inhibitory activity of whey hydrolysates

Whey hydrolysates from the fermentation were assayed for ACE inhibitory activity. Figure 2.7 shows the differences in ACE inhibitory activity among the hydrolysates produced by proteolytic activity of different *Lactobacillus* strains. Table 2.3 shows the summary of the ACE inhibitory activity derived from 9 samples. All hydrolysates generated a strong ACE inhibitory activity of more than 90%, among which *Lb. brevis* induced the highest activity (100 ± 0%), followed by *Lb. paracasei* (97.0 ± 0.5%), and the lowest activity was observed from the hydrolysate produced by *Lb. casei* (89.5 ± 2.5%). *Lb. helveticus*, which generated potent ACE inhibitory peptides from fermented sour milk (Nakamura et al., 1995a,b), also demonstrated high inhibition rate in this study. On the other hand, Pihlanto-Leppälä et al. (1998) failed to find ACE

inhibitory activity from fermentation of cheese whey with various lactic acid bacteria that are commonly used for the fermentation of dairy products. A number of factors may have attributed to such contradictory results, which include the use of different genus or species of LAB, the use of lower whey protein concentration as well as the addition of yeast extract in addition to glucose supplementation, and the application of longer fermentation time of 48 h, in contrast to 6 and 22 h in their study. All these factors may have induced higher proteolytic activity by LABs that were used in this experiment, thus producing higher amount of peptides with ACE inhibitory activity. Such assumption is supported by a study by Pihlanto-Leppälä et al. (1998), who observed the increase in proteolytic activity as well as ACE inhibitory activity of fermented whey that were further treated with pepsin and trypsin.

2.5 CONCLUSION

Fermentation of whey with lactic acid bacteria (LAB) was found to be effective in extracting ACE inhibitory peptides. Whey fermentation required the supplements, such as yeast extract and glucose, for higher biomass yield that would produce more proteolytic activity. Compared to yeast extract, the effect of glucose supplementation was insignificant. Whey hydrolysate induced by *Lb. brevis* showed the highest ACE inhibitory activity. Thus, whey fermentation can be utilized not only for the production of lactic acid and probiotic culture, but it can also be used to produce bioactive peptides.

Table 2.1 The *Lactobacillus* strains used for the fermentation of whey media.

Strains	Source	Sugar fermentation	Growth temperature
Lb. acidophilus	LAMA	Obligate homofermentative	37 °C
Lb. bifermentans	ATCC[1] 35409	Facultative heterofermentative	30 °C
Lb. brevis	KCTC[2] 3102	Obligate heterofermentative	37 °C
Lb. casei	LLG[3]	Facultative heterofermentative	30 °C
Lb. helveticus	ATCC 15009	Obligate homofermentative	37 °C
Lb. lactis	KCTC 2181	Homofermentative	37 °C
Lb. paracasei	ATCC 25302	Facultative heterofermentative	30 °C
Lb. plantarum	ATCC 14917	Facultative heterofermentative	30 °C
Lb. reuteri	ATCC 23272	Obligate heterofermentative	37 °C

[1] American Type Culture Collection.
[2] Korean Collection for Type Cultures.
[3] Cheese isolate identified by Arora and Lee (1995).

Table 2.2 Composition of fermentation media designed for determinination of optimal growth of *Lactobacillus* strains.

Media	Whey powder (%)	Yeast Extract (%)	Glucose (%)
1a[1]	1	0.5	0
1b[1]	2	0.5	0
1c[1]	5	0.5	0
1d[1]	10	0.5	0
2a[2]	2	0	0
2b[2]	2	0.5	0
2c[2]	2	1	0
3a[3]	2	0.5	0
3b[3]	2	0.5	1
3c[3]	2	0.5	2

[1] Media prepared to determine the effect of whey powder concentration.
[2] Media prepared to determine the effect of yeast extract concentration.
[3] Media prepared to determine the effect of glucose concentration.

Table 2.3 ACE inhibitory activity of whey hydrolysates obtained from whey fermentation with *Lactobacillus* strains.

Strains	Viable cell count (CFU/ml)	ACE inhibitory activity (%)*	IC_{50}[1] (mg/ml)*
Lb. acidophilus	1.8×10^8	93.0 ± 2.5	107.6 ± 2.9
Lb. bifermentan	3.2×10^8	92.0 ± 1.0	108.7 ± 1.2
Lb. brevis	2.6×10^8	100	100.0
Lb. casei	1.3×10^8	89.5 ± 2.5	111.8 ± 3.1
Lb. helveticus	1.2×10^8	93.3 ± 0.3	107.2 ± 0.4
Lb. lactis	6.0×10^8	95.4 ± 1.2	104.9 ± 1.4
Lb. paracasei	1.4×10^8	97.0 ± 0.5	103.1 ± 0.5
Lb. plantarum	1.7×10^8	93.2 ± 3.2	107.4 ± 3.7
Lb. reuteri	1.1×10^8	92.8 ± 3.8	108.0 ± 4.5

[1] Concentration of freeze-dried whey hydrolysates required to inhibit 50% of ACE activity.

*The values represent means of two different experiments with standard deviation less than 5%.

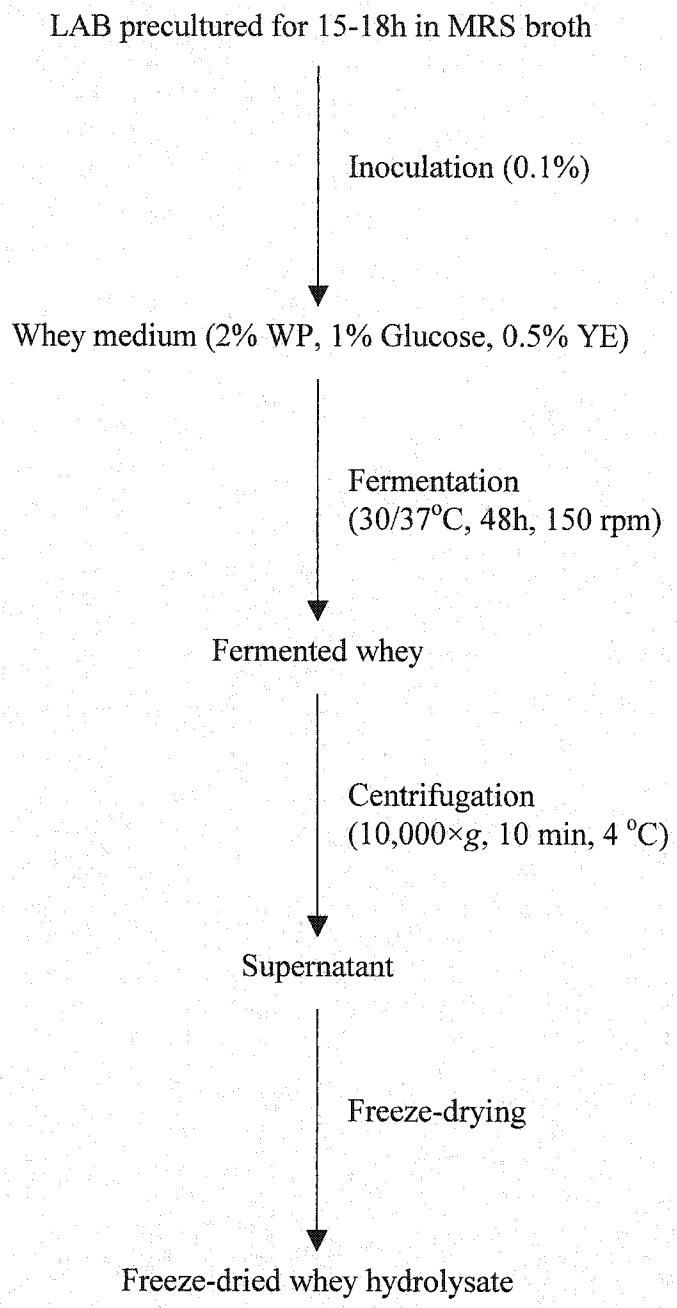

Figure 2.1 Procedure for whey fermentation with LAB and whey hydrolysate preparation.

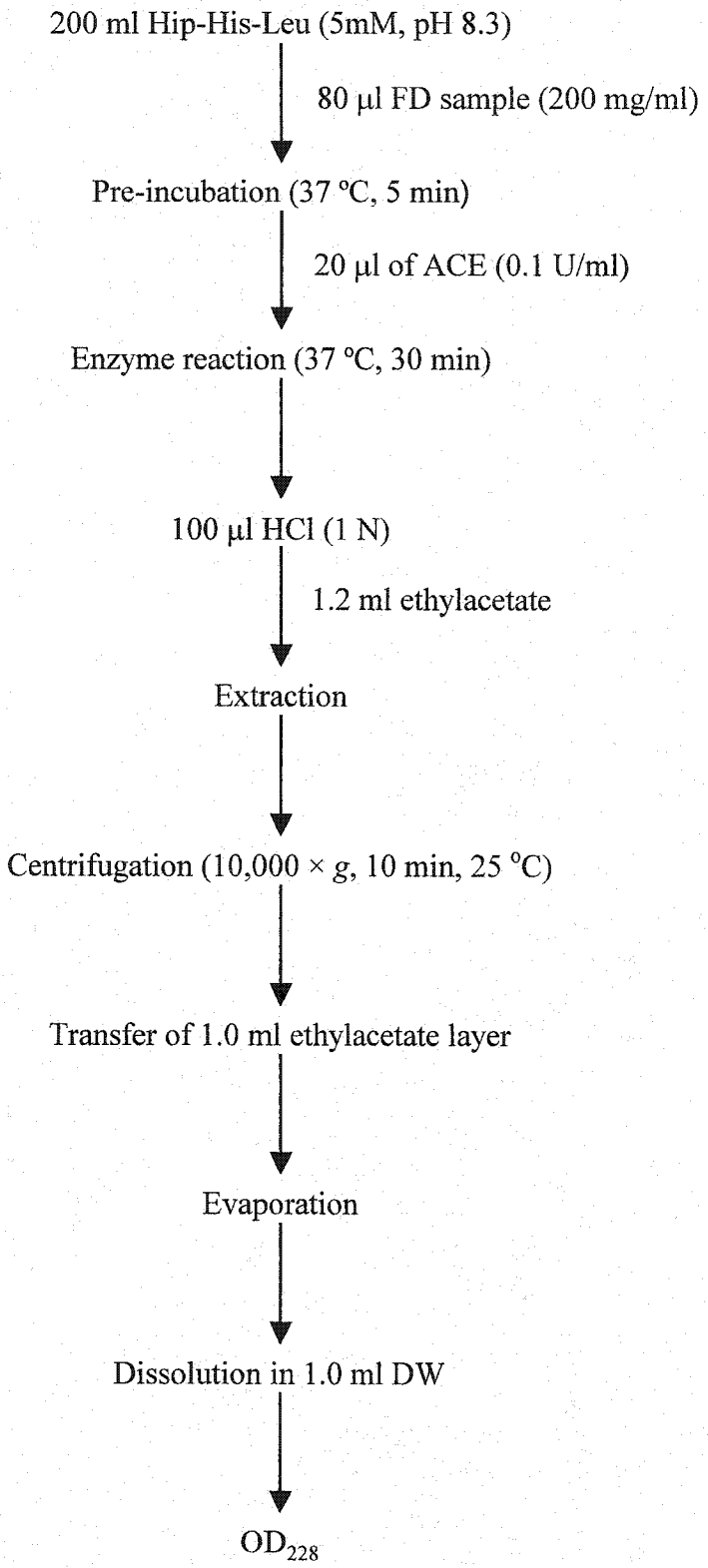

Figure 2.2 Procedure for *in vitro* ACE inhibitory assay.

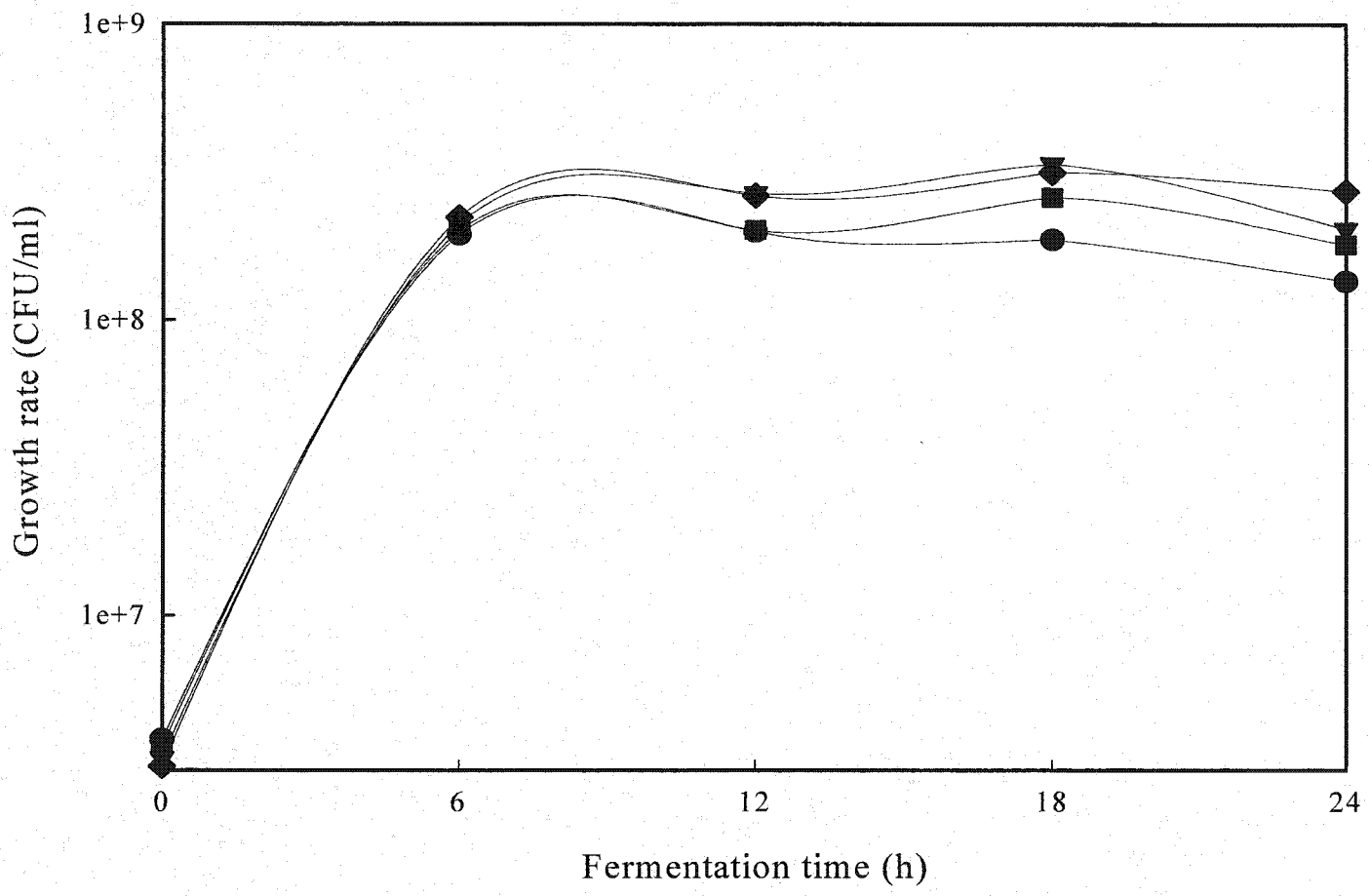

Figure 2.3 Growth of *Lb. brevis* in whey fermentation at different whey powder concentration; ● 1% WP, 0.5% YE; ■ 2% WP, 0.5% YE; ▼ 5% WP, 0.5% YE; ◆ 10% WP, 0.5% YE.

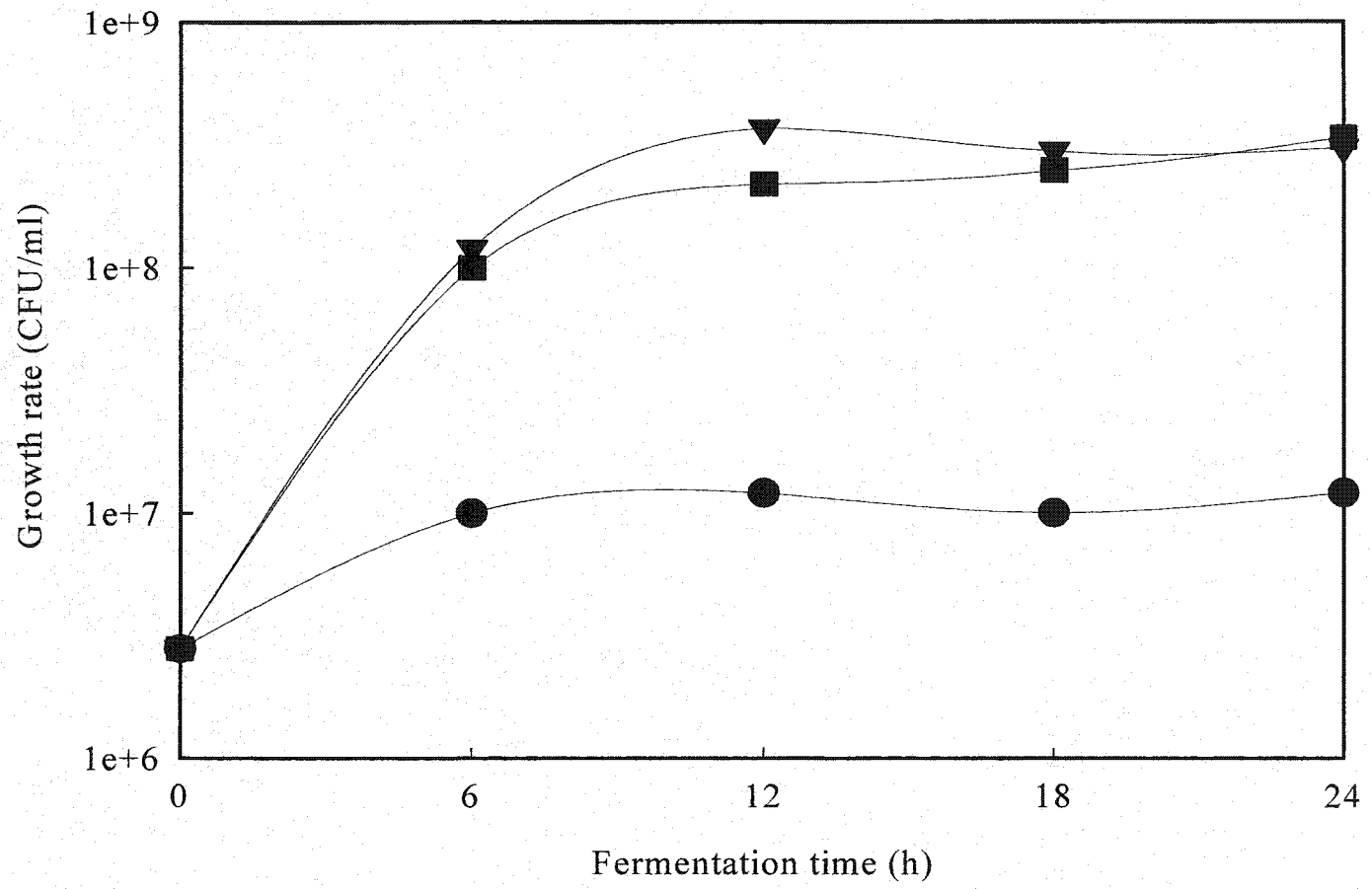

Figure 2.4 Growth of *Lb. brevis* in whey fermentation at different yeast extract concentration; ● 0% YE, 2% WP; ■ 0.5% YE, 2% WP; ▼ 1% YE, 2% WP.

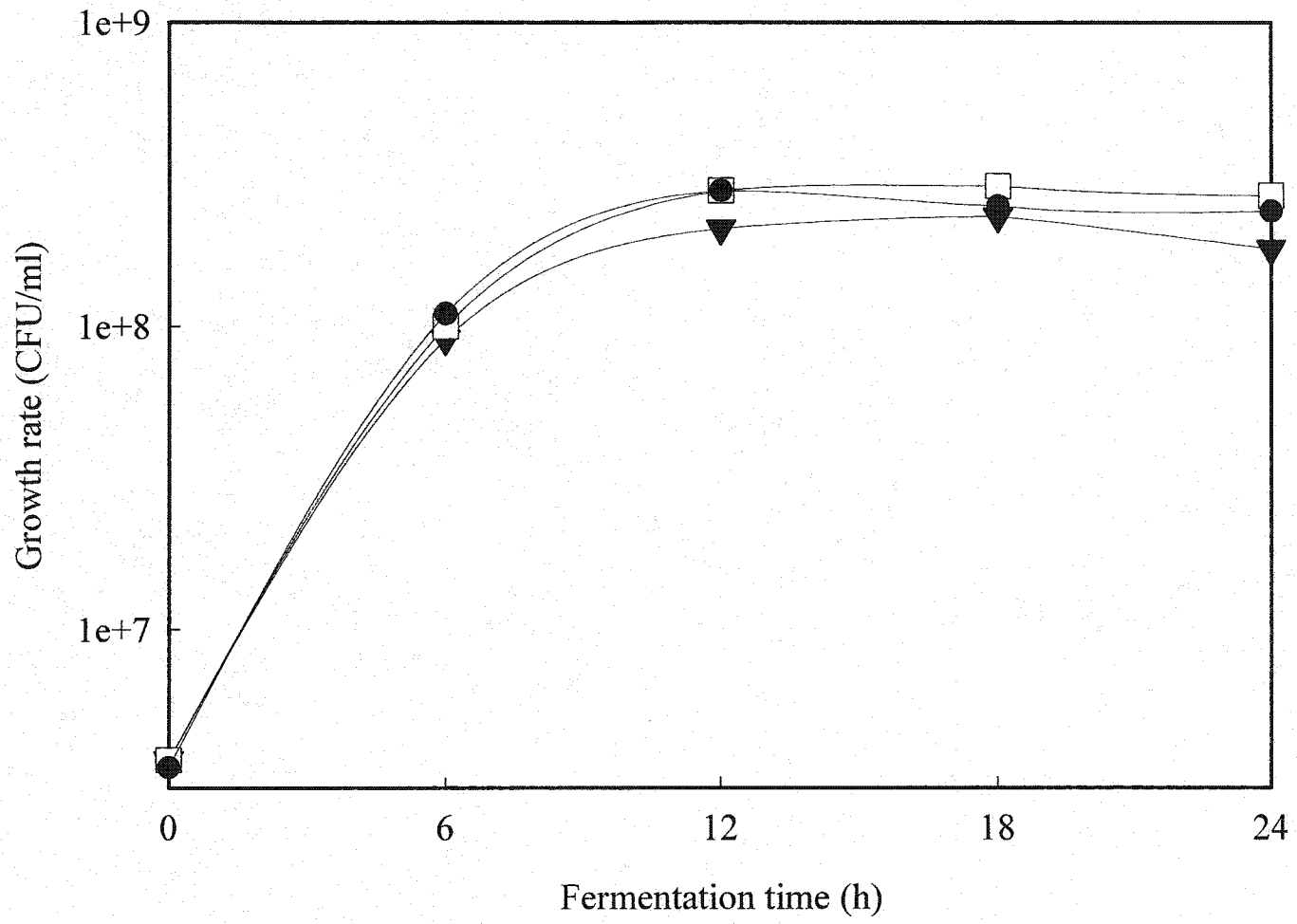

Figure 2.5 Growth of *Lb. brevis* in whey fermentation at different glucose concentration; ▼ 0% glucose, 2% WP, 0.5% YE; □ 1% glucose, 2% WP, 0.5% YE; ● 2% glucose, 2% WP, 0.5% YE.

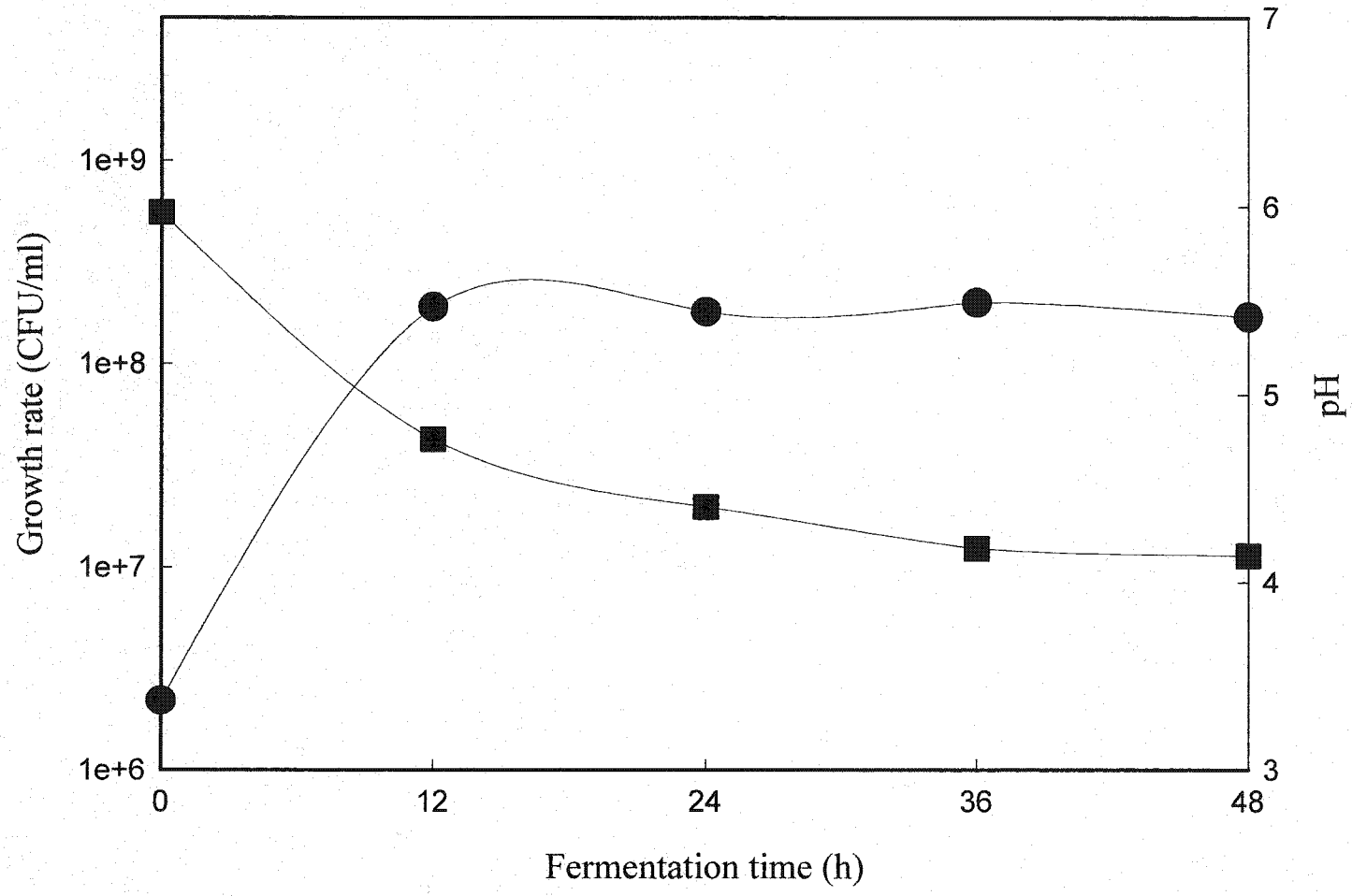

Figure 2.6 Growth rate and pH change during whey fermentation with *Lb. brevis*; ● viable cell count; ■ pH.

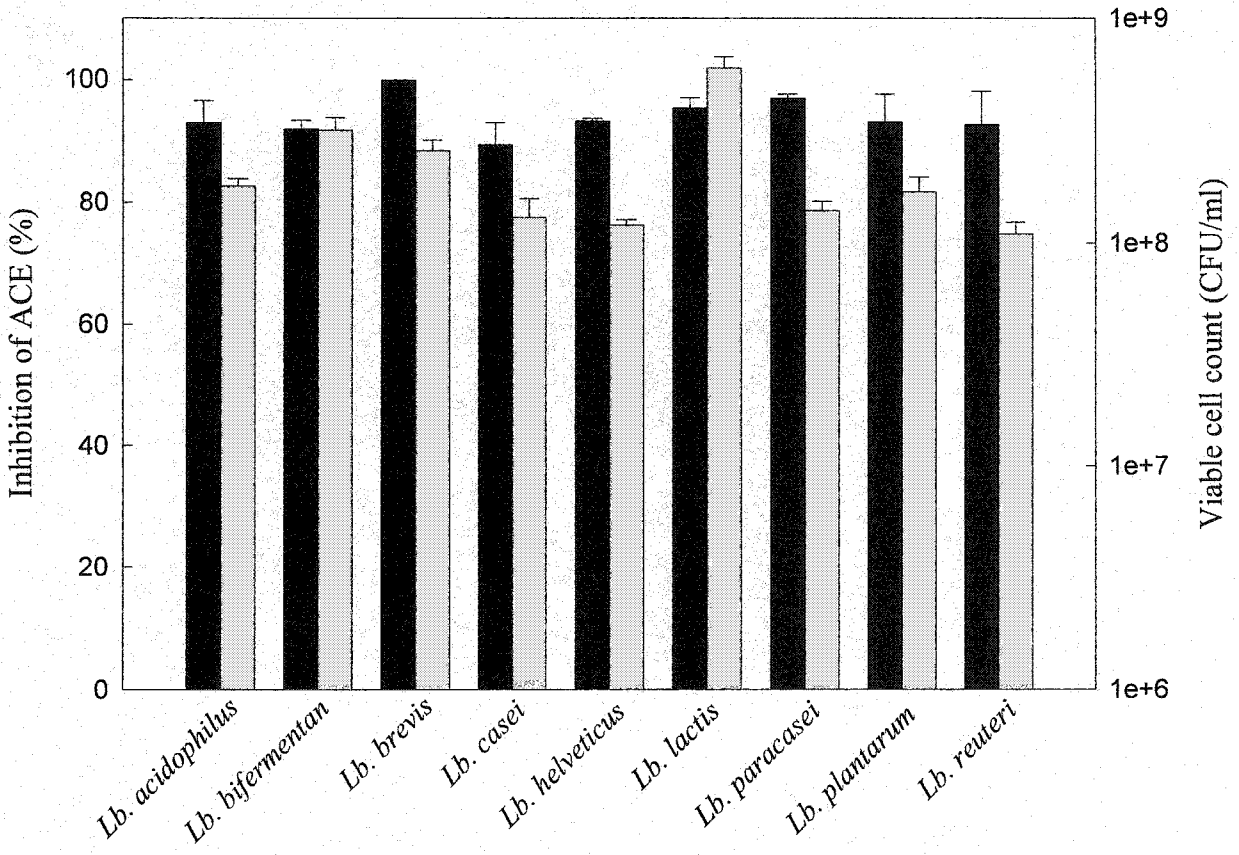

Figure 2.7 ACE inhibition of crude whey hydrolysates (at a concentration of 200 mg/ml), fermented with different strains of *Lactobacillus*, in relation to their cell count after 48 h of fermentation; ■ Inhibition of ACE; ▭ Cell count (Each error bar represents a standard deviation calculated from duplicate experiments).

CHAPTER 3

PURIFICTION AND CHARACTERIZATION OF WHEY-DERIVED ANGIOTENSIN I-CONVERTING ENZYME INHIBITORY PEPTIDES

In previous chapter whey was fermented with various strains of lactic acid bacteria (LAB) to produce whey hydrolysates with ACE inhibitory activity. The ACE inhibitory activities of these hydrolysates were compared in order to select *Lactobacillus* strains that induced whey hydrolysates with highest ACE inhibitory activity. In this chapter, potential angiotensin I-converting enzyme inhibitory peptides derived from whey, fermented with selected *Lactobacillus* strains, were purified and the amino acid sequence of these peptides was proposed.

The results of this study were summarized in the form of publication suitable for journal publication. The manuscript entitled "Purification and characterization of whey-derived angiotensin I-converting enzyme inhibitory peptides" was co-authored by Jae-Eun Ahn and Byong H. Lee. The amino acid sequencing of the peptides was conducted by Dr. Marcos DiFalco at Sheldon Biotechnology Centre (McGill University, Montreal, Quebec). The project was supervised by Dr. Byong H. Lee, while the actual experimental work and the manuscript writing were done by Jae-Eun Ahn. The manuscript was revised by Dr. Byong H. Lee.

3.1 ABSTRACT

Whey hydrolysates produced from fermenting whey with *Lb. brevis*, *Lb. helveticus*, and *Lb. paracasei* were partially purified by dialysis to remove molecules larger than 8,000 Da. The partially purified fractions were subjected to RP-HPLC, equipped with a Delta Pak C_{18} column. The RP-HPLC chromatograms showed three to seven peaks at the hydrophobic region of the elution profile. These peaks were subsequently purified for *in vitro* ACE inhibitory assay. All except one purified fractions corresponding to the peaks exhibited inhibitory activity to ACE, with IC_{50} ranging from 5.3 to 2565.8 μg/ml, but the fractions isolated from whey fermentation with *Lb. helveticus* were the most potent ACE inhibitor. The ACE inhibitory peptides in three purified fractions were characterized by Edman micro-sequencer (492 Procise®). These peptides were all composed of five amino acid residues, and most of them had either hydrophobic or aromatic amino acid at the C-terminal.

Key words: whey hydrolysates, *Lactobacillus*, ACE inhibitory peptides, purification, characterization

3.2 INTRODUCTION

Peptides that inhibit angiotensin I-converting enzyme (ACE) have been found in various food proteins (Ariyoshi, 1993; Okamoto et al., 1995; Yamamoto, 1997). Among these, milk proteins have been most extensively studied for isolation of ACE inhibitory peptides (Pihlanto-Leppälä, 1998; Takano, 1998, 2000; Yamamoto and Takano, 1999; Nurminen, 2000). Whey proteins, which represent about 20% of total milk proteins, are well known for their high nutritional value and versatile functional properties in food products (de Wit, 1998). In previous study, we reported that fermenting whey with various *Lactobacillus* strains resulted in the production of whey hydrolysates with potential ACE inhibitors. In addition, whey hydrolysates obtained from the fermentation with *Lb. brevis*, *Lb. helveticus*, and *Lb. paracasei* were found to exhibit higher ACE inhibitory activity. In this study, the whey hydrolysates produced from these three strains of *Lactobacillus* were purified and identified to investigate the potential ACE inhibitory peptides.

3.3 MATERIALS AND METHODS

3.3.1 Chemicals

Glucose ($C_6H_{12}O_6$), sodium chloride (NaCl), ethyl acetate ($C_4H_8O_2$), Hip-His-Leu, ACE from rabbit lung, Phe-Gly, *o*-phthaldialdehyde (OPA, $C_8H_6O_2$), sodium tetraborate ($Na_2B_4O_7 \cdot 10H_2O$), trifluoroacetic acid (TFA, $C_2HF_3O_2$), sodium dodecyl

sulfate (SDS, $C_{12}H_{25}O_4SNa$), and acetonitrile (CH_3CN) were purchased from Sigma Chemicals Co. (St. Louis, MO, USA), while hydrochloric acid (HCl) and sodium borate ($Na_2B_4O_7 \cdot 10H_2O$) were obtained from Fisher Scientific Company (Fair Lawn, NJ, USA). β-mercaptoethanol ($HSCH_2CH_2OH$) was purchased from Bio-Rad Laboratories (Richmond, CA, USA). Whey powder, yeast extract, and MRS were obtained from Pascobel Inc. (Longueuil, QC, Canada), Becton Dickinson Co. (Cockeysville, MD, USA), and Institut Rosell Inc. (Montreal, QC, Canada), respectively.

3.3.2 Substrates and cultures

The whey medium used in this study was composed of 2% (w/w) whey powder (12% protein), 1% glucose, and 0.5% yeast extract. Prior to fermentation, the medium was autoclaved at 121 °C for 15 min. The *Lactobacillus* strains used were *Lb. brevis*, *Lb. helveticus*, and *Lb. paracasei*.

3.3.3 Fermentation and whey hydrolysates preparation

For whey fermentation, each *Lactobacillus* strain was precultured in MRS broth for 15-18h and 0.1% (v/v) inoculum of each culture was inoculated to 200 ml of whey medium (pH 6) prepared in 500 ml Erlenmeyer flask. The fermentation was carried out at 30 °C (for *Lb. paracasei*) and 37 °C (for *Lb. brevis* and *Lb. helveticus*) for 48 h with mild agitation (150 rpm). Subsequently, the fermented whey was centrifuged (10,000 g for 10 min) to remove cell mass, insoluble denatured proteins, and other

components. After centrifugation, the supernatant was collected, frozen (48h), and freeze-dried (7 days) using Flexi-Dry MP-Microprocessor controlled bench top lyophilizer (FTS Systems, Inc., Stone Ridge, NY), which is equipped with a condenser and a vacuum pump. The freeze-dried hydrolysates were stored at –4 °C for further analysis.

3.3.4 Partial purification of whey hydrolysates

The crude whey hydrolysates (freeze-dried), obtained from whey fermentation with *Lb. brevis*, *Lb. helveticus*, and *Lb. paracasei* were separately dissolved in distilled water (200 mg/ml) and dialyzed in Spectrapor membrane tubing (6,000-8,000 Da cut-off, Spectrum Medical Industries Inc., Los Angeles, CA) to remove molecules larger than 8,000 Da. Each dialyzed portion was further freeze-dried into powder and subsequently reconstituted in distilled water at a concentration of 50 mg/ml. Each concentrate was assayed for ACE inhibitory activity in order to verify the presence of ACE inhibitors.

3.3.5 ACE inhibitory assay

The inhibitory activity against ACE was measured *in vitro* by the method of Cushman and Cheung (1971), modified by Nakamura et al. (1995a). For this assay, the Hip-His-Leu was dissolved in 0.1 M sodium borate buffer (pH 8.3) containing 0.3 M NaCl. Subsequently, 200 µl of 5 mM Hip-His-Leu solution was mixed with 80 µl of the partially purified whey hydrolysate (50 mg/ml), and then preincubated at 37 °C for 5 min. The reaction was initiated by the addition of 20 µl of ACE from rabbit lung dissolved in

distilled water (0.1 U/ml), and the mixture was incubated for 30 min at 37 °C. The reaction was stopped by the addition of 100 µl of 1 M HCl. The hippuric acid liberated by ACE was extracted from the acidified solution into 1.2 ml of ethyl acetate by vortex mixing. After a brief centrifugation, 1.0 ml of ethyl acetate layer was transferred to a clean tube. The ethyl acetate layer was evaporated and the hippuric acid was dissolved in 1.0 ml distilled water and the amount formed was measured spectrophotometrically at 228 nm. The amount of hippuric acid liberated from Hip-His-Leu under test conditions – but in the absence of an inhibitor – is defined as 100% ACE activity, and the extent of inhibition was calculated as follows:

$$(B - A)/(B - C) \times 100\%$$

where:

A = the optical density in the presence of ACE and ACE inhibitory component,

B = the optical density without ACE inhibitory component, and

C = the optical density without ACE.

3.3.6 OPA spectrophotometric assay

Peptide content was measured with *o*-phthaldialdehyde according to the method of Church et al. (1983), using a dipeptide, Phe-Gly (Sigma Chemical Co.) as a standard. This assay method is based on the reaction of *o*-phthaldialdehyde (OPA) and β-mercaptoethanol with primary amines, forming an adduct that absorbs strongly at 340 nm. For this assay, the OPA solution was prepared by combining the following reagents and diluting to a final volume of 50 ml with water: 25 ml of 100 mM sodium tetraborate

(Sigma Chemical Co.), 2.5 ml of 20% (w/w) sodium dodecyl sulfate (SDS, Sigma Chemical Co.), 40 mg of OPA dissolved in 1 ml of methanol, and 100 ml of β-mercaptoethanol. To measure the peptide content, 25 µl of purified fraction was added directly to 1.0 ml of OPA reagent in a 1.5 ml disposable cuvette; the solution was mixed briefly by inversion and incubated for 2 min at room temperature, and the absorbance at 340 nm was measured using a spectrophotometer.

3.3.7 RP-HPLC analysis

The ACE inhibitors were purified from the dialyzed concentrates by reversed-phase HPLC system (Waters, Milford, MA), consisting of 600 E system controller, a U6K injector, a 486 tunable absorbance UV detector, and a Millennium 2010 chromatography manager. For each HPLC analysis, an aliquot (100 µl) of the peptide solution were injected on a Delta Pak C_{18} column (100 Å, 30 mm × 150 mm). All samples were filtered through 0.2 µm nylon syringe filter (Chromatographic Specialties Inc., Brockville, Ontario) prior to injection. Samples were eluted for 60 min on the binary gradient mode with solvent A (100% H_2O, 0.1% trifluoroacetic acid) and solvent B (50% acetonitrile, 50% H_2O, 0.l% trifluoroacetic acid) at a flow rate of 0.5 ml/min. Gradient of solvents is shown in Table 3.1. The elution was monitored at A_{215} nm. After elution under the same conditions, a number of distinct peaks detected by HPLC were selected. The fractions corresponding to these peaks from 10 runs were collected and then lyophilized. Each lyophilized fraction was dissolved in 1 ml distilled water for the measurement ACE inhibitory activity and peptide concentration.

3.3.8 Peptide sequence

The peptide sequencing was carried out by the Edman sequencing reaction (Barrett and Elmore, 1998). Each purified fraction from HPLC analysis that was lyophilized was dissolved in 50% acetonitrile and 0.1% TFA. After the sample was diluted with 30 ml of 0.1% TFA, 20 ml was spotted onto biobrene pretreated micro TFA filters for sequencing using the pulsed-liquid method with 492 Procise® Sequencing system (Applied Biosystems, Foster City, CA).

3.4 RESULTS AND DISCUSSION

3.4.1 ACE inhibitory activity of the partially purified whey hydrolysates

Figure 3.1 shows the ACE inhibitory activity of the partially purified whey hydrolysates isolated from fermentation with *Lb. brevis*, *Lb. helveticus*, and *Lb. paracasei*, in relation to their peptide concentration. The ACE inhibition rates of the partially purified hydrolysates from *Lb. brevis*, *Lb. helveticus*, and *Lb. paracasei* were 64.7 ± 3.6%, 84.2 ± 2.9%, and 63.9 ± 3.3%, respectively. As predicted, these partially purified hydrolysates not only maintained their ACE inhibitory activity, but they also exhibited activity greater than the crude whey hydrolysates; the IC_{50} of the crude samples ranged from 100.0 to 107.2 mg/ml, whereas the IC_{50} of the partially purified samples ranged from 29.7 ± 1.1 to 39.1 ± 2.0 mg/ml (Table 3.2). This suggested that smaller peptide molecules were more responsible for the inhibitory activity than larger molecules.

Figure 3.1 also shows that despite almost equal amount of peptides in three partially purified hydrolysates (1.43 – 1.45 mg/ml), the hydrolysate from *Lb. helveticus* showed the highest inhibition rate. The IC_{50} of each hydrolysate in relation to its peptide concentration is summarized in Table 3.3. The results showed that not all peptides in the whey hydrolysates were responsible for ACE inhibitory activity, and that the hydrolysate from *Lb. helveticus* fermentation contained a high amount of bioactive peptides. The fact that *Lb. helveticus* generally has higher proteolytic activity than other lactic acid bacteria (Yamamoto et al., 1999) may have contributed to the results we obtained.

3.4.2 Purification of ACE inhibitors with RP-HPLC

Figures 3.2 through 3.4 show the HPLC elution profiles of partially purified whey hydrolysates obtained from *Lb. brevis*, *Lb. helveticus*, and *Lb. paracasei*, respectively. The sample fermented with *Lb. helveticus* gave the highest number of distinct peaks with seven peaks at the hydrophobic range of acetonitrile concentration from 10 to 30%, whereas samples fermented with *Lb. brevis* and *Lb. paracasei* produced three and four major peaks, respectively. The elution profile of hydrolysate from *Lb. helveticus* fermentation shows the highest number of major peaks, suggesting that *Lb. helveticus* has higher proteolytic activity than *Lb. brevis* and *Lb. paracasei*. In addition, the fact that certain peaks from different samples, especially the fractions B3 of *Lb. brevis*, H6 of *Lb. helveticus*, and P4 of *Lb. paracasei*, were eluted at the same retention time suggested that these peptides had similar hydrophobicity, and possibly similar amino acid compositions. Such results are in agreement with other reports, which indicated that peptides with

highly potent ACE inhibitory activity had Pro, Phe, or Tyr at the C-terminal and contain hydrophobic amino acids in the sequence (Saito et al., 1994b).

3.4.3 ACE inhibitory activity of the purified fractions

Figure 3.5 shows the ACE inhibitory activity of the purified peptide fractions that corresponded to the major peaks from the elution profiles. All peptide fractions, except P4 of *Lb. paracasei*, exhibited inhibitory activity with inhibition rate ranging from 7.6 ± 1.1 to 30.0 ± 0.1%. The highest inhibition rate was observed from the fraction H5 of *Lb. helveticus* (30.0 ± 0.1 %), followed by the fraction P1 of *Lb. paracasei* (25.9 ± 1.2%), and the lowest inhibition rate was obtained from the fraction B2 of *Lb. brevis* (7.6 ± 1.1%). However, the inhibition rate alone cannot be used to compare the ACE inhibitory activity among the peptide fractions since each peptide fraction had different peptide contents, which were determined by OPA assay (Church et al., 1983). According to the assay, the fraction P1 of *Lb. paracasei* had highest peptide content (847.9 μg/ml) (Table 3.4), thus making it one of the weakest ACE inhibitors with IC_{50} of 1640.4 ± 76.0 μg/ml even though it exhibited high ACE inhibition rate. On the other hand, the fraction H7 of *Lb. helveticus* with lower ACE inhibition rate than the fraction P1 of *Lb. paracasei* was determined as one of the most potent ACE inhibitor (IC_{50} = 7.8 ± 1.6 μg/ml) due to its lower peptide content (2.4 μg/ml). The most potent ACE inhibitor among the fractions was H5 of *Lb. helveticus* (IC_{50} = 5.3 ± 0.1 μg/ml), and the weakest ACE inhibitor was B2 of *Lb. brevis* (IC_{50} = 2637.8 ± 366.9 μg/ml). In general, peptide fractions isolated from *Lb. helveticus* fermentation were potent ACE inhibitors; except for the fraction H2, the

IC_{50} of these fractions ranged from 5.3 ± 0.1 to 358.3 ± 25.0 μg/ml. On the contrary, the fractions from *Lb. brevis* and *Lb. paracasei* were almost ten times less active. This finding indicates that fermenting whey with lactic acid bacteria, especially with *Lb. helveticus*, can produce ACE inhibitors that are as potent as casein-derived ACE inhibitors isolated from fermentation with *Lb. helveticus* and *Saccharomyces cerevisiae* (Nakamura et al., 1995a,b).

3.4.4 Peptide sequence

Table 3.5 shows the amino acid sequence of ACE inhibitors that were purified from fractionated whey hydrolysates. Sequence determination of only 3 fractions, i.e., B1 of *Lb. brevis*, H1 and H5 of *Lb. helveticus*, were possible. It was found that the peptide content in each fraction was not homogeneous. In other words, each fraction was composed of peptides with different sequences. The amino acid sequences of these peptides were Ala-Glu-Lys-Thr-Lys (β-lg f73-77) for B1, Ala-Gln-Ser-Ala-Pro (β-lg f34-38) for H1, and Ile-Pro-Ala-Val-Phe (β-lg f78-82), Ala-Pro-Leu-Arg-Val (β-lg f37-41), and Ala-His-Lys-Ala-Leu (α-la f106-110) for H5. All the peptides contained at least one alanine residue, and it was positioned at N-terminal, except for Ile-Pro-Ala-Val-Phe. The ubiquity of alanine residue in these peptides could be due to the abundance of this residue in whey proteins, specifically in β-lactoglobulin. As for the nature of C-terminal amino acids of these peptides, they were hydrophobic (Pro, Leu, and Val) or aromatic (Phe), except for lysine in B1. Such findings are consistent with previous reports by other researchers, who suggested that potent ACE inhibitors had aromatic or imino acid at the

C-terminal (Cheung et al., 1980; Saito et al., 1994b) and contained hydrophobic amino acids in the sequence (Saito et al., 1994b). The C-terminal Ala-Pro in H1 was also found in the first snake venom derived ACE inhibitor (Pyr-Lys-Trp-Ala-Pro), which was also a pentapeptide. However, unlike the snake venom derived pentapeptide with C-terminal Ala-Pro, which was the most potent snake venom derived ACE inhibitor, the pentapeptide in H1 was not highly potent. This could be due to the presence of polar serine residue in the antipenultimate position in contrast to aromatic tryptophan residue in the case of the snake venom derived pentapeptide.

These peptides were all composed of 5 amino acids that were in agreement with others, who reported that most of the potent ACE inhibitory peptides were composed of small number of amino acids. In addition to the first snake venom derived ACE inhibitor (Ferreira et al., 1970), one of the first milk casein derived ACE inhibitor was also pentapeptide (Maruyama et al., 1985). However, the potent whey protein derived ACE inhibitors so far reported were either a tetrapeptide or a heptapeptide (Mullally et al., 1996, 1997a). Two of the tetrapeptides had C-terminal phenylalanine residue, which was also found in the pentapeptide in the fraction H5. On the other hand, the N-terminal alanine residue in β-lactoglobulin derived heptapeptide (Ala-Leu-Pro-Met-His-Ile-Arg) was also found in one of the pentapeptides in H5. However, the potency of the peptides we isolated, in relation to the reported whey protein derived ACE inhibitors, is yet to be determined.

3.5 CONCLUSION

The present study demonstrated that partial purification of the crude whey hydrolysates obtained from fermenting whey with *Lb. brevis*, *Lb. helveticus*, and *Lb. paracasei* enhanced the ACE inhibitory activity, suggesting that peptides with smaller molecules were more potent ACE inhibitors. Several potential ACE inhibitory peptides were identified from the purified fractions. As expected, they were small peptides with 5 amino acid residues. Similar to many potent ACE inhibitory peptides reported so far, most of these peptides had either hydrophobic or aromatic amino acids at the C-terminal. However, these peptides were not as potent as the previously reported ACE inhibitors. The exact potency of the peptides we isolated, in comparison to other ACE inhibitors, is yet to be determined.

Table 3.1 HPLC gradient of solvents at 0.5 ml/min flow rate.

Time (min)	Solvent A[1] (%)	Solvent B[2] (%)	Ratio
0	100	0	Isocratic
5	100	0	Isocratic
40	60	40	Gradient
45	0	100	Gradient
50	0	100	Isocratic
55	100	0	Gradient
60	100	0	Isocratic

[1] Solvent A was composed of 100% water and 0.1% TFA.
[2] Sovent B was composed of 50% water, 50% acetonitrile, and 0.1% TFA.

Table 3.2 ACE inhibitory activity of whey hydrolysates obtained from whey fermentation with 3 *Lactobacillus* strains.

Sample type	Strains	Sample concentration (mg/ml)	ACE inhibitory activity (%)*	IC_{50}[1] (mg/ml)*
Crude				
	Lb. brevis	200	100	100.0
	Lb. helveticus	200	93.3 ± 0.3	107.2 ± 0.4
	Lb. paracasei	200	97.0 ± 0.5	103.1 ± 0.5
Partially purified				
	Lb. brevis	50	64.7 ± 3.6	38.8 ± 2.2
	Lb. helveticus	50	84.2 ± 2.9	29.8 ± 1.1
	Lb. paracasei	50	63.9 ± 3.3	39.3 ± 2.0

[1]Concentration of freeze-dried whey hydrolysates required to inhibit 50% of ACE activity.

*The values represent means of two different experiments with standard deviation less than 6%.

Table 3.3 ACE inhibitory activity of the partially purified whey hydrolysates in relation to their peptide concentration.

Strains	Peptide concentration[1] (mg/ml)*	ACE inhibitory activity (%)*	IC_{50}[2] (mg/ml)*
Lb. brevis	1.45 ± 0.01	64.7 ± 3.6	1.13 ± 0.07
Lb. helveticus	1.44 ± 0.01	84.2 ± 2.9	0.86 ± 0.03
Lb. paracasei	1.43 ± 0.01	63.9 ± 3.3	1.13 ± 0.06

[1] Peptide content of each fraction was determined by OPA spectrophotometric assay (Church et al., 1983).
[2]Concentration of peptides in the sample required to inhibit 50% of ACE activity.
*The values represent means of two different experiments with standard deviation less than 6%.

Table 3.4 ACE inhibitory activity of the purified fractions isolated from whey hydrolysates.

Strains	Purified fraction[1]	Peptide concentration[2] (μg/ml)	ACE inhibition (%)*	IC_{50}[3] (μg/ml)*
Lb. brevis				
	B1	83.5	8.2 ± 0.0	509.1 ± 0.0
	B2	390.6	7.6 ± 1.1	2637.8 ± 366.9
	B3	80.4	13.9 ± 1.2	291.4 ± 25.2
Lb. helveticus				
	H1	87.5	19.2 ± 4.0	238.6 ± 49.2
	H2	236.2	10.5 ± 0.6	1133.3 ± 59.7
	H3	7.2	12.3 ± 2.4	30.6 ± 3.0
	H4	6.4	18.6 ± 0.7	17.3 ± 0.7
	H5	3.2	30.0 ± 0.1	5.3 ± 0.1
	H6	82.0	11.5 ± 0.8	358.3 ± 25.0
	H7	2.4	16.1 ± 3.2	7.8 ± 1.6
Lb. paracasei				
	P1	847.9	25.9 ± 1.2	1640.4 ± 76.0
	P2	84.4	-	-
	P3	354.4	9.3 ± 0.3	1917.1 ± 51.8
	P4	47.3	12.9 ± 0.4	183.5 ± 5.7

[1] The purified fractions were obtained from RP-HPLC analysis.
[2] Peptide content of each fraction was determined by OPA spectrophotometric assay.
[3] Concentration of ACE inhibitor required to inhibit 50% of ACE activity.
* The values represent means of two different experiments with standard deviation less than 21%.

Table 3.5 Proposed amino acid sequence of potential ACE inhibitors purified from hydrolysates produced from whey fermentation with 3 strains of *Lactobacillus*.

Strains	Fraction	Peptide sequence	Fragment
Lb. brevis	B1	AEKTK	β-lg f73-77
Lb. helveticus	H1	AQSAP	β-lg f34-38
	H5	IPAVF	β-lg f78-82
		APLRV	β-lg f37-41
		AHKAL	α-la f106-110

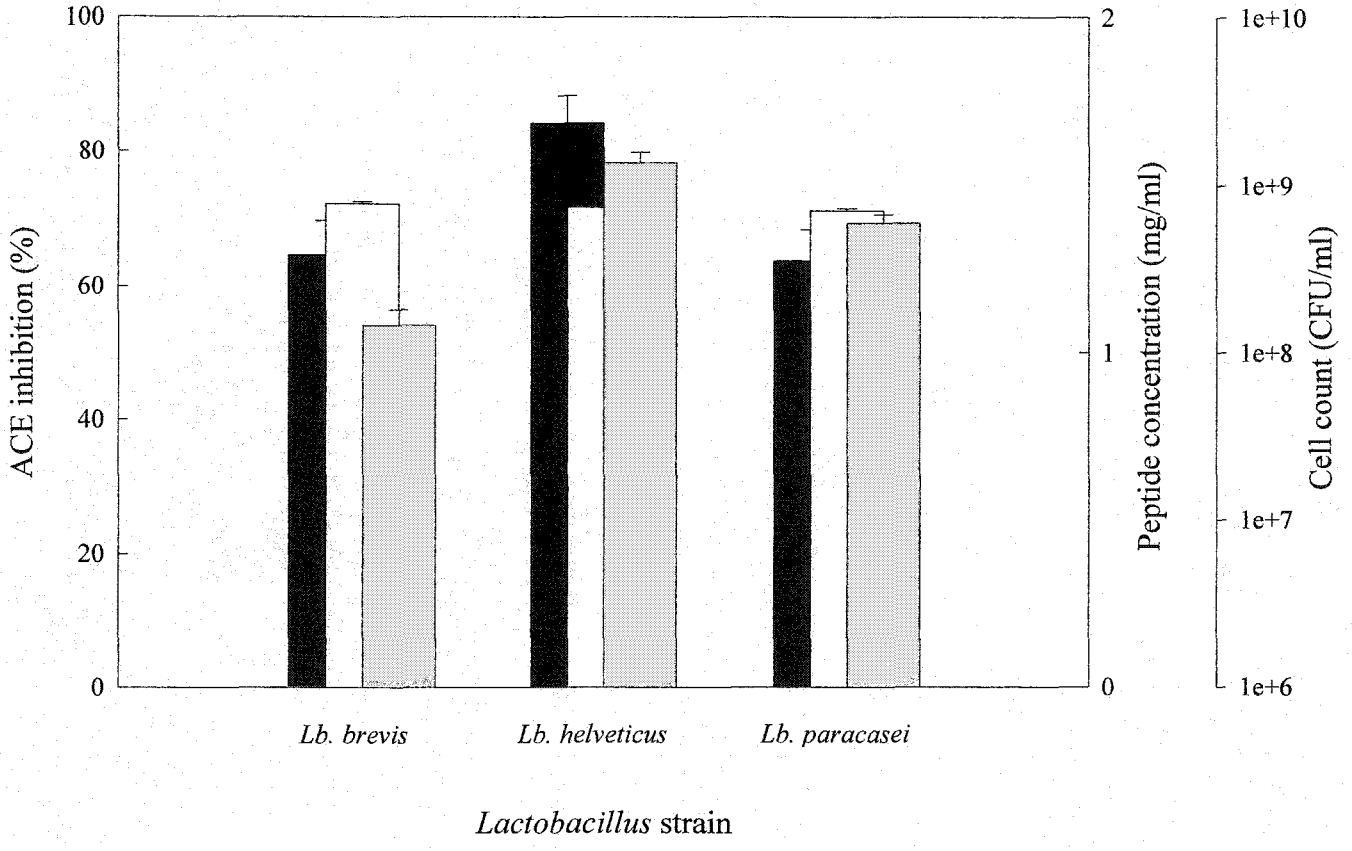

Figure 3.1 ACE inhibitory activity of partially purified whey hydrolysates in relation to their peptide concentration.and cell count; ■ ACE inhibition; □ Peptide concentration; ▭ Cell count (Each error bar represents a standard deviation calculated from duplicate experiments).

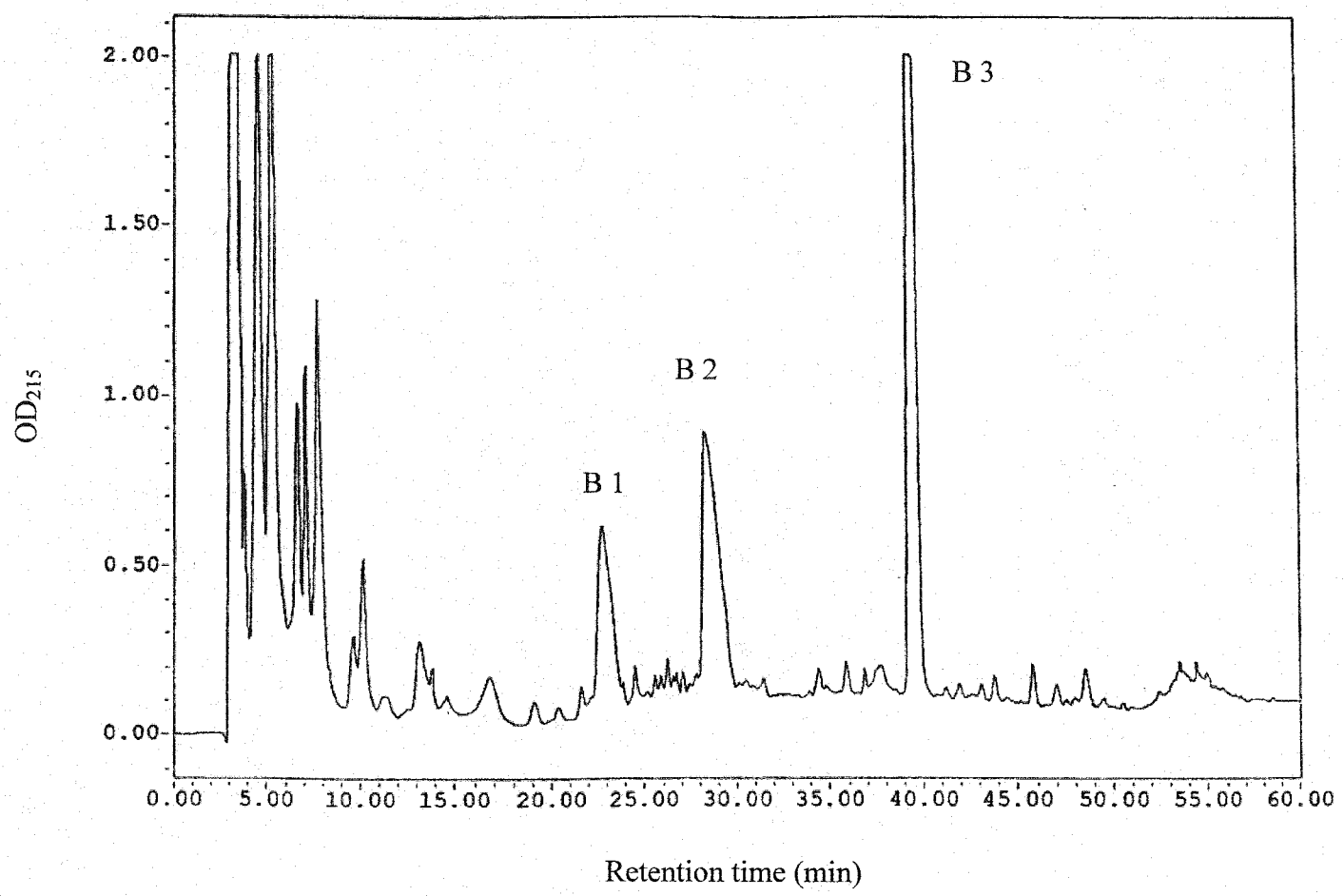

Fig. 3.2 HPLC chromatogram of partially purified whey hydrolysate isolated from fermentation with *Lb. brevis*; the peaks that are purified for ACE inhibitory assay are labeled.

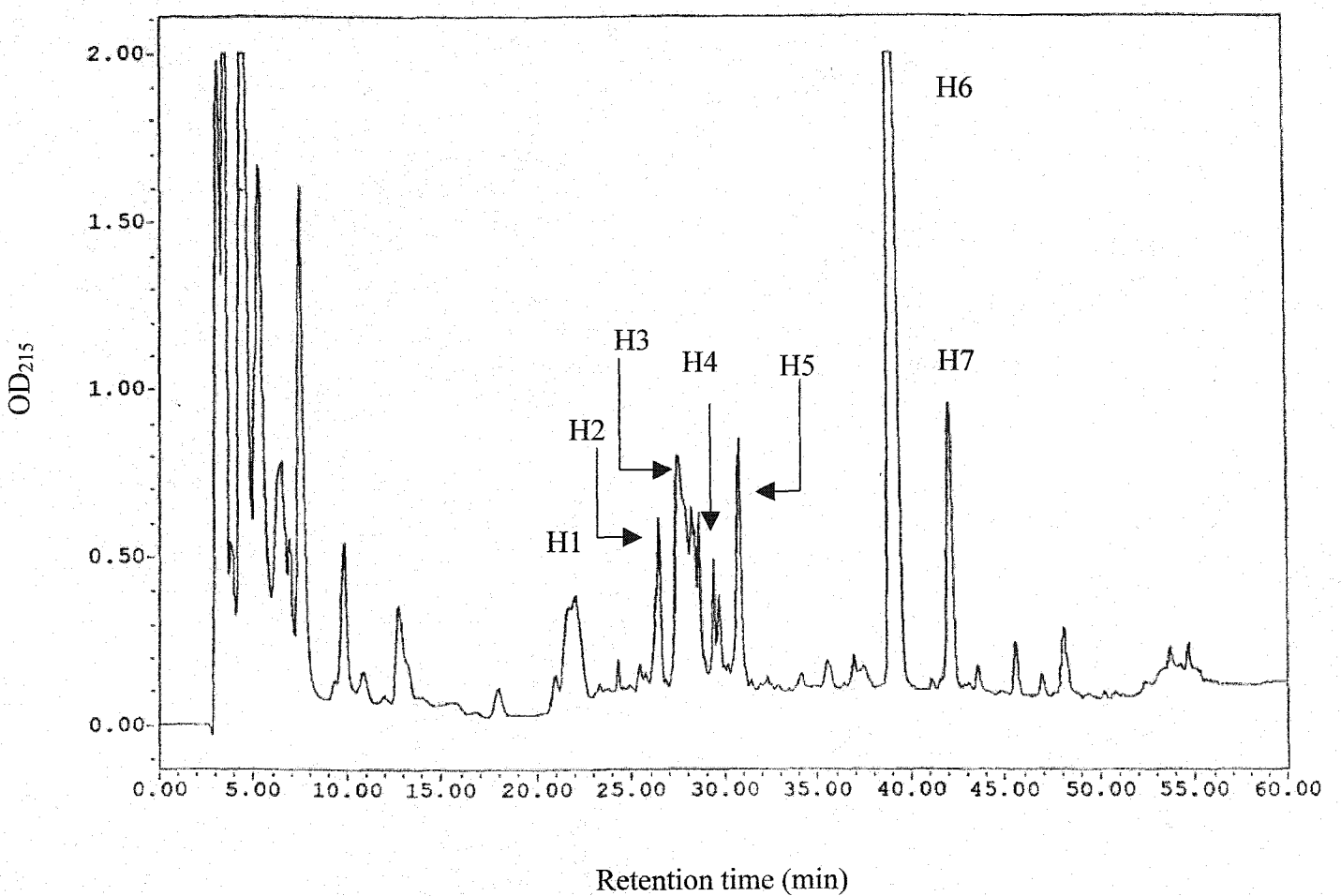

Fig. 3.3 HPLC chromatogram of partially purified whey hydrolysate isolated from fermentation with *Lb. helveticus*; the peaks that are purified for ACE inhibitory assay are labeled.

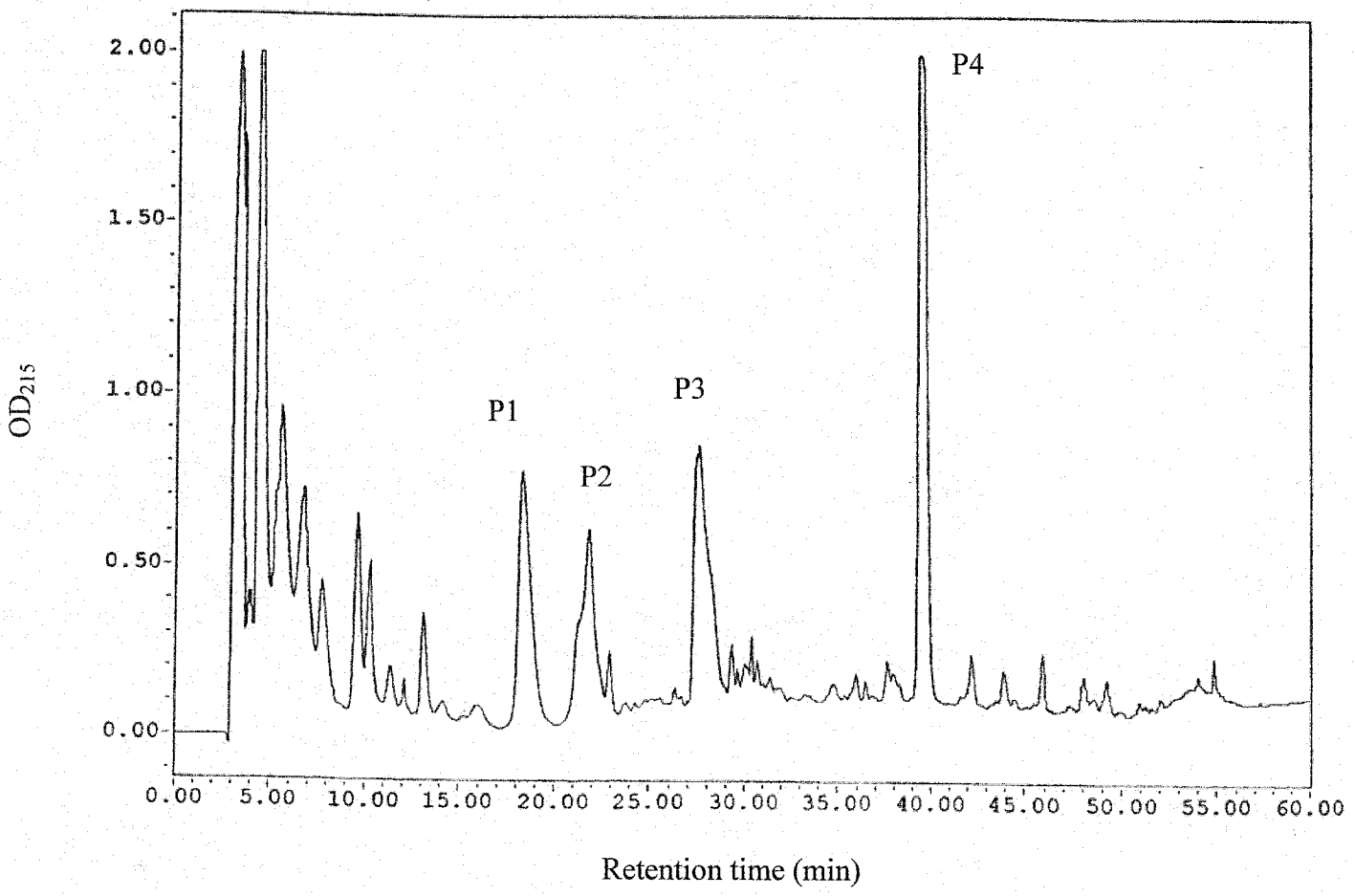

Fig. 3.4 HPLC chromatogram of partially purified whey hydrolysate isolated from fermentation with *Lb. paracasei*; the peaks that are purified for ACE inhibitory assay are labeled.

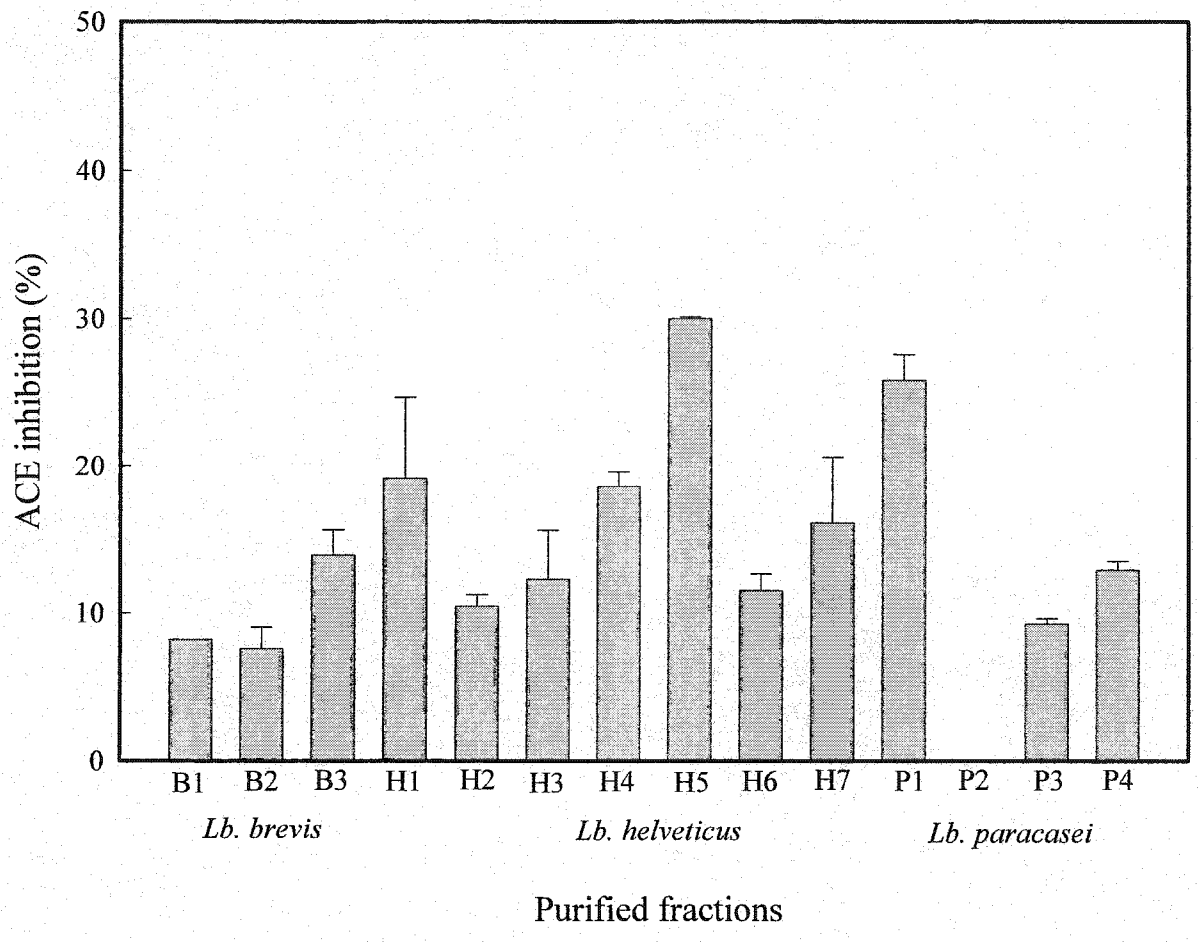

Fig. 3.5 ACE inhibitory activity of the purified fractions from reversed-phase HPLC (Each error bar represents a standard deviation calculated from duplicate experiments).

GENERAL CONCLUSION

Fermentation of whey by lactic acid bacteria was found to be effective in producing hydrolysates that contained potential ACE inhibitory peptides. Supplementing the whey with glucose and yeast extract increased biomass yield that would produce more proteolysis, hence higher number of peptides. The optimal bacterial growth was achieved in the medium containing 2% (w/v) whey powder, 1% (w/v) glucose, and 0.5% (w/v) yeast extract. However, glucose effect was not as significant as the yeast extract supplementation in improving the bacterial growth.

Among the nine *Lactobacillus* strains studied, *Lb. brevis*, *Lb. helveticus*, and *Lb. paracasei* were most effective in inducing ACE inhibitory peptides with inhibition rate ranging from 93.3 ± 0.3% to 100% at a concentration of 200 mg/ml. Partial purification of these hydrolysates by dialysis (6,000-8,000 Da) increased their inhibitory activity to more than two fold, indicating that smaller peptides were more potent ACE inhibitors.

Five ACE inhibitory peptides were identified from the purified fractions that were eluted at the hydrophobic region of HPLC chromatogram. These peptides were all composed of five amino acid residues, and most of them had either a hydrophobic (Pro, Val, and Leu) or aromatic (Phe) amino acid at the C-terminal. In fact, these peptides exhibited higher ACE inhibitory activities (IC_{50} = 5.3 ± 0.1 – 238.6 ± 49.2 μg/ml) than the peptide with hydrophilic amino acid (Lys) at the C-terminal (IC_{50} = 509.1 ± 0.0 μg/ml).

Even though the exact potency of the naturally occurring peptides we isolated, in comparison to commercial antihypertensive drugs, is yet to be determined, they may represent nutraceutical/functional ingredients with mild antihypertensive effect.

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APPENDIX I

Amino acid symbols

Amino acid	Three-letter code	One-letter code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V
Termination	End	U
Asn or Asp	Asx	B
Gln or Glu	Glx	Z
Unknown	Xxx	X