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Antihypertensive peptides from skimmed milk hydrolysate digested by cell-free extract of *Lactobacillus helveticus* JCM1004

Daodong Pan [a,*], Yongkang Luo [b], Masaru Tanokura [c]

[a] *Department of Food Science and Nutrition, Nanjing Normal University, Nanjing 210097, China*
[b] *Department of Food Science and Nutrition, College of China Agricultural University, Beijing 10083, China*
[c] *Laboratory of Food Engineering, Graduate School of Agricultural and Life Sciences, University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan*

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Abstract

In the present experiments, the proteinase, aminopeptidase and *x*-prolyl-dipeptidyl aminopeptidase activitives were measured at 513 ± 11.9, 376 ± 9.3 and 23.6 ± 1.6 units per gramme of cell-free extract of *Lactobacillus helveticus* JCM1004. ACE-inhibitory activity of skimmed milk hydrolysate produced by cell-free extract of *L. helveticus* JCM1004 was determined, and the optimum pH and hydrolysis time for the production of ACE-inhibition substances from skimmed milk were 6.5–7.0 and 6–10 h, respectively. Peptides of Val-Pro-Pro and Ile-Pro-Pro with potent ACE inhibitor and antihypertensive activitives were purified from the hydrolysate by three step-reverse-phase high-performance liquid chromatography. The IC_{50} values of Val-Pro-Pro and Ile-Pro-Pro were 9.13 ± 0.21 and 5.15 ± 0.17 μM, respectively. Significant decrease ($p < 0.01$) in SBP of SHR was measured after a single gastric intubation of Val-Pro-Pro or Ile-Pro-Pro at 8 or 4 h, respectively.

Keywords: *Lactobacillus helveticus*; Cell-free extract; Peptide; ACE inhibition; Antihypertensive

1. Introduction

Angiotensin-1-converting enzyme (ACE; peptidyldipeptide hydrolase, EC 3.4.15.1) is an exopeptidase that cleaves dipeptides from the C-terminal ends of various peptide substrates and regulates the activity of several endogenous bioactive peptides. Two basic reactions, conversion of inactive decapeptide angiotensin-I to the potent vasoconstrictor octapeptide angiotensin-II and inactivation of the vasodepressor nonapeptide bradykinin, catalysed by ACE, play key physiological roles in regulating blood pressure (Erdos & Skidgel, 1987; Johnston, 1992).

After the successful development of captopri as the orally active ACE inhibitor, a variety of potent inhibitors have been discovered from various types of food protein or have been synthesized (Brown & Vaughan, 1998; Kawasaki et al., 2000; Maruyama & Suzuki, 1982; Matsufuji et al., 1994; Matsui, Li, & Osajima, 1999; Matsui & Matsumoto, 2000; Miyoshi et al., 1991; Sugai, 1998). There are various substances originating in milk or milk products with diverse physiological activities, such as anti-microbial activity, anti-thrombotic activity, immunomodulatory activity and opioid activity (Abubakar, Satio, & Kitazawa, 1999; Daniel, Vohwinkel, & Rehner, 1990; Laffineur, Genetet, & Leonil, 1996; Loukas, Varoucha, Zioudrou, Streaty, & Klee, 1983; Yamauchi, 1992). Some physiologically active substances, such as bioactive peptides, are present in their inactive forms in the amino acid sequences

* Corresponding author. Tel.: +86-25-83598286; fax: +86-25-83707623.
E-mail address: pdd64@sina.com (D. Pan).

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(peptides) of milk proteins but can be released by enzymic proteolysis during, for example, fermentation or gastrointestinal digestion (FitzGerald & Meisel, 2000; Margaret, Hans, & Richard, 1997; Mullally, Meisel, & FitzGerald, 1997; Nakamura, Masuda, & Takano, 1996; Nakamura et al., 1995; Yamamoto, 1997). Identification and characterization of the enzyme involved in the production of physiologically active substances from milk proteins seems to be important for increasing the quality of processed food with dynamic functions, which the food exerts after ingestion.

Lactobacillus helveticus is widely used in the dairy industry to manufacture traditional fermented milk products, such as yogurt and cheese. However, these nutritional fastidious bacteria require a more free amino acids or peptides than are present in milk. Proteolytic activity is thus very important for lactobacillus growth in milk (Prichard & Coobear, 1993; Thomas & Pritchard, 1987). This proteolytic activity is also related to certain physiological functions in humans who have consumed cultured milk. Recently, a few of researchers have reported that milk proteins contain peptidic angiotensin I-converting (ACE) inhibitors, which can be released by proteolysis during milk fermentation by some strains of *L. helveticus* (Maeno, Yamamoto, & Takano, 1996; Yamamoto, Akino, & Takano, 1994a,b, 1999).

The aim of this work is to describe the production and purification of ACE inhibition peptides from skimmed milk hydrolysate produced by cell-free extract of *L. helveticus* JCM 1004, and the antihypertensive effects of purified peptides in spontaneously hypertensive rats.

2. Materials and methods

2.1. Materials

Hippuryl-L-histidyl-L-leucine (Hip-His-Leu), fluorescein isothiocyanated casein, L-lysine-*p*-nitroanilide, glycyl-prolyl *p*-nitroanilide and purified rabbit lung ACE were purchased from Sigma Chemical Co. Ltd. (St. Louis, MO); Other chemicals were from Wako Pure Chemicals Industries Co. Ltd. (Tokyo, Japan).

2.2. Bacterial strains, growth conditions and preparation of cell-free extract

Lactobacillus helveticus JCM1004, obtained from the Institute of Physical and Chemical Research (Hirosawa, Wako, Saitama, Japan), was used. The organisms were grown in 4 litres of MRS (1.0% yeast extracts, 1.0% polypeptone and 1.0% glucose) medium at 37 °C for 20 h, with shaking at 120 rpm.

Cells were harvested by centrifugation at 6000*g* for 10 min at 4 °C and washed three times with distilled water. The cells were suspended in 50 mM Tris hydrochloride buffer (pH 7.0) and sonicated with glass beads (0.3 mm diameter, 50 g) for 25 min (Insonator Model 200M, Kubota, Tokyo, Japan; 200 W, 1.5A) at 4 °C. The glass beads and cell debris were removed by centrifugation at 15,000*g* for 15 min at 4 °C. The supernatant was dialysed extensively against distilled water at 4 °C and the resulting insoluble materials were removed by centrifugation. Clear supernatant was lyophilized and used as cell-free extract.

2.3. Proteinase activity assay of cell-free extract

Proteinase activity was determined according to Twing (1984) with some modification. The reaction mixture contained 20 μl of 0.4% florescein isothiocyanated casein and 30 μl of cell-free extract in 50 mM Tris–HCl buffer pH 6.5. The reaction mixture was incubated at 42 °C for 60 min, then 120 μl of trichloroacetic acid (TCA) were added and the mixture was centrifuged at 16,000*g* for 10 min. 60 μl of the supernatant were neutralized with 3 ml of 500 mM Tris–HCl buffer (pH 8.5), and the fluorescence was measured using a fluorescence spectrophotometer 204 (Hitachi) with an excitation wavelength of 490 nm and an emission wavelength of 525 nm. One unit of proteolytic activity was defined as the amount of cell-free extract yielding 1% of total initial casein fluorescence as TCA-soluble fluorescence after 60 min of hydrolysis.

2.4. Aminopeptidase activity assay of cell-free extract

Aminopeptidase activity was measured during purification by the procedure of Soda (Soda & Desmazeaud, 1982) with L-lysine-*p*-nitroanilide as the substrates. The incubation mixture contained 0.05 ml (16.4 mM) of substrates, 2.85 ml of 50mM Tris-HCl buffer (pH 7.0), and 0.1 ml of cell-free extract in 50 mM Tris–HCl buffer (pH 7.0). Incubation was at 37 °C for 20 min. The reaction was stopped by the addition of 0.5 ml of 30% acetic acid, and the extent of hydrolysis was measured at A_{410}. One unit of aminopeptidase activity was defined as the amount of enzyme which produced a variation of 0.1 unit of A_{410} per min at 37 °C.

2.5. X-prolyl-dipeptidyl aminopeptidase activity assay of cell-free extract

X-prolyl-dipeptidyl aminopeptidase activity was assayed as described by (Khalid & Marth, 1990) with glycyl-prolyl *p*-nitroanilide (Gly-Pro-pNA) as the substrate. The incubation mixture contained 0.05 ml (6.4 mM) of substrates, 2.85 ml of 50 mM Tris–HCl buffer (pH 7.0), and 0.1 ml of cell-free extract in 50

mM Tris–HCl buffer (pH 7.0). Incubation was at 37 °C for 20 min. The reaction was stopped by the addition of 0.5 ml of 30% acetic acid, and the extent of hydrolysis was measured at A_{410}. One unit of enzyme activity was defined as the amount of enzyme which produced a variation of 0.1 unit of A_{410} per min at 37 °C.

2.6. Assay for ACE inhibitory activity

Activity, for the production of substances with ACE inhibition activity from skimmed milk, was assayed as follows: 10 ml of 10% (w/w) skimmed milk were mixed with 1.0 ml of cell-free extract solution and pH was adjusted to 7.0 unless otherwise stated. The mixture was then incubated at 37 °C for 6 h. The resulting mixture was heated at 90 °C for 5 min and pH was adjusted to 3.8 with 50% lactic acid solution. After centrifugation at 7000*g* for 10 min, supernatant was collected and pH was readjusted to 7.0; re-centrifugation at 7000*g* for 10 min, the clear supernatant was filtered using ultrafiltration on the basis of molecular weights (5 kDa MWCO) and lyophilized. The lyophilized material was dissolved in 1.0 ml of 0.1 M borate buffer containing 0.3 M NaCl (pH 8.3), and the resulting solution was centrifuged to remove the insoluble materials. The supernatant was filtered through 0.45 μm PVDF filters. Then the ACE-inhibition activity of the filtrate was measured according to (Cushman & Cheung, 1971) with some modification. Briefly, 200 μl of HHL buffer (5 mM Hip-His-Leu in 0.1 M borate buffer containing 0.3 M NaCl, pH 8.3) were mixed with 80 μl of sample solution and pre-incubated for 3 min at 37 °C. The reaction was initiated by adding 20 μl of ACE (dissolved in distilled water, 0.1 units/ml), and the mixture was incubated for 30 min at 37 °C. The reaction was stopped by adding 250 μl of 1.0 N HCl and mixed with 1.7 ml of ethyl acetate. The liberated hippuric acid was extracted with ethyl acetate, and 1.0 ml of ethyl acetate was removed from the ethyl acetate layer after centrifugation(8000*g*, 15 min) and lyophilized. The lyophilized material was redissolved in 1.0 ml of distilled water and the absorbance was measured at 228 nm using a spectrophotometer to measure the ACE activity. The average value from three determinations at each concentration was used to calculate the IC_{50} value and ACE-inhibitor rate. The extent of inhibition was calculated as follows :

$$\text{ACE} - \text{inhibitor rate } (\%) = [(B - A)/(B - C)] \times 100\%,$$

where A is the absorbance in the presence of ACE and with the ACE-inhibitory component, B is the absorbance with ACE and without the ACE-inhibitory component, C is the absorbance without ACE or ACE inhibitor component. The concentration of an ACE inhibitor needed to inhibit 50% of the ACE activity was defined as IC_{50} under these conditions.

2.7. Optimum-conditions assay for the production of ACE-inhibition materials from skimmed milk

5.0 ml skimmed milk (10%, w/w) were mixed with lyophilized cell free extract and the suspension was adjusted to various pH values from 5.5 to 8.0 for conducting a pH-dependent digestion of skimmed milk. The samples were then prepared by incubating the suspensions at 37 °C for 6 h as described above. In a separate experiment, a time-course analysis of skimmed-milk digestion was conducted by incubating the suspension (pH 7.0) at 37 °C for various times from 2.0 to 12.0 h. After incubation, samples were prepared as above.

2.8. Purification of ACE inhibition peptides

ACE inhibitors were purified by three-step reversed-phase high-performance liquid chromatography (RP-HPLC). Solvent A was acetonitrile-water-trifluoroacetic acid (100:900:1 by volume) and solvent B contained the same components in different proportions (900:100:0.8 by volume). In the first step, the lyophilized hydrolysate was dissolved in solvent A, and filtered through 0.45 μm PVDF filters; the filtered solution was applied on to a YMC-Pack ODS—AP—303 (4.6 × 250mm, YMC Co., Ltd, Japan) column and eluted with a linear solvent B gradient from 0% to 100% at a flow rate of 1.0 ml/min; fractions showing ACE inhibitory activitives were pooled and lyophilized. In the second step, the fractions with the most efficient ACE inhibitory activity were dissolved in solvent A, and then applied to a ODS-100S (3.9 × 150 mm, YMC Co., Ltd, Japan) column and eluted with a three segment linear solvent B gradient (0–40%, 40 min; 40–70%, 28 min; 70–100%, 22 min) at a flow rate of 1.0 ml/min. The fractions with the most efficient ACE inhibitory activity were further purified with a μBondasphere C_{18} (3.9 × 150 mm, Waters Inc., Tokyo, Japan) column and eluted with a three segment linear solvent B gradient (0–30%, 45 min; 30–65%, 25 min; 65–100%, 20 min) at a flow rate of 1.0 ml/min. Detection in all above RP-HPLC purifications was performed at 215 nm. Peptides with ACE inhibitory activity were collected and lyophilized. ACE inhibitory activity of each chromatographic peptide peak was tested three times to discriminate the most efficient peptides. Peptide content was measured with *o*-phthaldialdehyde, according to the method of Church et al. (Church, Swaisgood, Proter, Proter, & Catignai, 1983), using bactotrypton (Difco, Detroit, MI) as a standard.

2.9. Amino acid and sequence analysis of inhibition peptides

The amino acid composition was analyzed with an amino acid analyzer after hydrolysis of 6.0 N HCl for 24 h at 110 °C. The amino acid sequence was analyzed

by automated Edman degradation, using a Shimadzu PPSQ-21 protein sequencer (Shimadzu, Kyoto, Japan) according to the manufacturer's instructions.

2.10. Antihypertensive effects of VPP and IPP

Eight male spontaneously hypertensive rats (SHR, aged 18 weeks) purchased from Charles River Japan (Yokohama, Japan). Control, experimental groups of Val-Prp-Pro (VPP) and Ile-Prp-Pro (IPP) were each fed a laboratory diet. All rats were individually housed for 2 weeks at 22 ± 1 °C and 55 ± 5% humidity under controlled lighting from 8:30 to 20:30. Single gastric intubation administration of VPP or IPP sample was done in 20-week SHR, in which the doses of 1.80 and 3.60 mg of VPP or 1.20 and 1.80 mg of IPP per kg of body weight dissolved in 1.0 ml of 0.2 mM phosphate-buffered saline (PBS, pH 7.2) solution, were injected by gastric intubation. The rats of the control group were administered with the same volume of PBS solution. Systolic blood pressure (SBP) was measured at 0, 2, 4, 6, 8, 10 and 12 h after the administration. The blood pressure (BP) measurement was performed by the tail-pulse pick up method with a Softron BP system (Softron BP-98A, Tokyo, Japan) after warming the rat in a warm holder kept at 39 ± 0.5 °C for 10 min (Furushiro et al., 1990).

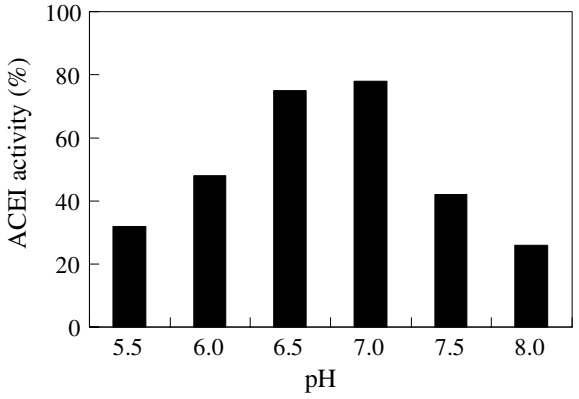


Fig. 1. Effect of pH on ACE-inhibition (ACEI) activity of skimmed milk hydrolysate digested with cell-free extract.

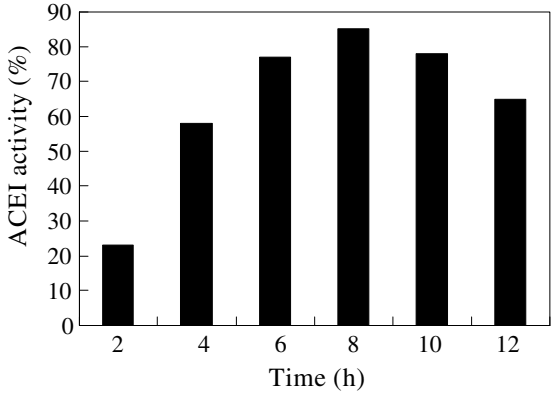
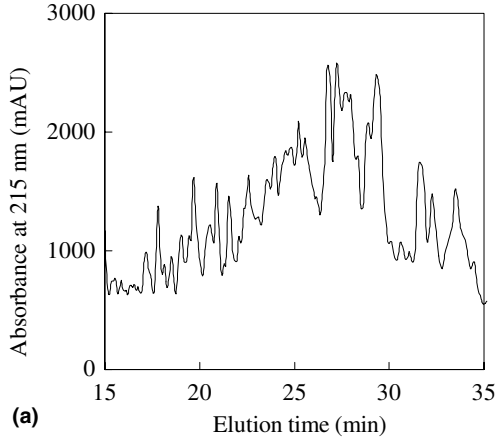
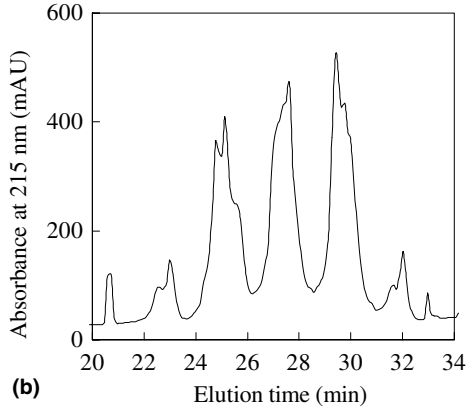


Fig. 2. Hydrolysis time on ACE-inhibition (ACEI) activity of skimmed milk hydrolysate digested with cell-free extract.





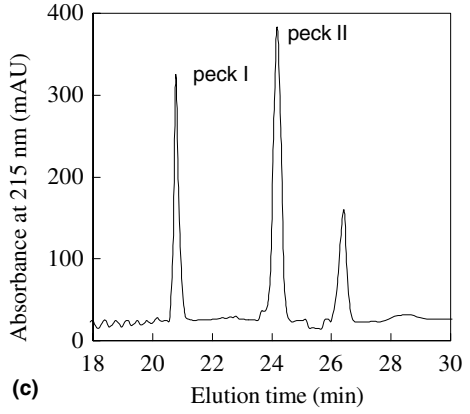


Fig. 3. Purification of the ACE inhibitors from skimmed milk hydrolysate digested with cell-free extract of *Lactobacillus helveticus* JCM 1004 by reverse-phase high-performance liquid chromatography (a, YMC-Pack ODS—AP—303 column; b, ODS-100S column; c, μBondasphere C_{18} column).

Table 1
Purification of ACE inhibitors from skimmed milk hydrolysate

Procedure	Total peptide (μg)	ACE inhibition (U)	Specific activity (U/μg)	Yield of activity (%)	Purification (-fold)
Hydrolysate	6243	312	0.05	100	1.0
YMC-Pack	198	262	1.32	83.97	26.4
ODS-100S	75	249	3.32	79.81	66.4
μBondasphere C_{18}					
Peak I	31	104	3.35	33.3	67.0
Peak II	28	130	4.64	41.7	92.8

2.11. Statistical analysis

Results of the experiment are given as means ± SEM. Statistical analysis of the results was done by using Student's *t*-test.

3. Results

3.1. ACE-inhibition activity of the hydrolysate prepared under different conditions

ACE-inhibitory activities of the samples formed at different pH and times upon digestion of skimmed milk with cell-free extract, were measured. The results indicated that pH 6.5–7.0 was optimum for the production of ACE-inhibition substances from skimmed milk (Fig. 1). It was also found that 6–10 h of hydrolysis time produced hydrolysate with higher ACE-inhibitory activities than did at 2, 4 or 12 h and activity decreased at 10 h of hydrolysis (Fig. 2).

3.2. Enzyme activity of cell-free extract

The proteinase, aminopeptidase and *x*-prolyl-dipeptidyl aminopeptidase activitives of cell-free extract from *L. helveticus* JCM1004 were determined at 513 ± 11.9, 376 ± 9.3 and 23.6 ± 1.6 unit per gramme.

3.3. Isolation and identification of ACE inhibition peptides

Elution profiles on reverse-phase high-performance liquid chromatography (RP-HPLC) are shown in Fig. 3. The angiotensin I-converting enzyme inhibition assay indicated that the fractions eluted between the elution times of 25 and 28 min, purified by first step RP-HPLC with the YMC-Pack column, had the most efficient inhibitory activities (Fig. 3a.). The above fractions were combined and concentrated, and then subjected to the second RP-HPLC separation step (ODS-100S column). Fractions eluted between 24 and 27 (Fig. 3(b)) indicated high ACE inhibitory activities. Among them, the fraction eluted at 24–26 min with the most efficient inhibitory activities was further purified by the final RP-HPLC step with the μBondasphere C_{18} column. By this procedure, fractions eluted at 20–21 (peak I) and 24 min (peak II) (Fig. 3(c)) with the highest ACE inhibitory activities were obtained.

The specific activity at each purification step is shown in Table 1. The ACE inhibitory substances of peaks I and II were purified about 67-and 92.8-fold; the yield of ACE inhibitory activity was 33.3% for peak I and 41.7% for peak II. The amino acid composition of peak I was Val, 1.0, and Pro, 2.06. By sequence analysis, peck I was found to be Val-Pro-Pro. The amino acid composition of peck II was Ile, 1.0, and Pro, 1.95, the sequence being Ile-Pro-Pro. The [^{1}H] nuclear magnetic resonace spectra and [^{13}C] nuclear magnetic resonance spectra showed that both active substances consisted of only three amino acids, and no other organic compound was found. The nuclear magnetic resonance signals supported the amino acid analysis of peaks I and II. The IC_{50} values of Val-Pro-Pro and Ile-Pro-Pro were 9.13 ± 0.21 and 5.15 ± 0.17 μM under the present assay conditions, respectively.

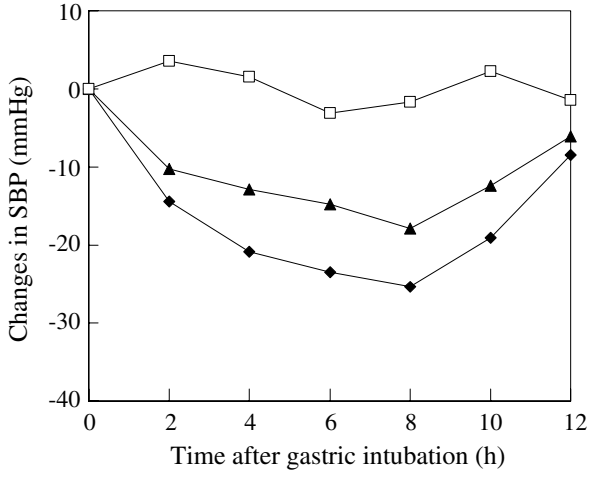


Fig. 4. Changes in mean SBP ($n = 8$) of SHR after a single gastric intubation of VPP. □, control (0.2 mM PBS); ▲ and ◆, the doses of 1.80 and 3.60 mg per kg body weight, respectively.

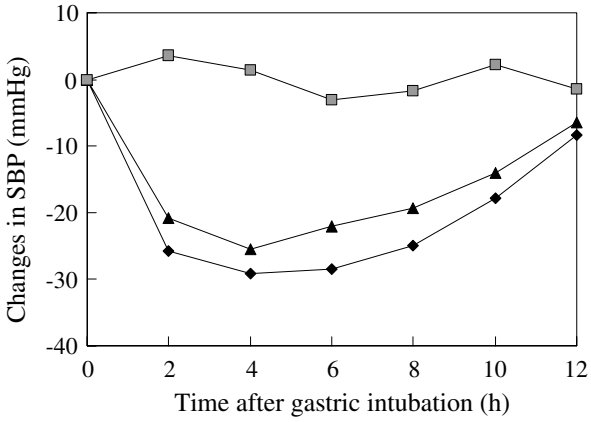


Fig. 5. Changes in mean SBP ($n = 8$) of SHR after a single gastric intubation of IPP. □, control (0.2 mM PBS); ▲ and ◆, the doses of 1.20 and 1.80 mg per kg body weight, respectively.

3.4. Antihypertensive effect of VPP and IPP on SHR

Figs. 4 and 5 show the changes in SBP measured in SHR during a 12-h observation period following a single gastric intubation of two different doses (1.80 and 3.60 mg per kg of body weight) of VPP or (1.20 and 1.80 mg per kg of body weight) of IPP. In the control groups, no significant changes were found in SBP during the 12 h after administration. However, the administration of the peptides caused potent decrease ($p < 0.05$) in SBP after 4, 6, 8 and 10 h (VPP groups) or 2, 4, 6 and 8 h (IPP groups) at both doses. Significant decrease ($p < 0.01$) in SBP was found at 8 h (VPP) or 4 h (IPP) for the two doses after gastric intubation.

4. Discussion

Some milk proteins have been treated with digestive enzymes such as pepsin and trypsin and ACE inhibitor activity has been identified in the hydrolysate. In this study, we used cell-free extract of *Lactobacillus helveticus* JCM1004 for the digestion of skimmed milk. Proteinase, aminopeptidase and *x*-prolyl-dipeptidyl aminopeptidase activitives were identified in the cell-free extract at 513 ± 11.9, 376 ± 9.3 and 23.6 ± 1.6 units per gramme. In a preliminary experiment, we found that the skimmed milk digested with cell-free extract of *L. helveticus* JCM1004 inhibited the ACE activity significantly, the optimum pH and hydrolysis time for the production of ACE-inhibition substances from skimmed milk were 6.5–7.0 and 6–10 h, respectively.

Two potent ACE inhibition peptides were purified, by three-step reverse-phase high-performance liquid chromatography, from the skimmed milk hydrolysate produced by cell-free extract of *L. helveticus* JCM1004, and 75.0% of the initial ACE inhibitory activity was recovered with these two peptides (Table 1), suggesting that most of the ACE inhibitory activity of the hydrolysate is attributed to these two peptides. By sequence analysis, the two peptides were found to be Val-Pro-Pro (VPP) and Ile-Pro-Pro (IPP). VPP and IPP showed higher ACE inhibitory activity than other reported ACE inhibitory peptides purified from milk products (Abubakar et al., 1999; Karaki, Doi, & Sugano, 1990; Margaret et al., 1997; Yamamoto et al., 1994a,b, 1999), and the IC_{50} values of them were 9.13 ± 0.21 and 5.15 ± 0.17 μM, respectively. Amino acid sequences of Val-Pro-Pro and Ile-Pro-Pro are found in the primary structure of bovine β-casein (84–86), β-casein (74–76), and κ-casein (108–110) (FitzGerald & Meisel, 2000; Yamamoto et al., 1994a, 1994b). These amino acid sequences may be cut off by proteinase from cell-free extract of *L. helveticus* JCM1004 during hydrolysis of shimmed milk. Further studies are necessary to elucidate the formation process of these ACE inhibitory peptides.

In the in vivo studies, a single gastric intubation administration of VPP and IPP showed blood pressure reduction in spontaneously hypertensive rats at doses of 1.80 and 3.60, or 1.20 and 1.80 mg per kg of body weight, respectively. The potent decrease in SBP was found at 4–10 h ($p < 0.05$ vs 0 h, $n = 8$) of VPP or 2–8 h of IPP ($p < 0.05$ vs 0 h, $n = 8$) after gastric intubation. From Figs. 4 and 5, dose-dependent lowering of blood pressure in SHR could be found in spontaneously hypertensive rats after administration of VPP or IPP.

Functional foods are foods that encompass potentially healthful products, including any modified food or food ingredient that may provide a health benefit beyond the traditional nutrients it contains. A nutraceutical has been defined as any substance that is a food or part of a food that provides medical and/or health benefits, including the prevention and treatment of disease (Losso, 2003). According to the present research results, the milk protein-derived peptides, VPP and IPP, appear to be the most interesting both as nutraceuticals and components of functional foods for prevention or treatment of hypertension.

Acknowledgements

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