Available online at www.sciencedirect.com
ScienceDirect
Food Chemistry 106 (2008) 552–558
Food Chemistry
www.elsevier.com/locate/foodchem

Antihypertensive effect of bioactive peptides produced by protease-facilitated lactic acid fermentation of milk

Jenn-Shou Tsai [a,*], Tai-Jung Chen [a], Bonnie Sun Pan [a], Shein-Da Gong [b], Mei-Yuh Chung [b]

[a] Department of Food Science, National Taiwan Ocean University, 2 Pei-Ning Road, Keelung 202, Taiwan, ROC
[b] Wei Chuan Foods Corporation, 23th Floor, 108, Section 1, Hsin Tai Wu Road, Hsichih, Taipei Hsien 221, Taiwan, ROC

Received 29 December 2006; received in revised form 30 March 2007; accepted 6 June 2007

Abstract

Milk was fermented for up to 5 h at 43 °C with two lactic acid bacteria (*Streptococcus thermophilus*, *Lactobacillus bulgaricus*). A protease, flavourzyme, was added at the beginning of fermentation. The whey fraction was separated from the fermented milk and freeze-dried. During the 5 h of fermentation, the soluble protein content increased from 4.9 to 57.4 mg/g and peptide content increased from 2.1 to 32.8 mg/g, while inhibition of angiotensin I-converting enzyme (ACE) increased by a decrease of IC_{50} from 0.708 to 0.266 mg/ml, respectively. The whey was fractionated into four fractions by size exclusion chromatography on a Sephadex G-15 column. The fourth fraction of the whey showed the highest inhibitory efficiency ratio (IER) being 1329%/mg/ml. The amino acid sequence of the inhibitory peptide was Tyr-Pro-Tyr-Tyr, of which the IC_{50} was 90.9 μM. The whey showed mixed-type inhibition kinetics, while Captopril, the positive control showed competitive inhibition on ACE. Their K_i values were 0.188 mg/ml and 0.0067 μg/ml, respectively. The systolic blood pressure (SBP) and diastolic blood pressure (DBP) was reduced to 15.9 and 15.6 mm Hg, respectively, in spontaneously hypertensive rat (SHR), after 8 weeks of oral administration of diluted whey (peptide concentration 4.9 mg/ml).

Keywords: Fermented milk; Angiotensin I-converting enzyme (ACE); Spontaneously hypertensive rat (SHR); Antihypertensive effect

1. Introduction

High blood pressure has been considered a risk factor for developing cardiovascular diseases (arteriosclerosis, stroke and myocardial infraction) and end-stage renal disease. Angiotensin I-converting enzyme (ACE; EC 3.4.15.1) plays an important physiological role in the regulation of blood pressure and electrolyte homeostasis. ACE hydrolyzed an inactive form of decapeptide, Angiotensin I, to an octapeptide, Angiotensin II, is a potent vasoconstrictor, and inactivated catalytic function of bradykinin, which is a depressor. Angiotensin II has been implicated in the regulation of cellular lipoxygenases (LOX), which plays a role in atherogenesis by catalyzing oxidative modification of low-density lipoproteins (LDL) (Abubakar, Saito, Kitazawa, Kawai, & Itoh, 1998). Angiotensin II and LOX-catalyzed oxidation of LDL are involved in hypertension and atherosclerosis. ACE inhibitors act as vasodilators, but the most obvious potential benefit is their effect on the renin–angiotensin–aldosterone system by reducing the levels of Angiotensin II. Clinical studies have demonstrated that ACE inhibitors significantly reduce the morbidity and mortality of patients with myocardial infraction or heart failure (Daemon, Lombardi, Bosman, & Schwartz, 1991; Geisterfer, Peach, & Owens, 1988).

Several ACE inhibitory peptides have been isolated from the enzymatic hydrolysis of milk proteins (Hernandez-Ledesma, Amigo, Ramos, & Recio, 2004; Maeno, Yamamoto, & Takano, 1996; Tauzin, Miclo, & Gaillard, 2002), and fermentation of milk with lactic acid bacteria (Gobbetti, Ferranti, Smacchi, Goffredi, & Addeo, 2000; Nakamura et al., 1995; Yamamoto, Maeno, & Takano,

* Corresponding author. Tel.: +886 2 2462 2192x5126; fax: +886 2 2462 0105.
E-mail address: tsaijs@mail.ntou.edu.tw (J.-S. Tsai).

0308-8146/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2007.06.039

1999), or by chemical synthesis according to milk protein sequences (Kohmura, Nio, & Ariyoshi, 1990; Mullally, Meisel, & Fitzgerald, 1996). Moreover, these ACE inhibitory peptides (e.g. Val-Arg-Tyr-Leu) have been isolated from the enzymatic hydrolysis of milk proteins (Hernandez-Ledesma, Miralles, Amigo, Ramos, & Recio, 2005), and Ile-Pro-Pro and Val-Pro-Pro from milk fermented with lactic acid bacteria (Gobbetti et al., 2000; Muguerza et al., 2006; Nakamura, Yamamoto, Sakai, & Takano, 1995). Ingestion of sour milk fermented by *Lactobacillus helveticus* that contained ACE inhibitory tripeptides (Ile-Pro-Pro and Val-Pro-Pro) seemed to lower blood pressure modestly in mild hypertensive subjects (Tuomilehto et al., 2004).

There are a number of products on the market manufactured by international food/food ingredients companies, aimed at exploiting the functional food potential of milk protein derived hypotensive peptides (IPP, VPP, FFVAP-FEVFGK and whey peptides) (Fitzgerald, Murray, & Walsh, 2004). These products are either in the form of fermented milk drinks or as milk protein hydrolysates. Therefore, the purpose of this study was to develop a combination method using lactic acid fermentation and proteolysis of milk to produce a milk product which is more abundant in bioactive peptides than the traditional fermentation or proteolysis products of milk. The bioactive peptides were examined on ACE inhibitory activity and stability by simulating gastrointestinal digestion. Furthermore, the inhibition type of the bioactive substances on ACE and the antihypertensive effect on spontaneously hypertensive rats were also evaluated.

2. Materials and methods

2.1. Materials and chemicals

Whole milk powder and skimmed milk powder were obtained from Wei Chuan Foods Corporation (Taiwan). Lactic acid bacteria (*Streptococcus thermophilus* and *Lactobacillus bulgaricus*) in powder form were purchased from Danisco Niebull Gmbh. (Alemanha, Germany). Flavourzyme (from *Aspergillus oryzae*, activity labeled 1160 LAPU/g) was purchased from Novo Nordisk A/S Co. (Denmark). Pepsin, Pancreatin, Hippuryl-L-histidyl-L-leucine (Hip-His-Leu), Angiotensin I-converting enzyme of rabbit lung, Captopril and chemicals of analytical grade were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Lactic acid fermentation facilitated by protease

The mixture of 4.5% (w/v) skimmed milk powder, 5.5% (w/v) whole milk powder and 7% (w/v) sucrose was pasteurized (95 °C, 30 min) then fermented with 0.1% (w/v) of lactic acid bacteria powder at 43 °C. At the beginning of fermentation, flavourzyme, filtered through a 0.2 μM membrane, was added to the milk at 0.14% (w/v) (the ratio of enzyme to milk protein was 0.45:100, w/w) and the fermentation continued for a total of 5 h. The fermented milk was then heated at 98 °C for 10 min to inactivate the protease and lactic acid bacteria. The insoluble material was removed by centrifugation at $6000\times g$ for 30 min. The supernatant was filtered (No. 2 filter paper; Toyo Roshi Kaisha, Ltd. Tokyo, Japan). The filtrate was defined as whey fraction (pH 4.47) and lyophilized to a powder form ready for analyses or fed to spontaneously hypertensive rats (SHR) at 150 ml/day with constant concentration.

2.3. Chemical analyses

The soluble protein content of the whey powder was determined by the Folin–Lowry method (Lowry, Resebrough, Farr, & Randall, 1951; Cooper, 1977). One millilitre of the sample was mixed with 1 ml of an alkaline-copper reagent and 3 ml of the Folin–Ciocalteu's phenol reagent (Merck, Germany) at tenfold dilution with deionized water. After the solution was allowed to stand for 30 min, the absorbance at 540 nm was measured with a spectrophotometer (Model UV-160A, Shimadzu, Kyoto, Japan). The soluble protein content was quantified using bovine serum albumin as standard.

2.4. Measurement of peptide content

The peptide content of whey was measured by the method of Church, Swaisgood, Porter, and Catignani (1983) with some modifications. The whey solution (50 mg/ml) was filtered by 0.2 μM membrane and the filtrate passed through ultrafiltration membrane with molecular weight (M.W.) cut-offs of 5000 Da (Millipore, Bedford, MA, USA). This permeate was defined as small peptides (M.W. $<$ 5000 Da). Fifty millilitres of reagent was prepared by mixing 25 ml of 100 mM borax, 2.5 ml of 20% (w/w) sodium dodecyl sulfate, 40 mg of *o*-phthaldialdehyde solution (dissolved in 1 ml of methanol) and 100 μl of β-mercaptoethanol and then adjusted to 50 ml with deionized water. Fifty microlitres of this permeate was mixed with 2 ml of reagent. The reaction mixture was incubated for 2 min at ambient temperature, and the absorbance at 340 nm was measured with spectrophotometer (Model UV-160A, Shimadzu, Kyoto, Japan). The peptide content was quantified using casein tryptone (Difco Laboratories, Sparks, MD, USA) as standard.

2.5. In vitro assay for ACE inhibitory activity

The inhibitory activity against ACE was determined using reverse-phase high performance liquid chromatography (RP-HPLC) modified for the spectrophotometric assay (Cushman & Cheung, 1971; Wu & Ding, 2002). Fifteen millimolar of Hippuryl-L-histidyl-L-leucine (HHL) was dissolved in 100 mM Na-borate buffer (pH 8.3) containing 300 mM NaCl. Rabbit lung ACE was dissolved in the same buffer at a concentration of 8 mU/ml. A mixture contain-

ing 75 μl of ACE solution and 75 μl of sample with 5000 Da molecular weight cut-off (Millipore, Bedford, MA, USA) was incubated at 37 °C for 10 min, to which was added 75 μl of HHL solution and incubated for 30 min. The reaction was stopped by adding 250 μl of 1 N HCl and 10 μl of the assay solution injected directly onto a Luna C_{18}column (4.6 × 250 mm, particle size, 5 μM; Phenomenex, Torrance, CA, USA) to separate the product and hippuric acid (HA) from HHL. The column was eluted with 50% methanol in water (v/v) containing 0.1% trifluoroacetic acid (TFA) at a flow rate of 0.8 ml/min using a pump (model L-7100, Hitachi, Tokyo, Japan) and a detector which was monitored at 228 nm by a UV spectrophotometer (UV/Visible detector 118, Gilson Medical Electronics, Villiers-le-Bel, France). The inhibition activity was calculated using the following equation:

$$\text{Inhibition activity } (\%) = [(E_c - E_s)/(E_c - E_b)] \times 100, \quad (1)$$

where E_c is the absorbance of the buffer (control), E_s is the absorbance of the reaction mixture (sample), E_b is the absorbance when the stop solution was added before the reaction occurred (blank). The IC_{50} value was defined as the concentration of peptide in mg/ml required to reduce 50% of the absorbance peak height of the hippuric acid (HA) (50% inhibition of ACE), which was determined by regression analysis of ACE inhibition (%) versus peptide concentration in duplicate.

2.6. In vitro gastrointestinal digestion

In vitro digestion was carried out in triplicate according to the method of Wu and Ding (2002). A 3.5 % (w/v) whey solution in 0.1 M KCl–HCl (pH 2.0) buffer, with pepsin (4% w/w) for 4 h at 37 °C, stopped by boiling water for 10 min and neutralized to pH 7.0 with addition of 2 N NaOH solution. Neutralized suspension (50 ml) was centrifuged (10,000×*g*, 30 min) and the supernatant was used for determination of ACE inhibitory activity. The remaining neutralized suspension was digested further by 4% (w/w) pancreatin at 37 °C for 4 h. The enzyme was inactivated by boiling for 10 min and centrifuged (10,000×*g*, 30 min), then the supernatant was used for ACE inhibitory activity (expressed as mg peptide/ml) determination.

2.7. Kinetic evaluation of ACE inhibition of whey

A Lineweaver–Burk plot was drawn to estimate the ACE inhibitory type of the whey. Maintaining the quantity of ACE constant at 50 mU/ml, the ACE inhibitory activities of the whey (from 0.108 to 0.430 mg/ml) and Captopril (from 0.0011 to 0.0044 μg/ml) were measured in various concentrations (5, 2.5, 1.25, 0.625 mM) of substrate (HHL). These activities were transformed into the velocity of ACE (μmol/min) catalysis. The resulting data were reciprocally plotted (substrate concentration on horizontal axis and velocity on vertical axis). The inhibition type of whey powder against ACE was estimated by comparing these data with those in the absence of the whey inhibitor. The inhibition constant (K_i) of the whey or the peptide inhibitor was obtained from the secondary plot of Lineweaver–Burk plot. The intercept on the horizontal axis is the absolute value of the K_i.

2.8. Size exclusion chromatography

The lyophilized whey powder (50 mg) was dissolved with 2 ml of deionized water. After filtration with a 5000 Da MWCO membrane, the resulting solution was fractionated by gel filtration on Sephadex G-15 column (1.6 × 90 cm; Amersham Pharmacia Biotech AB, Sweden), and eluted with 0.02 M phosphate buffer (pH 7.2). Fractions in 5 ml each were collected at a flow rate of 0.5 ml/min, and the absorbance was measured at 280 nm. Bacitracin (M.W. 1422.0 Da), penta-L-phenylalanine (M.W. 753.9 Da), and glycine (M.W. 75.0 Da) were used as the comparable standards of molecular weight.

2.9. Purification of ACE inhibitory peptide

The above fraction with the highest ACE inhibitory activity was collected, lyophilized and further separated by reverse-phase (RP) HPLC (model L-7100, Hitachi, Tokyo, Japan), using a Synergi 4u Hydro-RP 80A C_{18}column (10 × 250 mm; particle size, 4 μM; Phenomenex, Torrance, CA, USA). Solvent A was 0.1% (v/v) trifluoroacetic acid (TFA) in deionized water, and solvent B was 0.1% (v/v) TFA in 100% (v/v) acetonitrile (CH_3CN) solution. The separation was performed with a linear gradient from 0 to 60% of solvent B within 20 min, flow rate of 1.5 ml/min at room temperature and absorbance of the eluate was monitored at 220 nm with a UV spectrophotometer (UV/Visible detector 118, Gilson Medical Electronics, Villiers-le-Bel, France) that was linked to a data station (715 system controller, Gilson Medical Electronics, Villiers-le-Bel, France). The peak with the highest ACE inhibitory activity was collected, lyophilized and its amino acid sequence was identified.

2.10. Sequence analysis

The sequence of each peptide was identified by automated Edman degradation using a protein sequencer 492 Procise (Perkin Elmer, Applied Biosystem, Foster City, CA, USA).

2.11. Animals and in vivo measurement of blood pressure

Sixteen male spontaneously hypertensive rats (SHR), 7 weeks of age (National Laboratory Animal Center, Taipei, Taiwan) were housed in cages exposed to a cycle of 12 h of light and 12 h of darkness. The temperature and humidity in the animal room was controlled at 23 ± 1 °C and 55 ± 5%, respectively. The SHR were fed a standard laboratory diet (Rodent Laboratory Chow Diet 5001, PMI

Nutrition International, Brentwood, MO, USA). Tap water was freely available to the rats for one week before the experimental period. All SHR, 8 weeks of age (body weight: 215 ± 14 g) were divided into two groups: The experimental group ($n = 8$) where each rat was given 150 ml of diluted fermented milk whey (peptide concentration 4.9 mg/ml) containing 0.5% NaCl, while the control group ($n = 8$) were given 150 ml of distilled water containing 0.5% NaCl during the trial. Every other week, the rats were put in a thermostatic box at 45 °C for 5 min to measure systolic blood pressure (SBP), diastolic blood pressure (DBP) and heart rate using the tail-cuff method (model BP-98, Softron, Tokyo, Japan). Results are shown as means and standard errors.

2.12. Statistical analysis

Analysis of variances of results was carried out using the General Linear Model Procedure of SAS (1988). Multiple comparisons of means were carried out by Duncan's multiple range tests.

3. Results and discussion

3.1. Soluble protein, peptide content and IC_{50}

The effects of lactic acid bacteria fermentation and that facilitated by flavourzyme on soluble protein, peptide content and ACE inhibitory activity of IC_{50} were compared (Table 1). Soluble protein in milk whey (without fermentation and flavourzyme) was 4.9 mg/g, which increased slightly to 5.5 mg/g ($P < 0.05$) after it underwent fermentation with lactic acid bacteria for 5 h, while those of the flavourzyme-facilitated fermentation increased to 10 folds (57.4 mg/g) of that in the straight fermentation product. The peptide content was 2.1 mg/g initially. After 5 h of fermentation without the enzyme addition, 5.8 mg/g peptides were produced. Flavourzyme-facilitated fermentation, further increased the peptide content to 5.6 folds (32.8 mg/g).

ACE inhibitory activity was higher, indicated by a lower IC_{50} value being 0.226 mg/ml (flavourzyme-facilitated) in contrast to 0.515 mg/ml of that from straight fermentation for 5 h. These results indicated, as milk was fermented by lactic acid bacteria with protease, more active peptides were produced to inhibit the activity of ACE.

3.2. Isolation of ACE inhibitory peptide

The whey obtained from lactic acid fermentation facilitated with flavourzyme showed the strongest inhibitory activity on ACE. The molecular weight distribution of the ACE inhibitory peptides in this whey was fractionated by size exclusion chromatography on a Sephadex G-15 column. Four fractions were separated and designated as 1–4 (Fig. 1) of which the molecular weights ranged approximately from 1500 to 530 Da. ACE inhibitory activity was observed in all the fractions. Fraction 4 showed the highest inhibition capacity (91%) among all fractions. Its inhibitory efficiency ratio (IER) was the highest being 1329%/mg/ml (Table 2). The molecular weight of this fraction 4 was approximately 530 Da indicative of three or four amino acids residues. This coincides with the findings of the potent inhibitory tripeptides produced from fermented Calpis sour milk (Val-Pro-Pro, $IC_{50} = 5.1$ μM; Ile-Pro-Pro, $IC_{50} = 2.4$ μM) (Nakamura et al., 1995) which have been

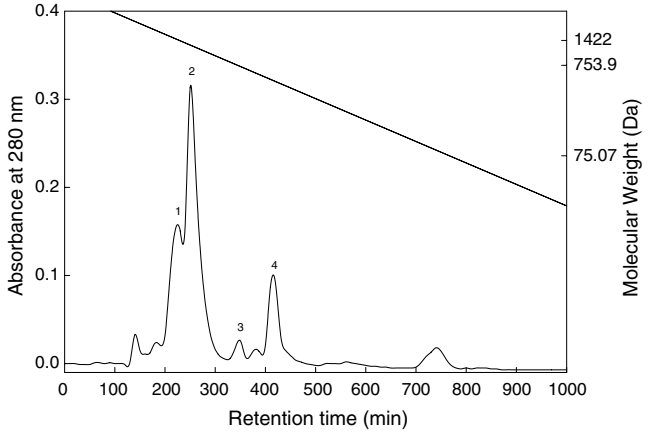

Fig. 1. Sephadex G-15 column chromatography of peptides separated from milk whey fermented with lactic acid bacteria for 5 h at 43 °C and facilitated with flavourzyme.

Table 1
Effects of lactic acid bacteria fermentation and flavourzyme-facilitated fermentation on chemical compositions and IC_{50} of ACE[A] of whey powder from fermented milk

Fermentation[B]	Flavourzyme[C] (%)	Soluble protein (mg/g)	Peptide content (mg/g)	IC_{50} (mg peptide/ml)
−	−	4.9 ± 0.1^{a}	2.1 ± 0.1^{a}	0.708 ± 0.008^{a}
+	−	5.5 ± 0.1^{b}	5.8 ± 0.1^{b}	0.515 ± 0.012^{b}
−	0.45	57.8 ± 0.7^{c}	36.5 ± 0.1^{d}	0.360 ± 0.037^{c}
+	0.45	57.4 ± 0.3^{c}	32.8 ± 0.6^{c}	0.266 ± 0.024^{d}

[A] ACE: Angiotensin I converting enzyme.
[B] −: without lactic acid bacteria or protease, flavourzyme. +: with lactic acid bacteria.
[C] Hydrolysis for 5 h.

Table 2
ACE[a] inhibitory efficiency ratio (IER) of the fractions from size exclusion chromatography of milk whey fermented with lactic acid bacteria and facilitated with flavourzyme

Fraction no.	M.W. (Dalton)	Inhibition (%)	Peptide content (mg/ml)	IER[b] (%/mg/ml)
1	1500	89	1.080	83
2	1390	84	0.256	332
3	840	87	0.246	354
4	530	91	0.069	1329

[a] ACE: Angiotensin I converting enzyme.
[b] IER: Inhibitory efficiency ratio = % inhibition/peptide content.

approved in Japan for use as a functional food for prevention of hypertension.

3.3. In vitro ACE inhibitory activities and inhibition type

The ACE Inhibitory activities and the inhibition types of whey were compared with that of Captopril (Table 3), the most widely used antihypertensive drug at present time. The IC_{50} of Captopril (1.55×10^{-3} μg/ml) was about 17,000 times lower than that of the whey (2.66×10^{2} μg/ml). The Captopril showed competitive inhibition competing with the substrate for binding to the active site of the enzyme, while whey showed a mixed type inhibition being able to bind at active site (competitive) and non-active site (noncompetitive) of ACE. The IC_{50} (0.0071 μM) of Captopril was in accordance with reported data (0.007 μM) (Pihlanto-Leppälä, Rokka, & Korhonen, 1998). The K_i value of Captopril (6.70×10^{-3} μg/ml or 30.9 nM) was almost 28,000 fold smaller than that of whey (1.88×10^{2} μg/ml) showing the drug has much stronger affinity to the ACE active site than the fermented whey. The K_i value of Arg-Met-Leu-Gly-Gln-Thr-Pro-Thr-Lys (isolated from peptic hydrolysate of porcine skeletal troponin C) (Katayama et al., 2004) and Val-Lys-Pro (isolated from freshwater clam muscle protein hydrolysate) (Tsai, Lin, Chen & Pan, 2006) were 60.6 and 2.6 μM, respectively. However, protein hydrolysates have rarely been reported with respect to its K_i value.

3.4. In vitro stability of whey derived ACE inhibitory peptides

In vitro gastric digestion provides a practical and easy process to imitate the oral administration of bioactive peptides. The ACE inhibitory activity of whey-derived peptides increased markedly by decreased IC_{50} from 0.266 to 0.149 mg peptide/ml by hydrolysis with pepsin, simulating stomach digestion (Table 4). However, the IC_{50} of ACE increased to 0.254 mg/ml after further digestion with pancreatin simulating small intestine digestion. This result indicated that the ACE-inhibitory peptides are released by fermentation and proteolysis, and could be survived or maintained in active form even by gastric digestion.

3.5. Purification of ACE inhibitory peptide

The most active peptide of fraction 4 was separated on a RP-HPLC column (ODS C_{18}) followed by repeated chromatography to confirm, as a single component by RP-phase HPLC column with same gradients of acetonitrile solution from 0 to 60%. The elution profile of peptide is shown in Fig. 2. One major peak was observed in the chromatogram, and collected, lyophilized and further analyzed for amino acid sequencing. This ACE inhibitory peptide consisted of four amino acid residues, Tyr-Pro-Tyr-Tyr, of which the ACE inhibitory activity IC_{50} value was 90.9 μM. This is the first report of an ACE inhibitory peptide being isolated and identified from whey of fermented milk with the sequence of κ-B casein 58–61 (Braunitzer, Chen, Schrank, & Stangl, 1973; Grosclaude, Mahé, Mercier, & Ribadeau-Dumas, 1972). Regarding to the relationship between structure and activity of ACE inhibitory peptides, Cheung, Wang, Sabo, and Chushman (1980) have reported that those peptides which had Pro, Phe or Tyr at the C-terminus, and Val and Ile at the N-terminus showed highly potent inhibitory activity. Sekiya, Kobayashi, Kita, Imamura, and Toyama (1992) has also reported that food-derived peptides having IC_{50} value ranged 100–500 μM had potential as antihypertensive agents.

Table 3
The inhibitory properties of milk whey fermented with lactic acid bacteria and facilitated with flavourzyme on ACE in reference to Captopril

Inhibitor	IC_{50}[a] (μg/ml)	K_i[b](μg/ml)	Inhibitory type
Whey	2.66×10^{2}	1.88×10^{2}	Mixed
Captopril[c]	1.55×10^{-3}	6.70×10^{-3}	Competitive

[a] The concentration of an inhibitor required to inhibit 50% of the ACE activity.
[b] Inhibition constant calculated from Lineweaver–Burk plots.
[c] Commercial ACE inhibitor purchased from Sigma Co.

Table 4
Effect of gastrointestinal protease hydrolysis on ACE inhibitory activity of milk whey fermented with lactic acid and facilitated with flavourzyme

Protease	IC_{50} (mg peptide/ml)
Control	0.266
Pepsin[a]	0.149
Pepsin + pancreatin[b]	0.254

[a] Hydrolyzed for 4 h.
[b] Pepsin hydrolyzed for 4 h followed by Pancreatin hydrolyzed for 4 h.

3.6. In vivo antihypertensive effect of fermented milk whey

The SHR aged 8 weeks fed the control and the fermented milk whey (peptide concentration 4.9 mg/ml) for

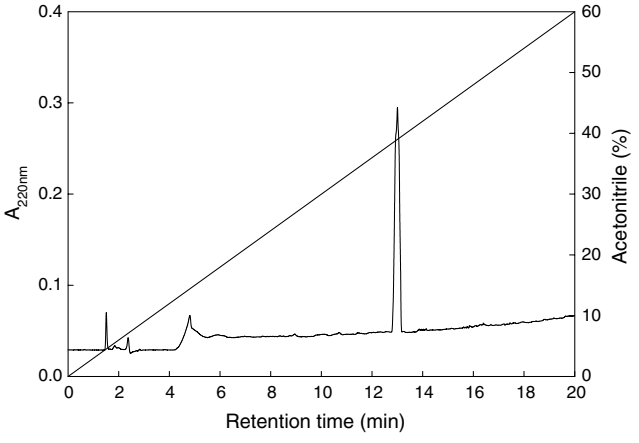

Fig. 2. An elution profile of the fraction 4 from Fig. 1 by reversed-phase HPLC. (Column: Jupiter 4u Proteo 90A, 4 μM, 10 × 250 mm, Phenomenex; flow rate: 1.5 ml/min; solvent system: A, H_2O/TFA 0.1%; B, CH_3CN/TFA 0.1%. Linear gradient from 0% to 60% B in 20 min).

two weeks aged 10 weeks showed no significant differences on SBP (Fig. 3). The systolic blood pressure (SBP) was 145.3 ± 6.9 and 145.5 ± 5.4 mm Hg, respectively, and SBP rose gradually to 161.6 ± 5.8 and 159.6 ± 6.7 mm Hg, respectively. The SHR aged 12 weeks showed SBP significantly different ($P < 0.05$) from the control group (173.4 ± 6.3 mm Hg) and the fermented whey group (164.3 ± 6.0 mm Hg). Oral administration of the fermented milk whey for 8 weeks of trial as the SHR aged 16 weeks resulted in a SBP of 168.43.4 mm Hg, being significantly lower ($P < 0.05$) than that of the control group (184.33.0 mm Hg). The average SBP decreased by 15.9 mm Hg after 8 weeks of oral administration of the diluted fermented milk whey. It was reported that lactic fermented milk containing ACE-inhibitory peptides fed to SHR (peptide concentration 5 mg/ml) for 8 weeks, the SBP was lowered by 22 mm Hg from 199 decreased to 177 mm Hg (Chen, Tsai, & Pan, 2007).

At the beginning of the trial, the diastolic blood pressure (DBP) of the SHR aged 8 weeks fed on the control and the fermented milk whey diet was 107.4 ± 8.5 mm Hg and 107.4 ± 8.9 mm Hg, respectively (Fig. 4). DBP rose gradually with age to 14 weeks in both groups and showed significant differences ($P < 0.05$) between the control (147.5 ± 7.8 mm Hg) and the fermented whey group (129.1 ± 5.9 mm Hg). Prolonging the trial to 16 weeks, the DBP of the control (146.2 ± 8.3 mm Hg) and the fermented whey group (130.6 ± 6.4 mm Hg) still maintained significant differences ($P < 0.05$). The average DBP decreased by 15.6 mm Hg after 8 weeks of oral administration of the fermented milk whey.

Oral administration of sour milks or milk protein hydrolysates (peptides) have been reported to reduce blood pressure of SHR significantly (Nakamura, Yamamoto et al., 1995; Sipola, Finchkenberg, Korpela, Vapaatalo, & Nurminen, 2002; Yamamoto, Akino, & Takano, 1994a, Yamamoto, Akino, & Takano, 1994b, 1999) and hypertensive humans (Hata et al., 1996; Kajimoto et al., 2001) after a single dose or several weeks. Yamamoto et al. (1994b) observed significant decrease in SBP (−15.2 to −29.3 mm Hg) of SHR by oral administration of milk fermented with *L. helveticus* strains CP790, CP611, CP615, JMC1006 or JMC1004. Whey isolated from milk fermented with *Lactobacillus helveticus* CPN4 also caused a reduction in SBP (−29.6 mm Hg) of SHR with gastric intubation after 6 h in vivo (Yamamoto et al., 1999).

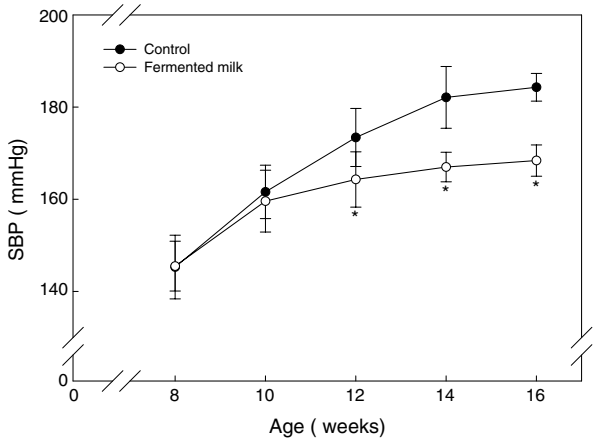

Fig. 3. Changes in systolic blood pressure (SBP) of SHR after oral administration of whey from flavourzyme facilitated fermented milk. –●–, 0.5% NaCl in water as control; –○–, whey from flavourzyme facilitated fermented milk added with 0.5% NaCl. Each point represents a mean value and the vertical bars represent the standard errors. ∗: significantly different from control, $P < 0.05$.

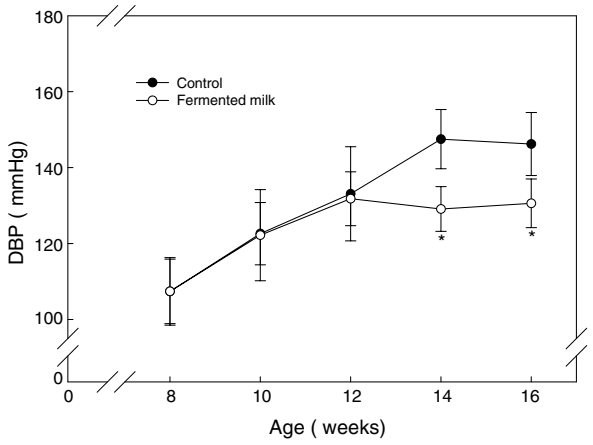

Fig. 4. Changes in diastolic blood pressure (DBP) of SHR after oral administration of whey from flavourzyme facilitated fermented milk. –●–, 0.5% NaCl in water as control; –○–, whey from flavourzyme facilitated fermented milk added with 0.5% NaCl. Each point represents a mean value and the vertical bars represent the standard errors. ∗: significantly different from control, $P<0.05$.

4. Conclusion

A new technique has been developed in this experiment using a combination of lactic acid bacteria fermentation for 5 h and flavourzyme hydrolysis to accelerate the production of bioactive peptides (32.8 mg/g of whey powder) in contrast to a 5.8 mg/g bioactive peptides from lactic fermentation alone. This fermented milk whey product inhibited ACE in vitro. The bioactivity was contributed mainly by peptide of Tyr-Pro-Tyr-Tyr which remained without being further hydrolyzed during in vitro gastrointestinal digestion. It was confirmed in vivo being preventive to hypertension in SHR (reduction in SBP by 15.9 mm Hg) by inhibition of ACE. Further research will be conducted to evaluate the blood pressure lowering effect in human trials to substantiate the clinical efficacy. Therefore, this fermented milk whey is expected to be a useful ingredient in physiologically functional foods for the prevention of hypertension.

References

Abubakar, A., Saito, T., Kitazawa, H., Kawai, Y., & Itoh, T. (1998). Structural analysis of new antihypertensive peptides derived from

cheese whey protein by proteinase K digestion. *Journal of Dairy Science, 81*, 3131–3138.

Braunitzer, G., Chen, R., Schrank, B., & Stangl, A. (1973). The sequence of β-lactoglobulin. *Hoppe-Seyler's Zeitschrift fur Physiologische Chemie, 354*, 867–878.

Chen, G. W., Tsai, J. S., & Pan, B. S. (2007). Purification of angiotensin I-converting enzyme inhibitory peptides and antihypertensive effect of milk produced by protease-facilitated lactic fermentation. *International Dairy Journal, 17*, 641–647.

Cheung, H. S., Wang, F., Sabo, E. F., & Chushman, D. W. (1980). Binding of peptides substrates and inhibitors of angiotensin-converting enzyme. *Journal of Biological Chemistry, 255*, 401–407.

Church, F. C., Swaisgood, H. E., Porter, H. D., & Catignani, G. L. (1983). Spectrophotometric assay using *o*-phthaldialdehyde for determination of proteolysis in milk and isolated milk proteins. *Journal of Dairy Science, 66*, 1219–1227.

Cooper, T. G., (1977). The tools of biochemistry. In J. Wiley, & Sons, (Eds.), *Spectrophotometry* (pp. 53–55). New York: Wiley.

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

Daemon, M., Lombardi, D., Bosman, F., & Schwartz, S. (1991). Angiotensin II induces smooth muscle cell proliferation in the normal and injured rat arterial wall. *Circular Research, 68*, 450–456.

Fitzgerald, R. J., Murray, B. A., & Walsh, D. J. (2004). Hypotensive peptides from milk proteins. *American Society Nutrition Science*, 980s–988s.

Geisterfer, A., Peach, M., & Owens, G. (1988). Angiotensin II induces hypertrophy of cultured rat aortic smooth muscle cells. *Circular Research, 62*, 749–756.

Gobbetti, M., Ferranti, P., Smacchi, E., Goffredi, F., & Addeo, F. (2000). Production of angiotensin-I-converting-enzyme-inhibitory peptides in fermented milk started by *Lactobacillus delbrueckii* subsp. *bulgaricus* SS1 and *Lactococcus lactis* subsp. cremoris FT4. *Applied and Environmental Microbiology, 66*(9), 3898–3904.

Grosclaude, F., Mahé, M. F., Mercier, J. C., & Ribadeau-Dumas, B. (1972). Characterization of genetic variants of α_{s1}- and β-bovine caseins. *European Journal of Biochemistry, 26*, 328–337.

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

Hernandez-Ledesma, B., Amigo, L., Ramos, M., & Recio, I. (2004). Angiotensin converting enzyme inhibitory activity in commercial fermented products. Formation of peptides under simulated gastrointestinal digestion. *Journal of Agricultural and Food Chemistry, 52*, 1504–1510.

Hernandez-Ledesma, B., Miralles, B., Amigo, L., Ramos, M., & Recio, I. (2005). Identification of antioxidant and ACE-inhibitory peptides in fermented milk. *Journal of Science and Food Agriculture, 85*, 1041–1048.

Kajimoto, O., Nakamura, Y., Yada, H., Moriguchi, S., Hirata, H., & Takahashi, T. (2001). Hypotensive effects of sour milk in subjects with mild or moderate hypertension. *Journal of Japanese Society of Nutrition and Food Science, 54*, 347–354.

Katayama, K., Tomatsu, M., Kawahara, S., Yamauchi, K., Fuchu, H., Kodama, Y., et al. (2004). Inhibition profile of nonapeptide derived from porcine troponin C against angiotensin I-converting enzyme. *Journal of Agricultural and Food Chemistry, 52*, 771–775.

Kohmura, M., Nio, N., & Ariyoshi, Y. (1990). Inhibition of angiotensin-converting enzyme by synthetic peptide fragments of various β-caseins. *Agricultural and Biological Chemistry, 54*(4), 1101–1102.

Lowry, O. H., Resebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry, 193*, 265–275.

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

Muguerza, B., Ramos, M., Sanchez, E., Manso, M. A., Miguel, M., Aleixandre, M. A., et al. (2006). Antihypertensive activity of milk fermented by *Enterococcus faecalis* strains isolated from raw milk. *International Dairy Journal, 16*, 61–69.

Mullally, M. M., Meisel, H., & Fitzgerald, R. J. (1996). Synthetic peptides corresponding to α-lactalbumin and β-lactoglobulin sequences with angiotensin-I-converting enzyme inhibitory activity. *Biological Chemistry Hoppe-Seyler, 377*, 259–260.

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

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

Pihlanto-Leppälä, A., Rokka, T., & Korhonen, H. (1998). Angiotensin I converting enzyme inhibitory peptides derived from bovine milk proteins. *International Dairy Journal, 8*, 325–331.

SAS (1988). *SAS user's guide: Statistics*. Cary, NC: SAS Institute Inc. (p. 584).

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

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

Tauzin, J., Miclo, L., & Gaillard, J. L. (2002). Angiotensin-I-converting enzyme inhibitory peptides from tryptic hydrolysate of bovine α_{s2}-casein. *FEBS Letters, 531*, 369–374.

Tsai, J. S., Lin, T. C., Chen, J. L., & Pan, B. S. (2006). The inhibitory effects of freshwater clam (*Corbicula fluminea*, Muller) muscle protein hydrolysates on angiotensin I converting enzyme. *Process Biochemistry, 41*, 2276–2281.

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

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

Yamamoto, N., Akino, A., & Takano, T. (1994a). Antihypertensive effect of the peptides derived from casein by an extracellular proteinase from *Lactobacillus*. *Journal of Dairy Science, 77*, 917–922.

Yamamoto, N., Akino, A., & Takano, T. (1994b). Antihypertensive effects of different kinds of fermented milk in spontaneously hypertensive rats. *Bioscience, Biotechnology and Biochemistry, 58*, 776–778.

Yamamoto, N., Maeno, M., & Takano, T. (1999). Purification and characterization of an antihypertensive peptide from a yogurt-like product fermented by *Lactobacillus helveticus* CPN4. *Journal of Dairy Science, 82*, 1388–1393.