Release of Short and Proline-Rich Antihypertensive Peptides from Casein Hydrolysate with an *Aspergillus oryzae* Protease

S. Mizuno, S. Nishimura, K. Matsuura, T. Gotou, and N. Yamamoto

R&D Center, Calpis Co., Ltd., 11-10, 5-Chome,
Fuchinobe, Sagamihara, Kanagawa 229-0006, Japan

ABSTRACT

Angiotensin-I converting enzyme inhibitory activities were measured after hydrolysis of casein by 9 different commercially available proteolytic enzymes. Among these enzymes, a protease isolated from *Aspergillus oryzae* showed the highest angiotensin-I converting enzyme inhibitory activity per peptide. The *A. oryzae* peptide also showed the highest antihypertensive effect in spontaneously hypertensive rats when the systolic blood pressure was measured 5 h after oral administration of 32 mg/kg of various enzymatic hydrolysates. Significant antihypertensive effects were observed with dosages of 9.6, 32, and 96 mg of the *A. oryzae* peptide/kg of body weight (BW), and the effects were dependent on these peptide dosages.

Analysis of peptide length showed the *A. oryzae* hydrolysate was the shortest of all tested casein hydrolysates; the peptide mixture had an average value of 1.4 amino acids (AA) in the sequence. To further characterize the *A. oryzae* hydrolysate, we analyzed the AA sequence of the whole peptide mixture. Various AA were detected at the first AA position, however, an increased number of Pro residues were observed at the second and third position of the *A. oryzae* hydrolysate. No strong signals were detected after the fourth AA position of the *A. oryzae* hydrolysate. These results suggest that the casein hydrolysate of *A. oryzae*, which expressed potent antihypertensive effects in spontaneously hypertensive rats, mainly contain short peptides of X-Pro and X-Pro-Pro sequences.

(Key words: antihypertensive peptide, angiotensin-I converting enzyme, *Aspergillus oryzae* protease, casein hydrolysate)

Abbreviation key: ACE = angiotensin-I converting enzyme, FOSHU = Food for Specified Health Use, SBP = systolic blood pressure, SHR = spontaneously hypertensive rats.

INTRODUCTION

Various physiologically functional peptides (bioactive peptides), such as opioid peptides (Teschemacher et al., 1997), antibacterial peptides (Lahov and Regelson, 1996), immunostimulating peptides (Lahov and Regelson, 1996), antioxidant peptides (Kim et al., 2001), and angiotensin-I converting enzyme (kininase II; EC 3.4.15.1)(ACE) inhibitory peptides, have been reported from protein hydrolysates, synthetic peptides, and fermented products and have been reviewed (Meisel and Bockelmann, 1999; Yamamoto et al., 2003). In the enzymatic preparation of bioactive peptides, casein is an appropriate protein source because of its safety and its long history in the human diet. It also has potential for easy processing because of the flexible and open structure. Various kinds of protease, such as trypsin, proteinase K, and thermolysin, have been used in the enzymatic hydrolysis of casein (Maruyama et al., 1985; Miyoshi et al., 1991; Yokoyama et al., 1992; Matsumura et al., 1993). Antihypertensive peptides have been studied extensively to determine biologically functional peptides; techniques include in vitro ACE inhibitory activity and in vivo studies using spontaneously hypertensive rats (SHR).

In our study, 2 kinds of ACE inhibitory peptides, Val-Pro-Pro and Ile-Pro-Pro, were generated from β - and γ -CN isolated from milk fermented with *Lactobacillus helveticus* (Nakamura et al., 1995a, b). The *L. helveticus*-fermented milk and peptide materials isolated from the fermented milk, which contained Val-Pro-Pro and Ile-Pro-Pro, showed antihypertensive effects in Japanese and Finish hypertensive subjects (Hata et al., 1996; Seppo et al., 2002). Generally, most of the reported ACE inhibitory peptides are short peptides with a Pro residue at the carboxyl terminal end (Yamamoto et al., 2003). The Pro residue at the C-terminus seems to have an important structural function in expressing strong inhibitory activity against ACE. Moreover, a short peptide is more readily absorbed from the gastrointestinal tract.

In this study, we prepared antihypertensive peptides from various commercially available proteases and studied their activity.

Received February 2, 2004.

Accepted May 31, 2004.

Corresponding author: N. Yamamoto; e-mail: naoyuki.yamamoto@calpis.co.jp.

Table 1. Comparison of angiotensin-I converting enzyme (ACE) inhibitory activities and antihypertensive effects of various enzymatic casein hydrolysates.

Protease	Origin	ACE inhibitory activity	Decrease in SBP ¹
		(% / μ g)	— (mm Hg) — $\bar{X}$ SD
Sumizyme FP	<i>Aspergillus oryzae</i>	4.1	-25.0 $\pm$ 4.3***
Trypsin	Porcine pancreas	0.8	-4.3 $\pm$ 5.3
Sumizyme CP	<i>Bacillus subtilis</i>	1.2	Not tested
Protease S	<i>Bacillus stearothermophilus</i>	1.4	-3.7 $\pm$ 6.4
Papain	<i>Carica papaya</i>	1.0	2.8 $\pm$ 5.2
Thermoase	<i>Bacillus stearothermophilus</i>	1.1	Not tested
Neurase F3G	<i>Rhizopus nivenus</i>	0.9	2.9 $\pm$ 5.4
Sumizyme RP	<i>Rhizopus delemar</i>	0.7	-5.1 $\pm$ 7.0
Bromeraine	Pineapple cannery	1.1	-4.6 $\pm$ 6.9
Not used (control)		Not tested	-2.5 $\pm$ 7.5

¹Mean of changes in systolic blood pressure at 5 h after an oral administration to spontaneously hypertensive rats (n = 5).

***Significant difference from the control (P < 0.001).

MATERIALS AND METHODS

Materials

Hippuryl-Histidyl-Leucine (Hip-His-Leu) and ACE from rabbit lung and casein were purchased from Sigma Aldrich Japan (Tokyo, Japan). Proteases, such as Sumizyme FP, Sumizyme CP, and Sumizyme RP, were purchased from Shinnihon Chemical Company (Anjyou, Japan). Trypsin, protease S, neurase F3G, thermoase, and bromeraine were obtained from Amano Enzyme Inc. (Nagoya, Japan). Trypsin was purchased from Higuchi Inc. (Tokyo, Japan), and papain was obtained from Nagase Chemtex Corporation (Kyoto, Japan). Casein was purchased from Takanashi (Hokkaidou, Japan). O-phthalaldehyde was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan)

Preparation of the Casein Hydrolysate

Casein was dissolved in hot water (80°C) to 15% (wt/vol), and the pH was adjusted to 7.0 by the addition of 1 N NaOH. After cooling to <20°C, various enzymes, listed in Table 1, were added directly to the casein solution at a final concentration of 0.6% (wt/vol). Each reaction mixture was incubated at 50°C for 14 and 24 h, after which the reaction was stopped by heating to 110°C for 10 min. The antihypertensive effect of each casein hydrolysate was measured by the method described subsequently. To characterize the peptides in the casein hydrolysates, nonhydrolysed protein and AA were removed by trapping the peptides in a tC18 Sep-pak cartridge (Waters, MA). The cartridge was washed with 10 mL of water, and peptides in the cartridge were eluted with 5 mL of 50% (wt/vol) ethanol. The eluted

peptides were lyophilized and dissolved in Tris-Cl (pH 8.0) for analysis of the ACE activity and peptide length.

Measurement of ACE Inhibitory Activity

The ACE inhibitory activity was measured according to the method of Cushman and Cheung (1971), with modification (Nakamura et al., 1995a). Twenty microliters of peptide solution were preincubated with Hip-His-Leu borate buffer (3.8 mM Hip-His-Leu, 0.1 M borate, and 0.3 M NaCl; pH 8.3) at 37°C for 5 min. Two milliunits of ACE were then added, and the mixture was incubated at 37°C for 30 min. The liberated hippuric acid was extracted with ethyl acetate. The activity of an ACE inhibitory peptide was defined as the amount needed to inhibit 50% of the ACE activity under these conditions.

Measurement of Blood Pressure

Male SHR, purchased from Charles River Japan (Yokohama, Japan), were divided into groups with each containing 5 rats fed laboratory chow (CE-2; Clea Japan, Tokyo, Japan). Systolic blood pressure (SBP) of the SHR at 21 to 23 wk old (BW, 310 to 330 g) was measured as follows. All rats were incubated at 45°C for 5 min, and the SBP was measured at the tail cuff with a programmed electro-sphygmomanometer (NARCO Bio-Systems, Austin, TX). Casein hydrolysates prepared by various proteases were orally administered by gastric intubation with nonhydrolysed casein as a control. Student's *t*-test was used for statistical analysis.

Evaluation of Peptide Length

The amount of N-terminal AA of the peptides isolated from the tC18 Sep-pak cartridge was measured by the *o*-phthalaldehyde method described previously, using casamino acid (Difco Co. Ltd., Detroit, MI) as a control. Casamino acid is an AA mixture with the same AA composition as casein because it is a casein hydrolysate with HCl. To evaluate the average peptide length, the number of N-terminal AA of each casein hydrolysate was measured, and the value was compared with the value for casamino acid. Average peptide length was calculated as follows: average peptide length = the number of N-terminal AA for casamino acid/the number of N-terminal AA for each casein hydrolysate.

AA Sequence Analysis

To characterize the enzymatic peptide hydrolysate, the AA sequence of the peptide mixture, prepared by a Sep-pak cartridge, was determined by a protein sequencer PPSQ-10 (Shimadzu, Japan).

Results

Selection of the Enzyme

To select potent ACE inhibitory peptides, various casein hydrolysates were prepared, as described in Materials and Methods, using 9 different commercially available proteolytic enzymes. The percentage of inhibitory ACE activity for 1 μ g of peptide was measured using the method described in Materials and Methods, and it is shown in Table 1. The ACE inhibitory activities were detected in all peptide samples listed in Table 1. However, among these enzymatic casein hydrolysates, a peptide with sumizyme FP, the protease from *Aspergillus oryzae*, showed the highest ACE inhibitory activity per peptide (Table 1). All results shown in Table 1 were obtained with hydrolysate when the enzyme reaction was stopped at 14 h; if enzyme reactions were prolonged to 24 h, there was little difference between the inhibitory activities (data not shown). During the enzyme reaction, precipitation was observed in all hydrolysates, but the amount of precipitate was lowest in the *A. oryzae* hydrolysate.

To compare the in vivo effects of selected casein hydrolysates listed in Table 1, the SBP of SHR was measured by a tail-cuff method, 5 h after oral administration of 32 mg of hydrolysate/kg of BW. As shown in Table 1, the casein hydrolysate from the *A. oryzae* protease (sumizyme FP) showed the highest activity in blood pressure, lowering among all 8 samples tested. The SBP of the SHR decreased by 25.0 mm Hg after oral administration of the *A. oryzae* peptide, compared with

Figure 1. Antihypertensive effect of different dosages of casein hydrolysate with *Aspergillus oryzae* protease, from 9.6 to 96 mg/kg of BW. Vertical bars represent standard error ($n = 5$). Asterisks indicate the level of significant difference from the control: ** $P < 0.01$ and *** $P < 0.001$.

2.5 mm Hg after oral administration of the control (casein). The antihypertensive effect of the *A. oryzae* peptide on the SHR was significant when compared with the control group ($P < 0.001$). There was high correlation between in vitro ACE inhibitory activity and in vivo blood pressure lowering activity for the tested samples, which suggests that the *A. oryzae* hydrolysate has a potential use as a blood pressure-lowering material. From these results, the *A. oryzae* hydrolysate showed the highest antihypertensive activity and was selected for further analysis.

Dose-Dependent Effect of the *A. oryzae* Peptide on Blood Pressure

The dose dependent effect of the *A. oryzae* peptide on SHR was examined. Significant antihypertensive effects of the *A. oryzae* peptide were observed at dosages between 9.6 and 96 mg/kg of BW. The changes in SBP at 9.6, 32, and 96 mg were -14.4 mm Hg ($P < 0.01$), -25.1 mm Hg ($P < 0.001$), and -31.0 mm Hg ($P < 0.001$), respectively, as shown in Figure 1. Moreover, a dose-dependent antihypertensive effect of the *A. oryzae* peptide was observed with dosages between 9.6 and 96 mg/kg of BW of SHR, and the effects were dependent on these peptide dosages (Figure 1).

Figure 2. Average peptide lengths of the casein hydrolysates after treatment with various proteases. The number of amino group of each casein hydrolysate was measured by the *o*-phthalaldehyde method described in Materials and Methods, using casamino acid as a control.

Characteristics of the *A. oryzae* Peptide

To further understand the *in vitro* and *in vivo* activities of the *A. oryzae* peptide among the other casein hydrolysates, the number of N-terminal ends of all samples was calculated by the *o*-phthalaldehyde method (Church et al., 1985), as described in Materials and Methods. Casein HCl hydrolysate was used as a control to evaluate average peptide length. As shown in Figure 2, the average peptide length of the *A. oryzae* peptide was 1.4, which was the shortest of all tested samples; most of the other peptides had >4.0 AA as the peptide length. We suggest that peptide length may be an important factor in ACE inhibition and antihypertensive activity.

AA Sequence

The amino terminal AA of the *A. oryzae* peptide mixture was analyzed to help understand its structure. Various AA residues were observed at the first amino terminal end of the peptide mixture. Interestingly, a Pro residue was mainly detected at the second and third AA position (Table 2). No strong signal was detected at the fourth, fifth, and sixth AA position. The number of AA released was calculated and is shown in Table 2. Amino acid signal released from the amino terminal end decreased gradually at the second and third AA positions; signals after the fourth AA position decreased to a basal level (Table 2). From these results, we suggest

that a high amount of di- and tripeptide in the *A. oryzae* hydrolysate exists. These findings support the results of the average peptide length listed in Table 1. Our results strongly suggest that the *A. oryzae* peptides contain mostly di- and tripeptides of X-Pro and X-Pro-Pro sequences.

DISCUSSION

In this study, casein hydrolysate prepared by *A. oryzae* showed a potent *in vitro* ACE inhibitory activity and *in vivo* blood pressure-lowering effect compared with other casein hydrolysates. From AA sequence analysis of the *A. oryzae* hydrolysate, the existence of short peptides of X-Pro-Pro and X-Pro peptides was suggested. These X-Pro-Pro and X-Pro peptides may be generated from excess proteolytic reactions catalyzed by a strong *A. oryzae* protease because there was a high content of AA in the hydrolysate (data not shown). Therefore, peptides remaining in the hydrolysate should have a high resistance to proteolytic enzyme action because the *A. oryzae* protease contains potent proteolytic enzyme activities.

Many reported ACE inhibitory peptides have a Pro residue at the C-terminal end (Nakamura et al., 1995a; Yamamoto et al., 1999). Short peptides containing Pro residues at the C-terminus are thought to be an appropriate ACE inhibitory peptide material. Recently, some kinds of antihypertensive peptides that had ACE inhibitory activities were isolated from some traditional cheese and proteolytic digests of whey proteins by HPLC (Abubakar et al., 1998; Saito et al., 2000). In that study, most of peptides purified from the whey protein hydrolysate prepared with a proteinase K treatment also contained Pro at the C-terminal end (Saito et al., 2000). The trypsin hydrolysate of whey protein showed a significant antihypertensive effect on SHR at 6 h after administration with a dosage of 8 mg/kg of BW (Saito et al., 2000). However, trypsin did not generate antihypertensive peptides from casein in this study, indicating the importance of the combination of protease and substrate protein to release active antihypertensive peptides.

Many kinds of ACE inhibitory peptides have been reported, however, pharmacokinetical studies of these peptides in SHR have not been comprehensively analyzed. In previous studies, the antihypertensive peptides, Val-Pro-Pro and Ile-Pro-Pro, showed potent blood pressure-lowering effects in animal (Nakamura et al., 1995b) and clinical studies (Hata et al., 1996). Moreover, the peptides were detected in a target organ, the aorta, in SHR after oral administration of these peptides (Kodama et al., 2002). Therefore, resistance of the ACE inhibitory peptide to proteolytic enzymes *in vivo*

Table 1. Number of released AA from the N-terminus of casein hydrolysate by an *Aspergillus oryzae* protease

AA	Position of N-terminus					
	1st	2nd	3rd	4th	5th	6th
						(pmol)
Asp	82	304	115	63	45	32
Glu	139	127	89	121	75	53
Asn	55	49	46	91	43	35
Gln	80	97	104	80	72	65
Ser	105	36	27	16	11	23
Thr	31	16	25	18	12	23
His	28	94	58	0	3	3
Gly	256	38	33	16	5	6
Ala	323	101	58	30	22	11
Tyr	725	114	52	28	19	23
Arg	13	7	6	8	7	12
Met	182	43	36	10	5	7
Val	869	127	196	64	42	22
Pro	42	1371	431	186	102	83
Trp	94	46	26	6	2	4
Phe	800	88	60	28	23	19
Lys	81	33	57	19	11	9
Ile	350	37	17	10	6	8
Leu	400	39	50	10	7	12
Total	4317	2767	1447	805	512	450

seems to be a very important characteristic in expressing a potent antihypertensive effect in SHR. Peptides having X-Pro-Pro and X-Pro sequences most likely exist in *A. oryzae* and may show resistance to proteolysis in SHR, especially after reaching the target organ. It has been suggested that strong in vitro ACE inhibitory activity and antihypertensive activity in SHR might be due to Pro-rich peptides. We suggest that the *A. oryzae* protease might be a suitable enzyme to generate potent ACE inhibitory activity and antihypertensive activity. To further understand the in vitro and in vivo activities, identification of each active component and confirmation of the Pro-rich sequence in the hydrolysate is essential. We attempted to isolate all peptides in the *A. oryzae* hydrolysate by HPLC; however, short peptides were eluted in such a narrow range that it was difficult to isolate each individual peptide. We suggest that the liquid chromatography-mass spectrometry method would be a useful tool in the quantification of X-Pro-Pro and X-Pro peptides in hydrolysate.

Aspergillus oryzae has a long history in the manufacturing of fermented food products in Japan, for example, rice wine, soy sauce, miso, and some traditional fermented food products (Masuda et al., 1996; O'Toole, 1999; Chiou and Cheng, 2001). *Aspergillus oryzae* plays an important role in accelerating ripening during fermentation because of rapid growth in protein sources caused by strong proteolytic activity and sugar utility. Casein and proteolytic enzymes of *A. oryzae* are well known as safe food materials. Therefore, it is likely that other *A. oryzae* peptides will also be safe as food products. Moreover, a recent toxicity study in rodents

and an antigenicity study proved the *A. oryzae* hydrolysate to be a safe food product (data not shown).

In Japan, regulation for functional food was established in 1991 as **FOSHU** (Food for Specified Health Use). To date, almost 400 kinds of FOSHU products are registered in Japan, and the market of FOSHU products is still expanding. For the application of this peptide material as a FOSHU product, a clinical study concerned with safety and efficacy would be necessary. Antihypertensive peptides prepared by casein hydrolysis with the *A. oryzae* protease used in this study might have potential for use in various types of food products as functional peptide materials.

REFERENCES

- Abubakar, A., T. Saito, H. Kitazawa, Y. Kawai, and T. Itoh. 1998. Structural analysis of new antihypertensive peptides derived from cheese whey protein by proteinase K digestion. *J. Dairy Sci.* 81:3131–3138.
- Chiou, R. Y., and S. L. Cheng. 2001. Isoflavone transformation during soybean koji preparation and subsequent miso fermentation supplemented with ethanol and NaCl. *J. Agric. Food Chem.* 49:3656–3660.
- Church, F. C., D. H. Porter, G. L. Catignani, and H. Swaisgood. 1985. An *o*-phthalaldehyde spectrophotometric assay for proteinases. *Anal. Biochem.* 146:343–348.
- Cushman, D. W., and H. S. Cheung. 1971. Spectrophotometric assay and properties of angiotensin-converting enzyme of rabbit lung. *Biochem. Pharmacol.* 20:1637–1648.
- Hata, Y., M. Yamamoto, M. Ohni, K. Nakajima, Y. Nakamura, and T. Takano. 1996. A placebo-controlled study of the effect of sour milk on blood pressure in hypertensive subjects. *Am. J. Clin. Nutr.* 64:767–771.
- Kim, S. K., Y. T. Kim, H. G. Byun, K. S. Nam, D. S. Joo, and F. Shahide. 2001. Isolation and characterization of antioxidative peptides from gelatin hydrolyzate of Alaska pollack skin. *J. Agric. Food Chem.* 49:1984–1989.

- Kodama, S., A. Yamamoto, A. Matsunaga, K. Nakagomi, and K. Hayakawa. 2002. Behaviors of D- and L-lactic acids during the brewing process of sake (Japanese rice wine). *J. Agric. Food Chem.* 50:767–770.
- Lahov, E., and E. Regelson. 1996. Antibacterial and immunostimulating casein-derived substances from milk: Casecidin, isracidin peptides. *Food Chem. Toxicol.* 34:131–145.
- Maruyama, S., K. Nakagomi, N. Tomizuka, and H. Suzuki. 1985. Angioten I-converting enzyme inhibitor derived from an enzymatic hydrolysate of casein. II. Isolation and bradykinin-potentiating activity on the uterus and the ileum of rats. *Agric. Biol. Chem.* 49:1405–1409.
- Masuda, O., Y. Nakamura, and T. Takano. 1996. Antihypertensive peptides are present in aorta after oral administration of sour milk containing these peptides to spontaneously hypertensive rats. *J. Nutr.* 126:3063–3068.
- Matsumura, N., M. Fujii, Y. Takeda, K. Sugita, and T. Shimizu. 1993. Angiotensin I-converting enzyme inhibitory peptides derived from bonito bowels autolysate. *Biosci. Biotechnol. Biochem.* 57:695–697.
- Meisel, H., and W. Bockelmann. 1999. Bioactive peptides encrypted in milk proteins: Proteolytic activation and tropho-functional properties. *Antonie van Leeuwenhoek* 76:207–215.
- Miyoshi, S., H. Ishikawa, T. Kaneko, F. Fukui, H. Tanaka, and S. Maruyama. 1991. Structures and activity of angiotensin-converting enzyme inhibitors in an alpha-zein hydrolysate. *Agric. Biol. Chem.* 55:1313–1318.
- Nakamura, Y., N. Yamamoto, K. Sakai, A. Okubo, S. Yamazaki, and T. Takano. 1995a. Purification and characterization of angiotensin I-converting enzyme inhibitors from sour milk. *J. Dairy Sci.* 78:777–783.
- Nakamura, Y., N. Yamamoto, K. Sakai, and T. Takano. 1995b. Antihypertensive effect of sour milk and peptides isolated from it that are inhibitors to angiotensin I-converting enzyme. *J. Dairy Sci.* 78:1253–1257.
- O'Toole, D. K. 1999. Characteristics and use of okara, the soybean residue from soy milk production. *J. Agric. Food Chem.* 47:363–371.
- Saito, T., T. Nakamura, H. Kitazawa, Y. Kawai, and T. Itoh. 2000. Isolation and structural analysis of antihypertensive peptides that exist naturally in Gouda cheese. *J. Dairy Sci.* 83:1434–1440.
- Seppo, L., O. Kerojoki, T. Suomalainen, and R. Korpela. 2002. The effect of a *Lactobacillus helveticus* LBK-16 H fermented milk on hypertension—A pilot study on humans. *Milchwissenschaft* 57:124–127.
- Teschemacher, H., G. Kock, and V. Brantl. 1997. Milk protein-derived opioid receptor ligands. *Biopolymers* 43:99–117.
- Yamamoto, N., M. Ejiri, and S. Mizuno. 2003. Biogenic peptides and their potential use. *Curr. Pharm. Des.* 9:1345–1355.
- Yamamoto, N., M. Maeno, and T. Takano. 1999. Purification and characterization of an antihypertensive peptide derived from a yogurt-like product fermented by *Lactobacillus helveticus* CPN4. *J. Dairy Sci.* 82:1388–1393.
- Yokoyama, K., H. Chiba, and M. Yoshikawa. 1992. Peptide inhibitors for angiotensin I-converting enzyme from thermolysin digest of dried bonito. *Biosci. Biotechnol. Biochem.* 56:1541–1545.