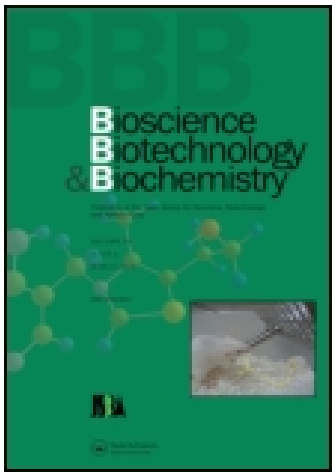

Bioscience, Biotechnology, and Biochemistry
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/tbbb20

Angiotensin I-converting Enzyme Inhibitory Peptides in an Alkaline Protease Hydrolyzate Derived from Sardine Muscle

Hiroshi Matsufuji[a], Toshiro Matsui[a], Eiji Seki[ab], Katsuhiro Osajima[ab], Masatoshi Nakashima[a] & Yutaka Osajima[a]

[a] Department of Food Science and Technology, Faculty of Agriculture, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812, Japan

[b] Research Laboratories, Senmi Ekisu Co., Hirano, Ozu 795, Japan

Published online: 12 Jun 2014.

To cite this article: Hiroshi Matsufuji, Toshiro Matsui, Eiji Seki, Katsuhiro Osajima, Masatoshi Nakashima & Yutaka Osajima (1994) Angiotensin I-converting Enzyme Inhibitory Peptides in an Alkaline Protease Hydrolyzate Derived from Sardine Muscle, Bioscience, Biotechnology, and Biochemistry, 58:12, 2244-2245, DOI: 10.1271/bbb.58.2244

To link to this article: http://dx.doi.org/10.1271/bbb.58.2244

Biosci. Biotech. Biochem., **58** (12), 2244–2245, 1994

Note

Angiotensin I-converting Enzyme Inhibitory Peptides in an Alkaline Protease Hydrolyzate Derived from Sardine Muscle

Hiroshi MATSUFUJI, Toshiro MATSUI, Eiji SEKI,* Katsuhiro OSAJIMA,* Masatoshi NAKASHIMA, and Yutaka OSAJIMA

Department of Food Science and Technology, Faculty of Agriculture, Kyushu University, 6–10–1 Hakozaki, Higashi-ku, Fukuoka 812, Japan

**Research Laboratories, Senmi Ekisu Co., Hirano, Ozu 795, Japan*

Received October 13, 1993

The ACE inhibitory activity of an alkaline protease hydrolyzate from sardine muscle did not change after being treated by gastrointestinal proteases (IC_{50} = 0.082 mg protein/ml). Eleven new ACE inhibitory peptides, constructed with 2 to 4 amino acid residues, were isolated from the hydrolyzate. The ACE inhibitory activity of each was mostly below 100 μM of IC_{50} value; the maximal inhibitory activity was observed for Lys-Trp (IC_{50} = 1.63 μM). The isolated peptides inhibited ACE competitively, except for Met-Tyr with non-competitive inhibition. As the result of sequence homology, Arg-Val-Tyr isolated from the hydrolyzate was found in the primary structure of angiotensins I, II, and III, and of des Asp[1]-angiotensin I.

Proteins[1–3] from natural sources can be considered as a "physiologically functional food" material, from which a number of angiotensin I-converting enzyme (ACE; peptidyl dipeptidase, exhibited EC 3.4.15.1) inhibitors[4–6] are derived. Some of them have antihypertensive activity by intravenous[7–9] and oral[10,11] administrations *in vivo*. However, judging from the preparations based on the hydrophobic affinity of peptides to the ACE active site, the inhibitors would have had strong bitterness and/or low solubility in water, in spite of their potent inhibitory activity. We have already reported[12] that the *Bacillus licheniformis* alkaline protease (2.4L, TYPE FG, Novo Co.) hydrolyzate (A-1) from sardine muscle, which had relative high ACE inhibitory activity (IC_{50} = 0.26 mg protein/ml), had little taste and fish odor, as well as high solubility in water. Further fractionation of A-1 in an ODS column with ethanol resulted in the production of a more potent inhibitor, the maximal ACE inhibitory activity being observed in the 10% ethanol-eluted fraction (Y-2; IC_{50} = 0.083 mg protein/ml). The Y-2 fraction also had little taste and fish odor, like A-1. In this report, we investigate the effectiveness of alkaline protease hydrolyzates as a "physiologically functional food" and attempt to isolate the ACE inhibitory peptides from the Y-2 fraction.

ACE inhibitory activity was determined by the 2,4,6-trinitrobenzene sulfonate (TNBS) method proposed elsewhere.[13] For each assay, the ACE inhibitor and Hip-His-Leu in a borate buffer (pH 8.3) containing 200 mM NaCl were incubated with purified rabbit lung ACE (12.5 mU/mg, Sigma Chemical Co.) at 37°C for 1 h. The reaction was stopped by adding 0.5 N HCl, the solution was adjusted to pH 9.12, and 0.1 M TNBS in a 0.1 M Na_2HPO_4 solution was then added. After incubating at 37°C for 20 min, 4 mM Na_2SO_3 in a 0.2 M NaH_2PO_4 solution was added, and the absorbance of the yielded TNP-His-Leu complex at 416 nm was measured with a Shimadzu UV-1200 spectrophotometer. The ACE inhibitor concentration required to inhibit 50% of the ACE activity is defined as the IC_{50} value.

A sardine muscle hydrolyzate with alkaline protease (A-1) was prepared after a pre-heat treatment (98°C for 10 min) as described in our previous paper.[1] This pre-heat treatment gave higher ACE inhibitory activity (IC_{50} = 0.083 mg protein/ml) than no heat treatment (IC_{50} = 0.26 mg protein/ml). To investigate the resistance of A-1 to digestion by gastrointestinal proteases, A-1 was digested by various proteases: pepsin (pH 2, 1.0 wt%, 4 h), chymotrypsin (pH 8, 1.0 wt%, 4 h), trypsin (pH 8, 1.0 wt%, 4 h), and the intestinal fluid from pigs (pH 7, one vial, Nihonkayaku Co., 4 h). Sugiyama *et al.*[14] have already reported that the ACE inhibitory activity of the alkaline protease hydrolyzate from defatted sardine meal was reduced by 40% after a digestion test with gastrointestinal proteases. However, as shown in Table I, the ACE inhibitory activity of A-1 was little changed after further digestion. Also, the average peptide length of A-1 calculated by the proportion of free amino groups before and after hydrolysis with HCl[15] was 2.90, and the peptides were constructed of 2 to 4 amino acid residues. Matsuda *et al.*[16] and Ogihira *et al.*[17] have reported that di- and tri-peptides would have low susceptibility to hydrolysis by any digestive enzymes. Hence, the high resistance of A-1 to digestion suggests that the peptides responsible for ACE inhibition would be small.

We then tried to isolate the inhibitory peptides from A-1. In this study, two isolation steps by GPC with reversed-phase columns were conducted to prevent a poor yield of the isolated peptide. In the first step, the Y-2 fraction with the most potent ACE inhibitory activity that was eluted from A-1 with 10% ethanol was applied to an Asahipak GS-320 column (7.6ϕ × 500 mm, Asahikasei Co.) for high-performance liquid chromatography (HPLC; Shimadzu LC-9A instrument) and eluted with 20% CH_3CN in 50 mM ammonium acetate (pH 6.7) at a flow rate of 1.0 ml/min, while monitoring the absorbance at 220 nm. The active fractions were collected and concentrated, and each fraction was rechromatographed with 20 mM ammonium acetate (pH 4.0). Next, the active fractions were put into an ODS-120T column (7.8ϕ × 300 mm, Tosoh Co.) and eluted with a linear CH_3CN gradient (10–50%, 80 min) in 10% ammonium formate (pH 6.3) at room temperature. The final separation was achieved in the same ODS column by linear-gradient elution with CH_3CN (0–30%, 90 min) in 0.1% trifluoroacetic acid, the isolated fractions being collected and

Table I. Resistance of the Alkaline Protease Hydrolyzate to Digestion by Gastrointestinal Proteases

Digesting protease	IC_{50} (mg protein/ml)
None	0.083
Pepsin (pH 2, 4 h)	0.073
Pepsin→Trypsin (pH8, 4 h)	0.080
Pepsin→Chymotrypsin (pH 8, 4 h)	0.082
Pepsin→Trypsin + Chymotrypsin (pH 8, 4 h)	0.079
Pepsin→Intestinal fluid (pH 7, 4 h)	0.077

Table II. ACE Inhibitors Derived from the Alkaline Protease Hydrolyzate

Amino acid sequence	Amino acid ratio in acid hydrolyzate	IC_{50} (μM)	Inhibition mode
Met-Phe	Met 1.00, Phe 1.21	44.7	Competitive
Arg-Tyr		51	Competitive
Met-Tyr		193	Non-competitive
Leu-Tyr	Leu 1.19, Tyr 1.00	38.5	Competitive
Tyr-Leu		82	Competitive
Ile-Tyr[a]	Ile 1.00, Tyr 0.64	10.5	Competitive
Val-Phe[a]	Val 1.00, Phe 1.16	43.7	Competitive
Lys-Trp	Lys 1.00, Trp —	1.63	Competitive
Gly-Arg-Pro		20.0	
Arg-Phe-His		330	
Ala-Lys-Lys	Ala 1.00, Lys 1.63	3.13	
Arg-Val-Tyr	Arg 1.00, Val 1.00, Tyr 0.91	205.6	Competitive
Gly-Trp-Ala-Pro		3.86	

[a] Reference 16.

dried.

Table II summarizes the isolated ACE inhibitory peptides from the alkaline protease hydrolyzate with the ACE inhibitory activity of each. The primary structure of each peptide was identified by a Shimadzu LC-6A amino acid analyzer and a Shimadzu PSQ-1 protein sequencer. Eleven inhibitory peptides, except for Val-Phe and Ile-Tyr, which have already been reported by other investigators,[9,18] were found for the first time in a natural protein hydrolyzate. Most of these peptides had more potent inhibitory activity, with IC_{50} values below 100 μM, than the reported ACE inhibitory peptides from natural sources; among them, Lys-Trp was the most potent inhibitor, with an IC_{50} value of 1.63 μM, of the ACE inhibitory dipeptides. All of the isolated peptides were constructed of 2 to 4 amino acid residues.

To determine the inhibition mode for the isolated peptides, the activity measurements while changing the concentration of the substrate and inhibitor were performed with an incubation time of 20 min. The inhibition mode to the ACE-catalyzed hydrolysis of Hip-His-Leu was evaluated by Lineweaver–Burk plots (Table II). As a result, Met-Tyr was found to act as a non-competitive inhibitor, while all of the other peptides were competitive. Although non-competitive ACE inhibitory peptides have already been reported by Kohama *et al.* (Pro-Thr-His-Ile-Lys-Trp-Gly-Asp)[19] and Saito *et al.* (Ile-Tyr-Pro-Arg-Tyr, Val-Tyr),[20] the inhibition site of these peptides on ACE was not specified. The inhibition mechanism of Met-Tyr is now under investigation by considering the possibility of inhibition due to the chelating effect on the zinc atom of ACE.

A homology investigation with the GENAS data base was carried out for the isolated peptides. Interestingly, the sequence of Arg-Val-Tyr with an IC_{50} value of 205.6 μM was highly homologous in its primary structure to a series of angiotensins in the pressor system: angiotensins I, II, and III, and des Asp[1]-angiotensin I. At present, we are attempting to confirm the existence of angiotensin fragments in blood which have ACE inhibitory activity.

Acknowledgment. This work was supported by a Grant-in-Aid for Scientific Research in Priority Areas from the Ministry of Education, Science, and Culture of Japan.

References

1) H. Ukeda, H. Matsuda, H. Kuroda, K. Osajima, H. Matsufuji, and Y. Osajima, *Nippon Nōgeikagaku Kaishi* (in Japanese), **65**, 1223–1228 (1991).
2) H. Chiba and M. Yoshikawa, *Kagaku to Seibutsu* (in Japanese), **25**, 396–405 (1987).
3) Y. Kawamura, T. Sugimoto, S. Takane, and M. Satake, *Biryou Eiyouso Kenkyu* (in Japanese), **6**, 117–121 (1989).
4) K. Suetsuna and K. Osajima, *Bull. Japan. Soc. Sci. Fish.* (in Japanese), **52**, 1981–1984 (1986).
5) T. Hazato and R. Kase, *Biochem. Biophys. Res. Commun.*, **139**, 52–55 (1986).
6) S. Maruyama, S. Miyoshi, and H. Tanaka, *Agric. Biol. Chem.*, **53**, 2763–2767 (1989).
7) S. Miyoshi, H. Ishikawa, T. Kaneko, F. Fukui, H. Tanaka, and S. Maruyama, *Agric. Biol. Chem.*, **55**, 1313–1318 (1991).
8) H. Ukeda, H. Matsuda, K. Osajima, H. Matsufuji, T. Matsui, and Y. Osajima, *Nippon Nōgeikagaku Kaishi* (in Japanese), **66**, 25–29 (1992).
9) K. Yokoyama, H. Chiba, and M. Yoshikawa, *Biosci. Biotech. Biochem.*, **56**, 1541–1545 (1992).
10) S. Maruyama, *Bioscience and Industry* (in Japanese), **47**, 1182–1186 (1989).
11) K. Suetsuna and K. Osajima, *Nippon Eiyo Shokuryo Gakkaishi* (in Japanese), **42**, 47–54 (1989).
12) T. Matsui, H. Matsufuji, E. Seki, K. Osajima, M. Nakashima, and Y. Osajima, *Biosci. Biotech. Biochem.*, **57**, 922–925 (1993).
13) T. Matsui, H. Matsufuji, and Y. Osajima, *Biosci. Biotech. Biochem.*, **56**, 517–518 (1992).
14) K. Sugiyama, K. Takada, M. Egawa, I. Yamamoto, H. Onizuka, and K. Oba, *Nippon Nōgeikagaku Kaishi* (in Japanese), **65**, 35–43 (1991).
15) T. Nakamura, Y. Syukunobe, R. Doki, K. Hirano, and H. Itoh, *Nippon Shokuhin Kogyo Gakkaishi* (in Japanese), **38**, 377–383 (1991).
16) H. Matsuda, T. Ishizaki, H. Morita, T. Nagaoka, K. Osajima, and Y. Osajima, *Nippon Nōgeikagaku Kaishi* (in Japanese), **66**, 1645–1647 (1992).
17) H. Ogihira and Y. Nakabou, *Taisha* (in Japanese), **27**, 993–1000 (1990).
18) H.-S. Cheung, F.-L. Wang, M. A. Ondetti, E. F. Sabo, and D. W. Cushman, *J. Biol. Chem.*, **255**, 401–407 (1980).
19) Y. Kohama, H. Oka, K. Yamamoto, T. Teramoto, M. Okabe, T. Mimura, Y. Nagase, Y. Chiba, and T. Fujita, *Biochem. Biophys. Res. Commun.*, **161**, 456–460 (1989).
20) Y. Saito, K. Nakamura, A. Kawato, and S. Imayasu, *Nippon Nōgeikagaku Kaishi* (in Japanese), **66**, 1081–1087 (1992).