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Angiotensin I-Converting Enzyme Inhibitory Activity of Protein Hydrolysates from Tuna Broth

JYH-SHENG HWANG[1] AND WEN-CHING KO[2]*

[1.] *Department of Food Nutrition, Hungkuang University, 34 Jhongci Rd., Shalu Township, Taichung County 433, Taiwan, R.O.C.*

[2.] *Department of Bioindustry Technology, Da-Tsuen University, 112 Shanjiao Rd., Dacun Township, Changhu County 515, Taiwan, R.O.C.*

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ABSTRACT

Angiotensin I-converting enzyme (ACE) inhibitors have a potent antihypertensive effect in both humans and animals. Various peptides derived from food proteins were found to have ACE inhibitory activity. For determining ACE inhibitory activity, protein hydrolysates were prepared from tuna broth (contain 4% water-soluble protein) using various commercial proteases. Orientase hydrolysate (OAH) exhibited the most potent inhibitory activity on ACE. The best hydrolysis conditions with respect to inhibition of ACE were 3hr of incubation at 50˚C, pH 6.5, with enzyme (1.0% orientase) to substrate (tuna broth) of 1/25 (v/v). Preparation under these conditions yielded activity (IC_{50} 12.52 ± 0.02 mg/mL). Fractionation of the orientase hydrolysate on a Sephadex G-25 gel filtration chromatograph resulted in the production of more active inhibitors. The active fractions were peptides of molecular weights < 1,000 Da. The most active fraction had an IC_{50} value of 0.21 ± 0.01 mg/mL. This fraction was rich in basic amino acids and aromatic amino acids [peptides with molecular mass (< 565 Da)].

Key words: angiotensin I-converting enzyme (ACE), protein hydrolysate, peptide, tuna broth

INTRODUCTION

Angiotensin I-converting enzyme (ACE, peptidyl dipeptidase, EC 3.4.15.1) is important in regulating blood pressure. In the rennin angiotensin system, ACE acts on angiotensin I to hydrolyze His-Leu from its C-terminal and produces a potent vasopressor aginotensin II[(1)]. In addition, in the kinin kallikrein system, ACE inactivates bradykinin, which performs a vasodilatory action[(2)]. Accordingly, ACE elevates blood pressure. Some inhibitors of ACE show antihypertensive effects *in vivo* and are used as pharmaceuticals[(3)]. Recently, ACE inhibitory peptides derived from hydrolystates of casein, fish muscle, and other food proteins were isolated[(4-7)]. The oral administration of these peptides reduced blood pressure in spontaneously hypertensive rats (SHR) and also inhibited the presser effect of intravenously administrated angiotensin-I in normotensive rats[(8)].

Tuna broth, a protein-rich stock (4% water-soluble protein) of the tuna canning industry is discarded as waste[(9)]. Canned tuna is the most valuable commercial canned fishery products in Taiwan, with an annual production of over 2,470 tons[(10)]. One tuna processing plant produces over 15 tons/day of tuna broth, which contains valuable proteins. This effluent causes not only water pollution but also loss of a potentially valuable protein resource[(11)]. Thus, the protein should be recovered and utilized. The authors prepare from tuna broth a hydrolysate with ACE inhibitory activity that could be applied as a new source of physiologically functional food material. The proteolysis conditions of the bioactive hydrolysates and their characteristics were investigated in this work.

* Author for correspondence. Tel: +886-4-8511888 ext. 4191; Fax: +886-4-22873208; E-mail: wcko@mail.dyu.edu.tw

MATERIALS AND METHODS

I. *Tuna Broth*

The tuna broth was obtained from a tuna-canning factory in Chiayi, Taiwan. Tuna fish meat was cooked with steam (100-105˚C) for 3hr. After the cooked meat was cooled, the soup base was filtered through two layers of gauze to remove any floating fat and solids. The collected liquid was vacuum-packed in 400-g polyethylene bags and stored at -30˚C.

II. *Enzymes and Chemicals*

Four commercial enzymes, orientase (derived from *Bacillus subtilis*, 70,000 Folin units/mg, Hankyu Co., Tokyo, Japan), protease XXIII (3.8 Folin units/mg, Sigma Chemical Co., LA, USA), papain (1.7 units/mg, one unit will hydrolyze 1.0 μmole of BAFE per min at 25˚C, pH 6.2, Sigma Chemical Co., LA, USA) and trypsin (protease activity: 24 IU/l, Sigma Chemical Co., LA, USA), were selected on the basis of their potential to hydrolyze fish protein in a preliminary hydrolysis assay. All chemicals used were of reagent grade and obtained from Sigma Co. (USA).

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III. *Preparation of Hydrolysates*

A 25 mL-sample of tuna broth (adjusted to pH 6.5 with 0.1N NaOH) containing about 4% protein (w/v) was hydrolyzed with individual protease at a ratio of substrate to enzyme of 25 (v/v). Hydrolysis was performed at pH 6.5. The pH was monitored and adjusted every 30 min by addition of 0.5 M NaOH or 1M HCl during the hydrolysis period. The content of soluble protein in tuna broth depended on pH and the highest solubility was observed at pH between 5.5 and 6.5[9]. The following experiments were performed on broth at pH 6.5 to maximize solubility (since it was close to 6.28, the pH value of tuna broth), although this pH value differs from the optimum pH value for the activity of the four enzymes (optimum pH of orientase is 7.0, protease XIII pH 7.5; papain pH 7.5; trypsin pH 7.5). The temperature of the reaction mixtures was adjusted to the optimum temperature of each enzyme, for the duration of hydrolysis. After the reaction proceeded for the set hydrolysis, it was terminated by immersing the reaction vessel into a water bath (100˚C) for 10 min with occasional stirring to inactivate enzymes. After cooling, the hydrolysate was centrifuged at 10,000 ×g for 10 min. The supernatant was collected, frozen at -70˚C and then freeze-dried.

IV. *Analysis of Free Amino Acids*

The free amino acids were characterized upon the enzymatic hydrolysates of tuna broth, according to the procedure of Moore and Stein[12]. The protein hydrolysate (15 mL) was added with 10 mL of 15% TCA for 30 min, and centrifuged (10 min at 10,000 ×g). The supernatants were freeze-dried after being frozen overnight at -30˚C, and placed into a 25-mL volumetric flask together with 10 mL of a 0.2 M citrate buffer (pH 2.2). The quantity and type of free amino acids present were determined with the ninhydrin regent with amino acid standards using a High Performance Amino Acid Analyzer (Beckman 6300, San Ramon, CA95483, USA), column: cation exchange resin, analysis temperature: 50-70˚C, cuvette length: 12 mm, citrate buffer (0.2 M, pH 2.2) flow rate: 20 mL/hr, ninhydrin flow rate: 10 mL/hr.

V. *Characterization of ACE Inhibition*

ACE inhibitory activity was measured spectrophotometrically using hippuryl-*L*-histidyl-*L*-leucine (Hip-His-Leu) as substrate according to the method of Cushman and Cheung[13]. A 50-μL sample solution and 100 μL of 2.5 mU ACE (from rabbit lung obtained from Sigma Co. USA) solution were added to 100 μL of 12.5 mM Hip-His-Leu solution in 1.0 M NaCl-borate buffer at pH 8.3. After incubation at 37˚C for 1 hr, the reaction was stopped by adding 250 μL of 0.5 N HCl. The liberated hippuric acid was extracted with 1.5 mL of ethyl acetate, and its absorbance at 228 nm was determined to evaluate the ACE inhibitory activity. The inhibition rate (%) is shown as {(Ec-Es/Ec-Eb)} × 100, where Es is the absorbance when the sample is added to the reaction mixture; Ec is the absorbance with buffer (instead of the sample) added, and Eb is the absorbance when the stop solution was added before the reaction occurred. The IC_{50} value was expressed in terms of mg protein/mL. The % of ACE inhibition versus log10 [hydrolysate] (mg/mL) curve was constructed using at least seven separate analyses (different concentrations). The IC_{50} value is the concentration when 50% of ACE activity was inhibited.

VI. *Protein Determination*

Protein concentrations were determined by the Lowry method[14]. Triplicate assays were performed for each sample and bovine serum albumin (100 μg/mL, 50 μg/mL, 25 μg/mL, 12.5 μg/mL) was used as the protein standard (r^2 = 0.9979).

VII. *Molecular Weight (MW) Distribution by Gel Filtration*

Freeze-dried powder (280 mg) obtained from protease hydrolysis was dissolved in 1 mL of distilled water and applied on a Sephadex G-25 column (2.5 × 70 cm), which was eluted with 0.05 M phosphate buffer, pH 6.5. Fractions in 5 mL were collected at a flow rate of 45 mL/hr. The absorbance at 280 nm and the ACE inhibitory activity of all fractions were measured. The distribution of MW of hydrolysate was estimated using β-endothein (M.W. 2,573), [Sar^1, Ala^8] angiotensin-II (M.W. 926), *N*-formyl-Met-Leu-Phe-Lys (M.W. 565.7) and tryptophan (M.W. 204) for calibration.

RESULTS

I. *Selection of Protease for Hydrolysis of Tuna Broth*

Table 1 summarizes the ACE inhibition indices obtained from the hydrolysates of tuna broth. The original tuna broth showed an extremely low (< 5%) ACE inhibition, which increased after hydrolysis with orientase, papain, trypsin, and protease XXIII. Among the four hydrolysates, orientase digest showed the strongest ACE

Table1. Inhibition of ACE by enzymatic hydrolyzates of tuna broth

Enzyme	ACE-inhibition rate (%)[a]
Orientase	62.2 ± 2.1
Protease XXIII	24.5 ± 2.5
Papain	34.1 ± 2.1
Trypsin	25.9 ± 1.9
None	3.0 ± 1.3

[a]Tuna broth was hydrolyzed with individual enzyme preparations (at a ratio of each enzyme solution (1.0%) to substrate of 1/25 (v/v) for 2 hr at the optimum temperature for each enzyme, pH 6.5). Orientase at 50˚C, protease XXIII at 37˚C, papain at 25˚C, trypsin at 37˚C. Values are mean ± standard deviation (n = 3).

inhibition (around 60%). Orientase was therefore selected to prepare hydrolysate in the following experiments.

II. *Inhibition of ACE Using Orientase Hydrolysates*

Figure 1 shows the effect of proteolysis time and orientase concentration on the inhibition of ACE at 50˚C at an enzyme (0.1, 0.5, 1.0, 2.0% orientase)/substrate (tuna broth) ratio of 1/25 (v/v). With the addition of 1.0 and 2.0% enzyme, the activity reached the maximal value (about 77.45%) after 3 hr of proteolysis and then leveled off, while at 0.1% and 0.5%, the maximal activity of 33% and 51%, respectively, were obtained after 6 hr of hydrolysis. Hydrolysates with ACE inhibition index values > 50% were selected for the determination of the IC_{50} value, which is the amount of hydrolysate that inhibits ACE activity by 50%. Table 2 summarizes the IC_{50} value of orientase hydrolysates prepared under optimal conditions to show the maximal inhibitory activity at each concentration (0.5, 1.0, 2.0%) of enzyme added shown in Figure 1. The IC_{50} value associated with the maximum inhibitory activity was 12.52 ± 0.02 mg/mL for 1.0% orientase and 12.50 ± 0.02 mg/mL for 2.0% orientase after 3 hr incubation. The two hydrolysates differ insignificantly in IC_{50} values. Table 3 indicates similar pattern in the amino acid composition for the two hydrolysates prepared.

III. *Fractionation of Hydrolysate and ACE Inhibitory Activity*

The apparent molecular weight distribution of peptides in hydrolysate prepared with 1.0% orientase for 3 hr was estimated by gel filtration on a Sephadex G-25 column (Figure 2). The gel filtration showed four major fractions, OA1 (no. 13-15, retention time 81-100 min, M.W. > 2573), OA2 (no. 31-33, retention time 201-230 min, 926 > M.W. > 565), OA3 (no. 39-41, retention time 254-273 min, M.W. < 565) and OA4 (no. 46-48, retention time 300-320 min, M.W. < 565). The active fractions were those that contained

Table 2. IC_{50} values of orientase hydrolysates prepared from tuna broth under various conditions

Enzyme (%)	Hydrolysis time[a] (hr)	IC_{50}[b] (mg/mL)
0.5	6	26.58 ± 0.01
1.0	3	12.52 ± 0.02
2.0	3	12.50 ± 0.02

[a]Hydrolysis was performed at 50˚C at pH 6.5 at an enzyme (0.5, 1.0, 2.0% orientase)/substrate (tuna broth) ratio of 1/25 (v/v) for the specified time to yield the maximum activity shown in Figure 1.
[b]Values are mean ± standard deviation (n = 3).

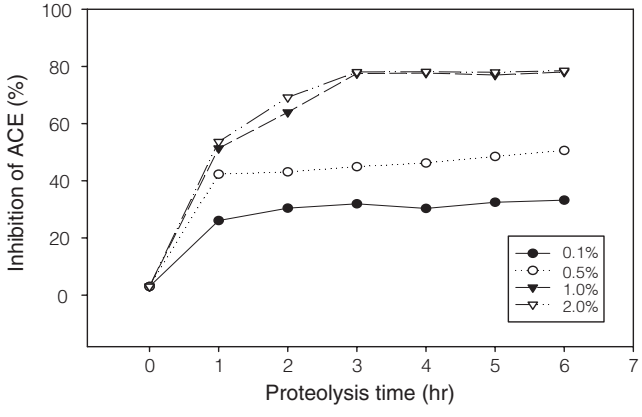

Figure 1. Effect of proteolysis time on inhibition of ACE as a function of orientase concentration.
Tuna broth was hydrolyzed by orientase at 50˚C, pH 6.5 and a ratio of enzyme solution (0.1, 0.5, 1.0, 2.0% orientase) to substrate of 1/25 (v/v).

Table 3. Amino acid contents of orientase hydrolysates of tuna broth

Amino acids (μmol/mL)	Tuna broth	1.0%-3 hr proteolysis[a]	2.0%-3 hr proteolysis[a]
Asp	258.52 (0.82)[b]	257.16 (1.36)[b]	257.06 (1.46)[b]
Tau	156.51 (7.58)	144.53 (11.98)	144.65 (11.86)
Thr	129.63 (0.78)	128.35 (1.28)	128.19 (1.44)
Ser	128.01 (0.88)	126.55 (1.46)	126.37 (1.64)
Glu	317.27 (1.19)	315.22 (2.05)	314.96 (2.31)
Pro	289.91 (0.61)	288.96 (0.95)	288.73 (1.18)
Gly	946.05 (0.84)	944.4 (1.65)	944.21 (1.84)
Ala	431.42 (1.73)	428.2 (3.22)	427.75 (3.67)
Val	148.13 (1.11)	146.32 (1.81)	146.1 (2.03)
Hcy	92.10 (0.09)	91.87 (0.23)	91.87 (0.23)
Met	58.04 (0.47)	57.1 (0.94)	57.1 (0.94)
Ile	55.26 (0.65)	54.09 (1.17)	53.97 (1.29)
Leu	112.66 (1.41)	110.25 (2.41)	110.06 (2.60)
Tyr	37.91 (0.57)	37.05 (0.86)	36.99 (0.92)
Phe	63.90 (0.62)	61.78 (2.12)	61.88 (2.02)
Trp	3.75 (0.06)	3.68 (0.07)	3.65 (0.10)
Lys	171.13 (1.05)	169.39 (1.74)	169.24 (1.89)
His	215.15 (5.81)	205.75 (9.4)	205.13 (10.02)
Arg	28.12 (0.90)	26.89 (1.23)	26.74 (1.38)
Total	3643.47 (27.17)	3597.54 (45.93)	3594.65 (48.82)

[a]Proteolysis was performed at a ratio of enzyme solution (1.0% , 2.0% orientase) to substrate of 1/25 (v/v) at 50˚C for 3hr.
[b]Figure in parentheses shows the contents of free amino acid in tuna broth or hydrolysates.

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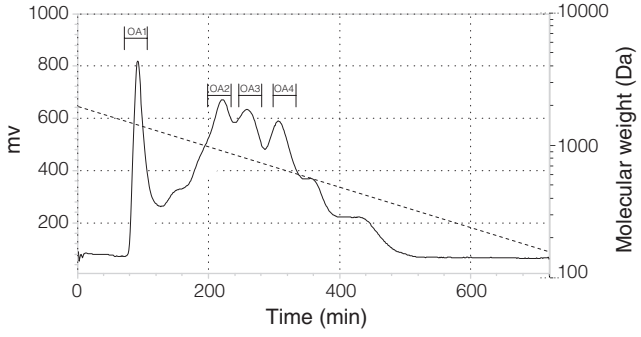

Figure 2. Sephadex G-25 gel filtration profile of hydrolysate obtained from tuna broth by 1.0% orientase.
Eluent: 0.05M phosphate buffer (pH 6.5); flow rate: 45 mL/hr.
MW was estimated using β-endothein (M.W. 2,573), [Sar^1, Ala^8] angiotensin-II (M.W. 926), *N*-formyl-Met-Leu-Phe-Lys (M.W. 565.7) and tryptophan (M.W. 204).

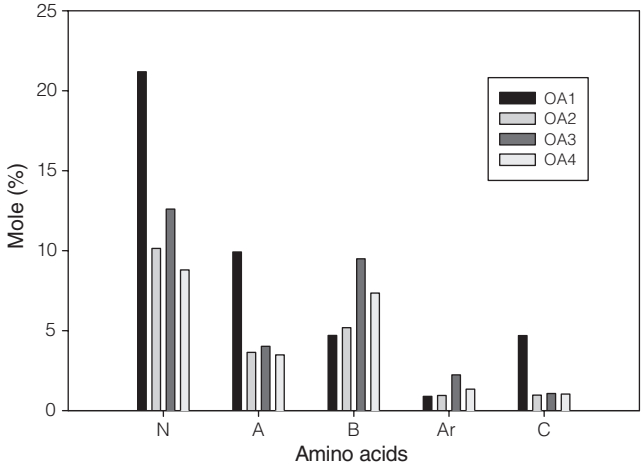

Figure 3. Amino acid contents of fractions OA1, OA2, OA3 and OA4.
N: neutral amino acids, including Gly, Ala, Ser, Thr, Val, Leu, ILe.
A: acidic amino acids, including Asp and Glu.
B: basic amino acids, including Lys, His, Arg.
Ar: aromatic amino acids, including Phe, Tyr and Trp.
C: cyclic amino acids, including Pro.

peptides of molecular weights < 1,000 Da (Table 4). OA1, having higher molecular weight, did not affect ACE. Among the active fractions, OA3 inhibited ACE most strongly (IC_{50} = 0.21 ± 0.01 mg protein/mL), and the IC_{50} values for OA2 and OA4 were 25.26 ± 0.02 and 0.52 ± 0.03 mg protein/mL, respectively.

IV. *Amino Acid Content in Hydrolysates*

Table 4 summarizes the amino acid content in OA1, OA2, OA3 and OA4 fractions. The mole % of basic amino acids (Lys, His, Arg) for OA1, OA2, OA3 and OA4 were 4.70, 5.18, 9.49 and 7.35, and aromatic amino acids (Phe, Tyr, Trp) were 0.98, 0.95, 2.23 and 1.34, respectively. OA3 contained more basic and hydrophobic amino acids than the other fractions (Figure 3).

DISCUSSION

Peptides that inhibit ACE have been found in many different food protein hydrolysates[15]. For each enzyme treatment in this study, all hydrolysates were capable of inhibiting ACE, indicating that ACE inhibition was brought about by any peptides. These peptides were inactive in the proteins existing in tuna broth and were released during enzymatic hydrolysis. Consequently, proteins in tuna broth are a source of ACE inhibitory peptides.

Orientase treatments were applied to determine the best proteolysis conditions. The results (Figure 1) showed that the development of activity on ACE with the addition of 1.0% orientase was similar to that of 2%. The hydrolysis curve is similar to that reported for sardine muscle hydrolysate[5]. A slight difference between the IC_{50} values for 1.0 and 2.0% enzyme addition indicates the appropriate proteolysis conditions for yielding inhibitory activity, which required 3hr of incubation with 1.0% orientase. The activity of the alkaline protease hydrolysate derived from sardine muscle (IC_{50} = 3.15 mg/mL)[5] was about 4.1 fold higher than that of the prepared hydrolysate (IC_{50} = 12.50 ± 0.02 mg/mL). This difference may result from the proteases used.

The orientase hydrolysate was fractionated to determine the distribution of molecular weight of the active peptides. The results indicated that peptides with molecular mass of 204 to 926 Da were responsible for the ACE inhibition. This finding was consistent with the literature, in which ACE inhibitory peptides were shown to have molecular weights usually of 2-14 amino acids[16].

Antihypertensive effects of food protein-derived hydrolysates/peptides in rats and humans have been studied[17-21]. Astawan *et al.*[22] reported that a pepsin hydrolysate of dried-salted fish with an IC_{50} value of 0.63 mg/mL had an antihypertensive effect on SHR. The IC_{50} value of the tuna broth hydrolysates (OA3 and OA4, IC_{50} = 0.21± 0.01 and 0.52 ± 0.03 mg/mL, respectively) was thus within the range of effective IC_{50} of the food protein hydrolysates reported in literature (Table 4), but had lower ACE inhibiting potencies than the synthetic antihypertensive drug captopril (IC_{50} = 0.0013 μg/mL)[23]. However, the application of cooking juice hydrolysates in treating/preventing hypertension unlike captopril, are expected to have no undesirable side effects.

Comparison of the amino acid compositions and the properties of the fractions OA1, OA2, OA3, OA4 (Figure 3 and Table 4) reveals that OA3, the most active fraction is apparently had an abundance of basic and aromatic amino acids. Cheung *et al.*[16] reported that a peptide had aromatic amino acid residues at its C-terminal and basic or hydrophobic ones at its N-terminal were essential for strong and competitive inhibition on ACE. Seki *et al.*[24] also reported aromatic amino acids and basic amino acids are important to ACE inhibition of peptides. In this study, a greater mole

Table 4. Comparison of IC_{50} values, amino acid contents of hydrolysate fractions from tuna broth prepared with orientase comparing to the sardine muscle hydrolysate (SMH)

Amino acids (mole %)	OA1	OA2	OA3	OA4	SMH[a]
Asp	5.87	1.89	1.90	1.71	8.97
Tau	0.02	N.D	N.D	N.D	
Thr	1.97	0.95	0.96	0.72	4.49
Ser	1.83	1.15	1.04	0.95	5.05
Glu	4.05	1.75	2.12	1.77	12.79
Pro	4.69	0.97	1.07	1.03	3.70
Gly	9.17	2.89	5.22	2.48	8.75
Ala	3.22	1.97	1.93	1.66	8.19
1/2Cys	0.17	0.12	0.12	0.22	1.01
Val	1.87	1.15	1.14	1.12	7.52
Met	0.27	0.16	0.05	0.08	4.38
Ile	1.24	0.82	0.88	0.73	4.38
Leu	1.88	1.21	1.43	1.14	8.64
Tyr	0.32	0.36	0.84	0.18	2.25
Phe	0.44	0.50	0.98	0.86	2.02
Trp	0.13	0.09	0.41	0.30	1.57
Lys	1.29	0.75	1.27	0.86	7.86
His	0.53	0.85	0.54	0.79	4.83
Arg	2.88	3.58	7.68	5.70	3.59
NH_3	58.16	78.84	70.42	77.79	
IC_{50} (mg /mL)	--[b]	25.26 ± 0.02	0.21 ± 0.01	0.52 ± 0.03	026

[a]Data from Matsui *et al*.[5]

[b]---: no inhibition activity on ACE.

OA1, OA2, OA3 and OA4: fractions of hydrolysates prepared from tuna broth using 1.0% orientase at a ratio of enzyme to substrate of 1/25 (v/v) incubated at 50˚C for 3 hr.

(%) fraction of aromatic amino acids or basic amino acids in the fractions obtained from orientase hydrolysate was associated with a greater observable inhibition on ACE (Table 4).

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