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Isolation and Inhibitory Activity of Angiotensin 1 −Converting Enzyme Inhibitor in Enzymatic Hydrolyzates of Extracts from Skin and Bone of Croaker **Argyrosomus argentatus.**

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ABSTRACT

Inhibitors which inhibit the angiotensin 1-converting enzyme(ACE) were prepared from trypsin digests of croaker extracts. The inhibitory activity of ACE detected in the trypsin degests of croaker extracts was fractionated into two major phosphopeptides fractions of P-1 and P-2 by gel filtration chromatography on sephadex G-50.

The inhibition of ACE of the two kinds of phosphopeptides fractions of P-1 and P-2 was investigated in vitro.

The IC_{50} values of p-1 and p-2 of phosphopeptides for ACE were 0.18 and 10.2mg protein/$m\ell$, respectively.

In addition, the ACE inhibitor of P-1 and P-2 were further purified by ultrafiltration and by sephadex G-15 and G-25 chromatography. ACE inhibitory activity was fractionated into three major phosphopeptides fractions of P−1−1, P−1−2 and P−1−3 from the trypsin digests of the P-1, and into three major fractions of P-2-1, P-2-2 and P-2-3 from the trypsin digests of the P-2 by gel filtration rechromatography on sephadex G-15 and G-25, respectively.

The inhibition of ACE of the six kinds of phosphopeptides fractions (P−1−1, P−1−2, P−1−3, P−2−1, P−2−2 and P−2−3) was analyzed in vitro.

The IC_{50} values of P−1−1, P−1−2, P−1−3, P−2−1, P−2−2 and P−2−3 of phosphopeptides for ACE were 1.12, 1.84, 0.15, 0.70, 4.80 and 1.98mg protein/$m\ell$, respectively.

The P−1−3 fraction had most inhibition activity and showed 0.15mg protein/$m\ell$ inhibition against ACE at IC_{50} value.

The amino acid compositions of the phosphopeptides fractions (P−1−1, P−1−2, P−1−3, P−2−1, P−2−2 and P−2−3) were characterized by relatively high percentage for Glu, Asp, Gly, Ala, Val, Leu, Tyr, Lys and Arg.

INTRODUCTION

Our interest has been focused on the preparation and isolation of phosphopeptides from natural resources that are involved in blood pressure regulation.

Many investigators have tried to prepare and isolate the ACE inhibitory peptides[1~10].

To date, there is still no report on the ACE inhibitory activity of phosphoproteins and phosphopeptides in nature.

In previous paper[11~16], auther et. al. have reported that the inhibitory activity of ACE detected from digests by proteolytic enzymes such as pepsin, trypsin and chymotrypsin of oyster extracts was fractionated into two major fractions of high molecular weight and low molecular weight by gel filtration chromatography on Sephadex G—50, G—25 and G—15.

In this investigation, the ACE inhibitors was detected two major phosphopeptides of P-1 and P-2 in the trypsin digests of guchi extracts by Sephadex G-50 column chromatography. The P-1 and P -2 had high inhibitory activity against ACE at IC_{50} value.

Therefore, P-1 and P-2 of ACE inhibitors were further purified by ultrafiltration and by Sephadex G-25 and G-15 chromatography.

MATERIALS AND METHODS

Materials :

Sephadex G-15 was a product of Pharmacia. Proteolytic enzymes (trypsin) were obtained from Boehringer Co. ACE from rabbit lung acetone powder was obtained from Sigma Chemical Co. (U.S. A.).

Hippuryl-L-histidyl-leucine (HHL) as a substrate was obtained from the Peptide Institute (Osaka, Japan).

All other reagents were obtained from Nacalai Tesque (Kyoto, Japan).

Assay of ACE inhibitory activity :

The activity of ACE inhibition was assayed by the method of Yamamoto et. al.[17].

For each assay, 100μl of ACE inhibitor and 50μl of Hip-His-Leu (2.5 mM in a borate buffer containing 200 mM NaCl at pH 8.3) were incubated with 100μl of 2.5mu／mℓ of ACE at 37℃for 30 min. The reaction was stopped by adding 250μl of IN HCl, and 1.5mℓ of ethyl acetate was then added to the mixture, and the mixture was shaked for 30 sec. The mixture was centrifuged at 2500 rpm for 10min., and 1.25mℓ from the supernatant was transfered to test tube, and then heated at 120℃ for 45min., 0.5mℓ of 1 M NaCl was then added to the dried material, and the solution was shaked for 30 sec., and the absorbance of the yielded hippuric acid at 228 nm was measured with a Hitachi 101 spectrophotometer.

The ACE inhibitor concentration required to inhibit 50 % of the ACE activity is defineed as the IC_{50} value.

Fractionation of inhibitor from croaker extracts by column chromatography :

After the precipitate of croaker extracts had been removed by centrifugation at 3000 rpm for 25 min., the supernatant was then applied to a 2.5x 40cm column of sephadex G-50.

The column was eluted with 500mℓ of distillated water. The eluate was collected in 10mℓ frac-

tions, while monitoring the absorbance at 280nm for phosphopeptides and at 820nm for total phosphorus. The peaks were denoted as P-1 and P-2. The each peak was collected, and determined the amino acid and ACE inhibitory activity.

Isolation and purification of ACE inhibitors from trypsin digests of croaker extracts :

The freeze-dried powder about 450mg of P-1 and P-2 obtained by Sephadex G-50 chromatography were dissolved in 10$m\ell$ of 0.01 M Tris-HCl buffer(pH 8.0), and then 10mg of trypsin was added to the mixture, and the mixture incubated at 37℃ for 24 hrs. After reaction, the incubation mixture was then heated for 15min. in a boiling-water bath.

After the precipitate had been removed by centrifugation at 14000 rpm for 15min., the supernatant was then ultrafiltrated with membrane filter(MW Cutoff 2000; Amicon).

The filtrate was then applied to a 2.5 x 40cm column of Sephadex G-25 and G-15(prewashed with water). The column was eluted with 250$m\ell$ of distillated water.

The eluate was collected in 5$m\ell$ fractions, while monitoring the absorbance at 280nm for phosphopeptides and at 820nm for total phosphorus. Each fraction was collected, and determined the amino acid and ACE inhibitory activity.

Quantitative analysis :

Total phosphorus was estimated by the method of Chen et. al[18]. Amino acid analyses were carried out with a JTC-200A amino acid analyzer. The sample for amino acid analyses was hydrolyzated in 6 N HCl at 110℃ for 24hrs. The nitrogen and protein were analyzed in the usual way(% protein=%Nx 6.25).

RESULTS AND DISCUSSION

Fig 1 showed the Sephadex G-50 column chromatogram of the tryptic hydrolyzates of croker. The experimental details are described in the text.

The entire effluent was collected in tubes on a fraction collector for quantitative analysis of the total phosphorus and for qualitative analysis of the phosphopeptides at 280nm. Phosphorus-containing two peaks and two peaks of peptides at 280nm were eluted from the column in the case of tryptic hydrolyzates. The first peak and second peak designated as P-1 and P-2, respectively.

The maximum total-phosphorus content was observed in fraction No.7 in the case of P-1, and in fraction No.22 in the case of P-2 as shown in the Fig.1.

In addition, the maximum absorption at 280nm was observed in fraction No.7 in the case of P-1, and in fraction No.20 in the case of P-2. Fraction No.3～10 of the P-1 and fraction No.11～26 of the P-2 were pooled, and analysed for ACE inhibitory activity and amino acids.

The inhibition of ACE of the P-1 and P-2 was investigated in vitro. It has been demonstrated that P-1 and P-2 had high inhibitory activity and showed 0.18 and 10.24mg protein/$m\ell$ inhibition against ACE at IC_{50} value.

Therefore, the P-1 and P-2 of ACE inhibitors were further purified by ultrafiltration and

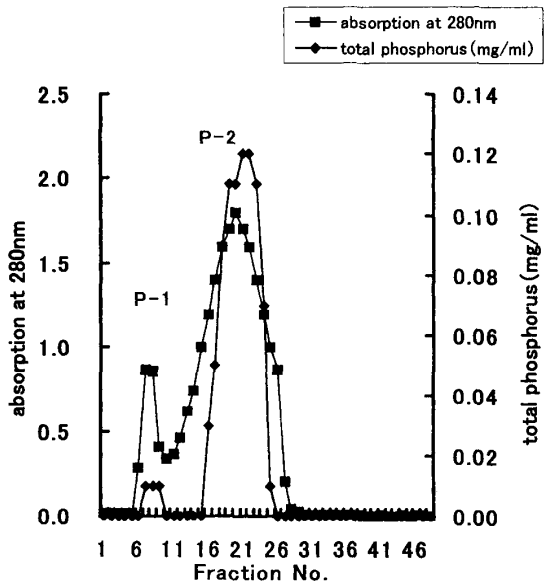

Fig. 1. Chromatographic Profile of Phosphopeptides Eluted by Sephadex G-50 Column Chromatography from the Enzymatic Hydrolyzates of Extracts of Croaker, Argyrosomus argentatus.
The experimental details are described in the text.
◆-◆ indicate total-phosphorus and ■-■ indicate phosphopeptides at 280nm in the collected fractions. P-1 and P-2 represent combined fractions 3～10 and fractions 11～26, respectively.

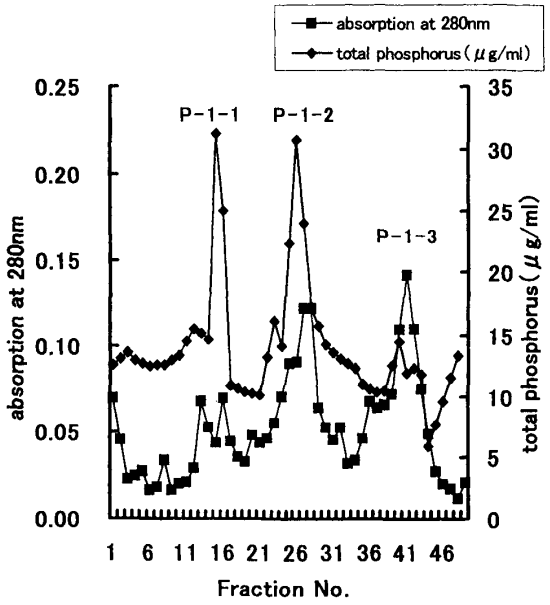

Fig. 2. Chromatographic Profile of Phosphopeptides by Rechromatography on Sephadex G-15 Column of Trypsin digests of Active Fraction (P-1) Obtained by Sephadex G-50 Chromatography.
The experimental details are described in the text.
◆-◆ indicate total-phosphorus and ■-■ indicate phosphopeptides at 280nm in the collected fractions. P-1-1, P-1-2 and P-1-3 represent combined fractions No.12～19, fractions No.22～31 and fractions 37～45, respectively.

Sephadex G-15 and G-25 chromatography.

Fig.2 shows the chromatographic profile of phosphopeptides by rechromatography on Sephadex G-15 column of trypsin digest of most active fraction(P-1) obtained from Sephadex G-50 chromatography.

The experimental details are described in the text. The entire effluent was collected in tubes on a fraction collector for quantitative analysis of the total-phosphorus and for qualitative analysis of the phosphopeptides at 280nm.

Phosphorus-containing three peaks and three peaks of peptides at 280nm were eluted from the column in the case of tryptic hydrolyzates of P-1. The first peak, second peak and third peak designated as P-1-1, P-1-2 and P-1-3, respectively.

The maximum total-phosphorus content was observed in fraction No.15 in the case of P-1-1, and in fraction No.26 in the case of P-1-2, and in fraction No.40 in the case of P-1-3 as shown in the Fig 2.

In addition, the maximum absorption at 280nm was observed in fraction No.16 in the case of P-1-1 in fraction No.27 in the case of P-1-2 and in fraction No.41 in the case of P-1-3. Fraction No.12～19 of the P-1-1, fraction No.22～31 of the P-1-2 and fraction No.37～45 of the P-1-3 were

pooled, and analysed for amino acids and ACE inhibitory activity.

Fig.3 show the chromatographic profile of phosphopeptides by rechromatography on Sephadex G-15 column of trypsin digest of active fraction (P-2) obtained from Sephadex G-50 chromatography. The experimental details are described in the text.

The entire effluent was collected in tubes on a fraction collector for quantitative analysis of the total-phosphorus and for qualitative analysis of the phosphopeptides at 280nm.

Table1. Phosphorus Contents of the P-1-1, P-1-2, P-1-3, P-2-1, P-2-2 and P-2-3 Fractionated by Rechromatography on Sephadex G-15 and G-25 Columu of Trypsin Digests of P-1 and P-2 obtained by Sephadex G-50 Chromatography of Extracts of Croaker, Argyosomus Argentatus.

Fraction	T-P ($\mu g/m\ell$)
P-1	37.6
P-2	839.9
P-1-1	141.57
P-1-2	150.95
P-1-3	99.03
P-2-1	46.83
P-2-2	201.05
P-2-3	55.21

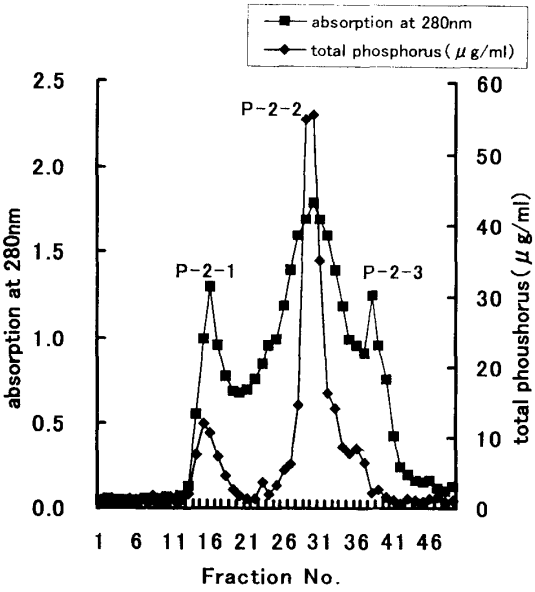

Fig. 3. Chromatographic Profile of Phosphopeptides by Rechromatography on Sephadex G-25 Column of Trypsin digests of Active Fraction (P-2) Obtained by Sephadex G-50 Chromatography. The experimental details are described in the text.
◆-◆ indicate total—phosphorus and ■-■ indicate phosphopeptides at 280 nm in the collected fractions. P-2-1, P-2-2 and P-2-3 represent combined fractions No.13～19, fractions No.20～32 and fractions 33～43,respectively.

The phosphorus-containing three peaks and three peaks of peptides at 280nm were eluted from the column in the case of tryptic hydrolyzates of P-2.

The first peak, second peak and third peak designated as P-2-1, P-2-2 and P-2-3, respectively.

The maximum total-phosphorus content was observed in fraction No.16 in the case of P-2-1, and in fraction No.30 in the case of P-2-2 and in fraction No.36 in the case of P-2-3 as shown in the Fig.3.

In addition, the maximum absorption at 280nm was observed in fraction No.16 in the case of P-2-1, and in fraction No.30 in the case of P-2-2 and in fraction No.38 in the case of P-2-3. Fraction No.13～19 of the P-2-1, fraction No.20～32 of the P-2-2 and fraction No.33～43 of the P-2-3 were pooled, and analyzed for amino acids and ACE inhibitory activity.

In Table 1 is reported the phosphorus contents of the P-1, P-2, P-1-1, P-1-2, P-1-3, P-2-1, P-2-2, P-2-3 eluted by chromatography on Sephadex G-50, G-25 and G-15 column from croaker extracts.

The total-phosphorus content in the P-1, P-2, P-1-1, P-1-2, P-2-1, P-2-2 and P-2-3 fractions were 37.6, 839.9, 141.6, 150.9, 99.0, 46.7, 201.1 and 55.2 μg per $m\ell$ of the fraction, respectively.

Table2. Amino Acid Composition of Phosphopeptides in the P-1, P-2, P-1-1, P-1-2, P-1-3, P-2-1, P-2-2 and P-2-3 Fractionated by Rechromatography on Sephadex G-15 and G-25 Column of Trypsin Digests of the P-1 and P-2 Obtained by Sephadex G-50 Chromatography.

(μg／mg・N)

Amino Acid	P-1-1	P-1-2	P-1-3	P-2-1	P-2-2	P-2-3
Asp	13.71	20.14	5.88	53.11	1.76	2.28
Thr	6.49	8.05	3.35	10.11	0.68	1.09
Ser	11.39	14.57	12.65	14.51	0.80	1.28
Glu	11.13	16.33	7.35	34.04	3.22	2.42
Gly	10.05	20.81	8.90	51.18	8.80	25.75
Ala	7.43	13.95	2.94	15.89	2.59	7.01
Cys	0.00	0.00	0.00	1.20	0.00	0.30
Val	6.69	21.24	6.78	6.84	1.34	1.96
Met	0.00	1.05	0.00	0.20	0.44	5.14
Ileu	6.02	12.43	8.16	3.53	0.74	1.46
Leu	11.76	12.90	3.51	6.78	1.31	4.54
Tyr	15.09	23.43	29.63	13.80	0.44	4.25
Phe	0.00	7.62	0.00	6.11	0.52	14.39
His	9.01	25.19	15.51	6.93	0.26	4.24
Lys	4.77	46.19	2.78	11.42	1.61	0.56
Arg	2.45	4.48	2.04	7.40	0.86	0.95
Pro	7.53	22.00	2.20	31.62	4.48	10.35
Total	123.52	270.38	111.68	274.67	29.85	87.97

Table 2 summarises the amino acid composition of the P-1, P-2, P-1-1, P-1-2, P-1-3, P-2-1, P-2-2 and P-2-3 fractions. Of note in P-1, P-2, P-1-1, P-1-2, P-1-3, P-2-1, P-2-2 and P-2-3 is the very high acidic amino acids(Asp and Glu), Gly, Ala, Val, Leu, Tyr, Phe, Lys and Arg content.

Cheung et. al[19]. and Matsui et.al[4]. have reported that it was essential for strong and competitive ACE inhibition that a peptide had aromatic amino acid residues at the C-terminal (i.e., Try. Tyr. Pro.), and hydrophobic or basic ones at the N-terminal.

Table 3. Angiotensin-1 Converting Enzyme Inhibitory Activity of the P-1, P-2, P-1-1, P-1-2, P-1-3, P-2-1, P-2-2, and P-2-3 of Phosphopeptides obtained by Rechromatography on Sephadex G-15 and G-25 Column of tryptic Hydrolyzates of P-1 and P-2 obtained by Sephadex G-50 Column Chromatography.

Fraction	Protein (mg/mℓ)	IC_{50} (mg protein/mℓ)
P-1	0.150	0.179
P-2	17.938	10.240
P-1-1	0.372	1.116
P-1-2	0.262	1.840
P-1-3	0.153	0.152
P-2-1	0.563	0.700
P-2-2	9.988	4.802
P-2-3	2.125	1.980

Table 3 shows angiotensin converting enzyme inhibitory activity of the P-1, P-2, P-1-1, P-1-2, P-1-3, P-2-1, P-2-2 and P-2-3 phosphopeptides eluted by Sephadex G-50, G-25 and G-15 column chromatography.

The IC_{50} values for P-1, P-2, P-1-1, P-1-2, P-1-3, P-2-1, P-2-2 and P-2-3 were 0.18, 10.24, 1.12, 1.84, 0.15, 0.70, 4.80 and 1.98 mg protein/mℓ, respectively.

The P-1-3 fraction had most inhibition activity and showed 0.15 mg protein/mℓ inhibition against ACE at IC_{50} value, and the IC_{50} value in the P-1-3 fraction was 1.2, 67, 7, 12, 4.6, 31 and 13 times higher than those in the P-1, P-2, P-1-1, P-1-2, P-2-1, P-2-2 and P-2-3 fractions, respectively.

These results suggest that the phosphopeptide fractions are a mixture of several phosphopeptides.

In addition, the results above, it has been demonstrated that the isolated peptide inhibitors occurs as the phosphate-containing phosphopeptides, but more experiments will be needed to prove the ACE inhibition of the phosphopeptides isolated from Croaker, argyrosomus argentatus.

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