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Isolation and Inhibitory Activity of Angiotensin 1-Converting Enzyme Inhibitor in Enzymatic Hydrolyzate of Hot-Water-Extract from Sea Anemone, Actinia equinia.

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ABSTRACT

Author describe a method of extraction and partial purification of phosphoproteins from Sea anemone, actinia equinia. The extraction of the phosphoproteins was carried out with water and hot water.

The sea anemone contained two kinds of phosphoproteins (W-P-1, W-P-2) in the water extract and two kinds of phosphoproteins (H-P-1, H-P-2) in the hot water extracts by gel filtration chromatography on Sephadex G-50, respectively.

In addition, the ACE inhibitor phosphopeptides of H-P-1 was further purified by ultrafiltration and by Sephadex G-15 chromatography.

Inhibitors which inhibit the angiotensin 1-converting enzyme (ACE) were prepared from the pepsin digests of the hot-water-extracts of seaanemone. The inhibitory activity of ACE in the pepsin digests of the hot water extracts (H-P-1) was fractionated into two major phosphopeptides fractions of H-P-1-1 and H-P-1-2 by gel filtration chromatography on Sephadex G-15.

The IC_{50} values of H-P-1 and H-P-2 of phosphopeptides for ACE were 23.2 and 15.9 mg protein / ml, respectively. The inhibition of ACE of the two kinds of phosphopeptides fractions (H-P-1-1, H-P-1-2) were analyzed in vitro.

The IC_{50} values of H-P-1, H-P-2, H-P-1-1 and H-P-1-2 of phosphopeptides for ACE were23.26, 15.91, 0.55 and 0.33 mg protein / ml, respectively. The H-P-1-2 fraction had most inhibition activity and showed 0.33 mg protein / ml inhibition against ACE at IC_{50} value.

The amino acid compositions of the phosphopeptides (H-P-1, H-P-1-1 and H-P-1-2) were characterized by relatively high percentage for Glu, Asp, Gly, Arg and Lys.

It has been demonstrated that the H-P-1, H-P-1-1 and H-P-1-2 contained about 85.8%, 74.2% and 3.1% as phosphonate-phosphorus of total phosphorus. The results above, the SephadexG-15 gel filtration patterns of the active fractions obtained from the Sephadex G-50 column chromatography indicated that the molecular weight of the phosphopeptide was about 200~3000.

INTRODUCTION

Our interest has been focused on the preparation and isolation of phosphopeptide and phosphonopeptide 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 ~ 18], 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 extract 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 were detected two major phosphopeptides of H-P-1 and H-P-2 in the hot water extract of sea anemone, *Actinia equinia* by Sephadex G-50 column chromatography. The H-P-1 and H-P-2 had high inhibitory activity against ACE at IC_{50} value.

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

MATERIALS AND METHODS

Materials :

Sephadex G-15 was a product of Pharmacia. Proteolytic enzymes (trypsin, pepsin) 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 obtainedfrom 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[19]. For each assay, 100 μl of ACE inhibitor and 50 μl of Hip-His-Leu (2.5mM in a borate buffer containing 200mM NaCl at pH 8.3) were incubated with 100 μl of 2.5mu / ml of ACE at 37℃ for 30min. The reaction was stopped by adding 250 μl of 1N HCl, and 1.5 ml of ethyl acetate was then added to the mixture, and the mixture was shaked for 30sec. 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 45 min., 0.5ml of 1M 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 228nm was measured with a Hitachi 101 spectrophotometer.

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

Fractionation of phosphopeptide in sea anemone extracts by column chromatography:

After the precipitate of sea anemone extracts with hot water had been removed by centrifugation at 3000 rpm for 25 min., the supernatant was then applied to a column (2.5 x 40cm) of Sephadex G-50. The column was eluted with 500 ml of distillated water. The eluate was collected in 10 ml fractions, while monitoring the absorbance at 280 nm for

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

Isolation and Purification of ACE Inhibitors in Pepsin Digests of Sea anemone extracts :

The freeze-dried powder about 765 mg of H-P-1 obtained by Sephadex G-50 chromatography were dissolved in 50ml of HCl-KCl buffer(pH2.0), and then 15mg of pepsin was added to mixture, and the mixture incubated at 37℃ for 24hrs. After reaction, the incubation mixture was then heated for 15min in a boiling water bath. After the precipitate had been removed by centrifugation at 14,000 rpm for 15min, the supernatant was then ultrafiltrated with membrane filter (MW Cutoff 3000: Amicon).

The filtrate of H-P-1 was then applied to a 2.5x40cm column of Sephadex G-15 (prewashed with water). The column was eluted with 250ml of distillated water. The eluate was collected in 5ml 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[20]. Amino acid analyses were carried out with a amino Acid analyzer (JTC-200A). The sample for amino acid analyses was hydrolyzated in 6N HCl at 110℃ for 24hrs. The nitrogen and protein were analyzed in the usual way (% protein = % N x 6.25).

RESULTS AND DISCUSSION

Fig.1 showed the Sephadex G-50 column chromatogram of the hot water extracts of sea anemone. The experimental details are described in the text.

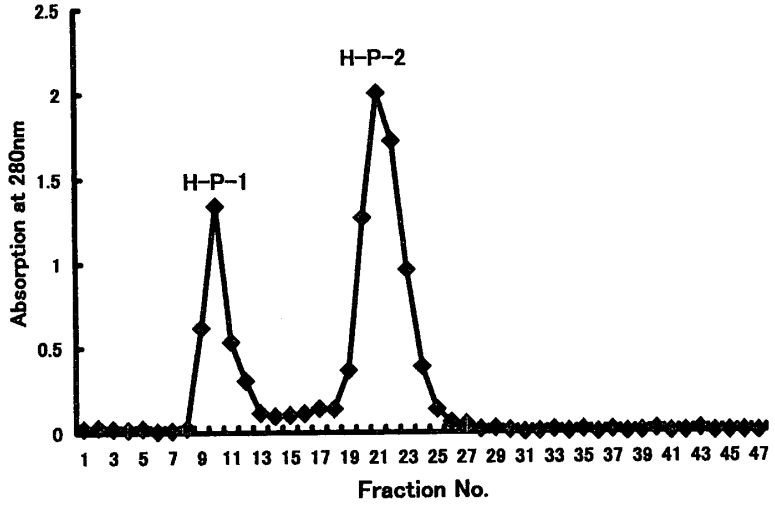

Fig.1 Chromatographic profile of phosphopeptides eluted by Sehadex G-50 collumn Chromatography of the hot water extracts of sea anemone, actinia equinia. The experimental details are described in the text. ◆—◆ indicate phosphopeptides at 280mm in the collected fraction H-P-1 and H-P-2 represent combined fractions No.8 ~ 13 and 19 ~ 25, respectively.

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 hot water extracts. The first peak and second peak designated as H-P-1 and H-P-2, respectively.

The maximum absorption at 280 nm was observed in fraction No.9 in the case of H-P-1, and in fraction No.21 in the case of H-P-2. The fraction No.8～13 of the H-P-1 and the fraction No.19～25 of the H-P-2 were pooled, and analyses for ACE inhibitory activity and amino acids. The inhibition of ACE of the H-P-1 and H-P-2 was investigated in vitro. It has been demonstrated that H-P-1 and H-P-2 had high inhibitory activity and showed23.3 and 15.9 mg protein / ml inhibition against ACE at IC_{50} value. Therefore, the H-P-1 and H-P-2 of ACE inhibitors were further purified by ultrafiltration and Sephadex G-15 column chromatography.

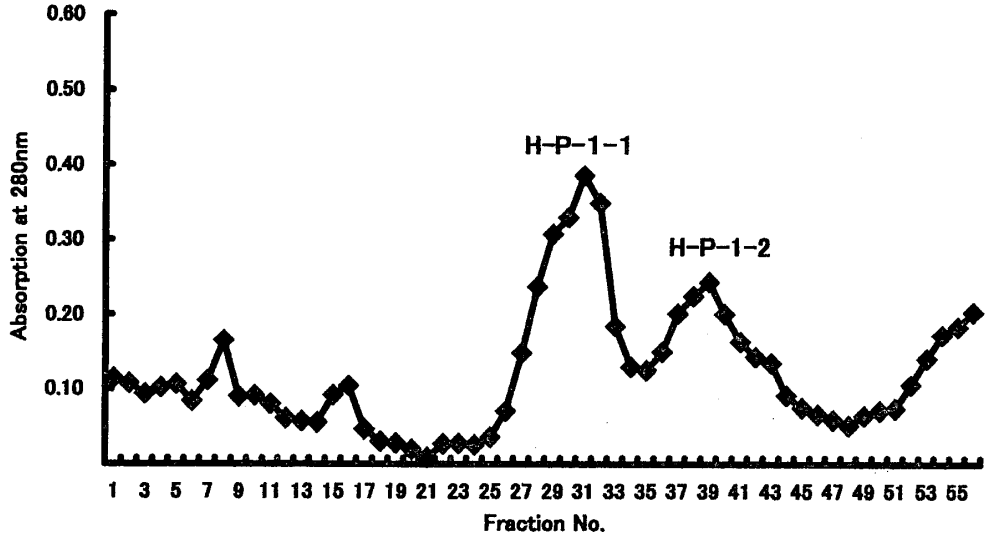

Fig.2 Chromatographic profile of phosphopeptides by rechromatography on Sephadex G-15 column of pepsin digest of active fraction H-P-1 obtained from Sephadex G-50 chromatography.
Active fraction H-P-1 obtained from Sephadex G-50 chromatography was applied on the Sephadex G-15 column (2.5x40cm), pre-exuilibrated with water, and eluted with water. The elute was collected in 5 ml fractions, while monitoring the absorbance at 280nm for peptides.
◆—◆ indicate phosphopeptides at 280nm in the collected fractions. H-P-1-1 and H-P-1-2 represent combined fractions 25 ～ 34 and 35 ～ 45, respectively.

Fig.2 shows the rechromatographic profile of phosphopeptides by rechromatography on Sephadex G-15 column of trypsin digest of active fraction (H-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.

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

peak designated as H-P-1-1 and H-P-1-2, respectively.

The maximum absorption at 280nm was observed in fraction No.30 in the case of H-P-1-1, and in fraction No.39 in the case of H-P-1-2. The fraction No.25～34 of the H-P-1-1 and the fraction No.35～45 of the H-P-1-2 were pooled, and analyzed for amino acids and ACE inhibitory activity.

Table 1. Phosphorus content of the phosphopeptides eluted by column chromatography on Sephadex G-50 and G-15 from the the proteolytic hydrolysates of the hot-water-extracts of sea anemone, Actinia equinia

Fraction	T-P(μg/tube)	C-P(μg/tube)	C-P/T-P(%)
H-P-1	744.2	638.6	85.8
H-P-1-1	89.1	66.1	74.2
H-P-1-2	96.3	2.9	3.1

Abbreviations used are; H-P : peak eluted from hot-water-extracts,
C-P ; phosphonate-phosphorus, T-P ; total-phosphorus

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

The total-phosphorus content in the H-P-1, H-P-1-1 and H-P-1-2 fractions were 744, 89 and 96μg per tube of the peaks, respectively.

The amounts of phosphonate -phosphorus (c-p) in the H-P-1, H-P-1-1 and H-P-1-2 were 638, 66 and 2.9 μg per tube of the peaks.

In addition, about 85.8, 74.2 and 3.1 % of the total-phosphorus in the H-P-1, H-P-1-1 and H-P-1-2 fractions were found to be phosphonate, which was primarily 2-aminoethyl-phosphonic acid.

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

Cheung et.al[21]. 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 shows angiotensin converting enzyme inhibitory activity of the phosphopeptides eluted by Sephadex G-50 and G-15 column chromatography.

The IC_{50} values for H-P-1, H-P-1-1, H-P-1-2 and H-P-2 were 23.2, 0.6, 0.3 and 15.9 mg protein / ml, respectively. The H-P-1-2 fraction had most inhibition activity and showed 0.33 mg protein / ml inhibition against ACE at IC_{50} value.

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

Table 2. Amino acid composition of the phosphopeptides eluted by column chromatography on Sephadex G-50 and G-15 from hot-water-extracts of sea anemone, Actinia equinia.

(μ g/mg protein)

Amino acid	H-P-1	H-P-1-1	H-P-1-2
Asp	1.64	4.57	2.54
Thr	0.95	3.13	1.25
Ser	0.79	5.06	2.31
Glu	2.15	5.90	3.19
Pro	0.74	2.35	1.56
Gly	1.45	4.44	3.71
Ala	0.70	3.80	1.42
Val	0.53	2.73	1.75
Ileu	0.42	8.34	3.26
Leu	0.73	13.09	1.86
Tyr	1.00	2.31	0.50
Phe	0.72	3.07	2.32
His	0.14	0.91	0.47
Lys	0.97	10.35	11.25
Arg	0.78	7.80	4.11
C-P	0.99	77.47	6.82
Total	14.77	155.32	48.32

Abbreviations used are ; C-P ; phosphonate – phosphorus ; H-P ; peak eluted from hot-water-extracts.

Tabel 3. Angiotensin converting enzyme inhibitory activity of the phosphopeptides obtained by rechromatography on Sephadex G-15 column of proteolytic hydrolysates of the H-P-1 eluted from Sephadex G-50 column.

Fraction	Inhibition(%)	Protein(mg/ml)	IC_{50}(mgProtein/ml)
H-P-1	34.8	16.19	23.261
H-P-2	38.4	12.25	15.919
H-P-1-1	103.0	1.15	0.558
H-P-1-2	65.8	0.44	0.334

In addition, the results above, it has been demonstrated that the isolated peptide inhibitors occurs as the phosphorus containing phosphopeptides, but more experiments will be needed to prove the ACE inhibition of the phosphopeptides isolated from sea anemone, Actinia equinia.

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