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Inhibition of Angiotensin 1-Converting Enzyme by Phosphopeptides in Proteolytic Hydrolysates Derived from Oyster, *Crassostrea gigas.*

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ABSTRACT

Hydrolysates which inhibit the angiotensin 1-converting enzyme(ACE) were prepared from oyster with three kinds of proteases.

The inhibitory activity of ACE detected in the hydrolysates by three kinds of proteases of oyster was fractionated into two major phosphopeptides fractions of P-1 and P-2 in peptic hydrolysates, T-1 and T-2 in tryptic hydrolysates, C-1 and C-2 in chymotryptic hydrolysates by gel filtration chromatography on Sephadex G-50, respectively.

The inhibition of ACE of the six kinds of phosphopeptides fractions(P-1, P-2, T-1, T-2, C-1 and C-2) was investigated in vitro.

The IC_{50} values of P-1, P-2, T-1, T-2, C-1, and C-2 of phosphopeptides for ACE were 0.3, 2.9, 2.7, 2.6, 1.5 and 1.4mg protein/ml, respectively.

The pepsin treated fraction P-1 had most inhibition activity and showed 0.3mg protein/ml inhibition against ACE at IC_{50} value.

The phosphono-compounds was found in the phosphopeptides fractions of hydrolysates with three kinds of protease.

It has been demonstrated that the P-1, P-2, T-1, T-2, C-1 and C-2 contained about 79.13%, 79.19%, 11.07%, 4.71%, 15.26% and 4.49% as phosphonate-phosphorus of total phosphorus.

The amino acid compositions of the phosphopeptides fractions (P-1, P-2, T-1, T-2, C-1 and C-2) were characterized by relatively high percentage for glutamic acid, aspartic acid, alanine, lysine and threonine.

INTRODUCTION

Protein from natural resources are important for supplying nutrient and energy. Recently, it has become apparent that they also have many functions relating to physio-

logical regulation[1,2]. A number of functional peptides derived from milk, soybeen and fish have been found, which may serve to promote Ca absorption[3], lowering of blood pressure[2,4~9], regulation of cholesterol in serum[10] and other benefits[11].

The ACE inhibitory abilities of foods have recently been investigated[12]. ACE(EC. 3, 4, 15, 1) play an important physiological role inregulating blood pressure, ACE catalyzes the conversion of inactive angiotensin I to a potent vasoconstrictor angiotensin II[13,14].

Our interest has been focused on the preparation and isolation of phosphopeptides from natural resources that are involved in blood pressure regulation. In previous papers[15~17], phosphoproteins and phosphopeptides that inhibited the ACE were isolated from edible shellfish.

Many investigators have tried to prepare and isolate the ACE Inhibitory peptides. In this report, we investigate the effectiveness of three kinds of proteolytic hydrolysates as a physiologically functional food and attempt to isolate the ACE inhibitory phosphopeptides from the three kinds of proteolytic hydrolysates of Oyster.

MATERIALS AND METHODS

Materials: Edible Oyster, *Crassostrea gigas* was purchased on the localmarket. Sephadex G-50 was a product of Pharmacia. Proteolytic enzymes(pepsin, trypsin, chymotrypsin) was obtained from Boehringer Co. ACE from rabbit lung acetone powder were 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[18].

For each assay, 100μl of ACE inhibitor and 50μl of Hip-His-Leu(2.5mM in a borate buffer containing 200mM NaCl at pH 8.3) were incubated with 100μl of 2.5mu/ml of ACE at 37℃ for 30min. The reaction was stopped by adding 250μl of 1N HCl, and 1.5ml 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 30min., 0.5ml of 1M NaCl was then added to the dried material, and the solution was shaked for 30sec., 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.

Isolation and preparation of ACE inhibitors from proteolytic hydrolysates :

① Hydrolysis by pepsin:

The freeze-dried material(26.4 g) of Oyster, *Crassostrea gigas* was extracted with 550ml of 0.01M Tris-HCl buffer(pH 8.0) by an efficient magnetic stirrer in a 1000ml beaker at room temperature for 2hrs. The extracts were centrifuged at 3000rpm for 15min.

The supernatant was freeze-dried and approximately extract 15g was obtained. The freeze-dried extract(4.9 g) was dissolved in 100ml of HCl-KCl buffer (pH 2.0), and then 100mg of pepsin was added to the mixture, and the mixture incubated at 37℃ for 24hrs. After proteolysis, the solution was then heated for 15min. in a boiling-water bath.

After the precipitate had been removed by centrifugation at 3000rpm for 15min, the supernatant was then dialyzed for 24hrs. to remove inorganic salts. Dialyzed solution was centrifuged again, and supernatant was freeze-dried.

② Hydrolysis by trypsin and chymotrypsin:

The freeze-dried extract(4.9 g) was dissolved in 100ml of 0.01M Tris-HCl buffer(pH 8.0), and then 100mg of trypsin or chymotrypsin was added to mixture, and the mixture incubated at 37℃ for 24hrs. After proteolysis, the solution was then heated for 15min. in a boiling-water bath.

After the precipitate had been removed by centrifugation at 3000rpm for 15min., the supernatant was then dialyzed for 24hrs. to remove inorganic salts. Dialyzed solution was centrifuged again, and supernatant was freeze-dried.

Fractionation of inhibitor by column chromatography:

The freeze-dried hydrolysate of pepsin, trypsin and chymotrypsin was dissolved in 15ml of 0.01M Tris-HCl buffer and then applied to a 2.5×40cm column of Sephadex G-50 (prewashed with 0.01M Tris-HCl buffer). The column was eluted with 500ml of 0.01M Tris-HCl buffer. The eluate was collected in 10ml fractions,while monitoring the absorbance at 280nm for peptides and at 820nm for total phosphorus.(Peptic, tryptic and chymotryptic fractions were denoted as P-1, P-2, T-1, T-2, C-1 and C-2, respectively).

Each fraction was collected, and determined the phosphonate phosphorus, amino acid and ACE inhibitory activity.

Quantitative analysis:

Total phosphorus was estimated by the method of Chen et.al[19]. Phosphonate-phosphorus was estimated by the method of Tamari et. al[20] . Amino acid analyses were carried out with a JTC-200A amino acid analyzer. The sample for amino acid analyses was hydrolyzed in 6N HCl at 110℃ for 24hrs. The nitrogen and protein were analyzed in the usual way(% protein=% N×6.25).

RESULTS

Fig. 1 showed the Sephadex G-50 column chromatogram of the peptic hydrolysates of Oyster, *Crassostrea gigas*. 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.

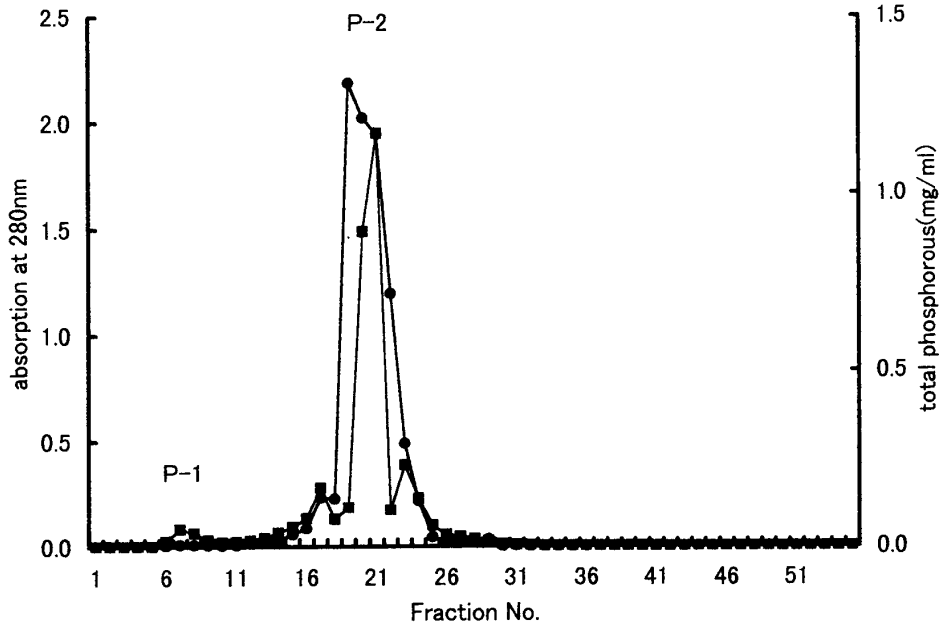

Fig. 1. Chromatographic Profile of Phosphopeptides Eluted by Sephadex G-50 Column Chromatography from the Hydrolysates Obtained by Peptic Hydrolysis of Oyster, *Crassostrea gigas*.
The experimental details are described in the text.
●−● indicate total-phosphorus and ■−■ indicate phosphopeptides at 280nm in the collected fractions. P-1and P-2 represent combined fractions 6～10 and fractions 18～24, respectively.

Two phosphorus-containing peaks and two peptides peaks were eluted from the column in the case of peptic hydrolysates. 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.19 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.21 in the case of P-2. Fraction No. 6～10 of the P-1 and fraction No.18～24 of the P-2 were pooled, and analysed for phosphonate-phosphorus(C-P), amino acids and ACE inhibitory activity.

Fig. 2 shows the Sephadex G-50 column chromatogram of the tryptic hydrolysates of

Oyster, *Crassostrea gigas*. 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.

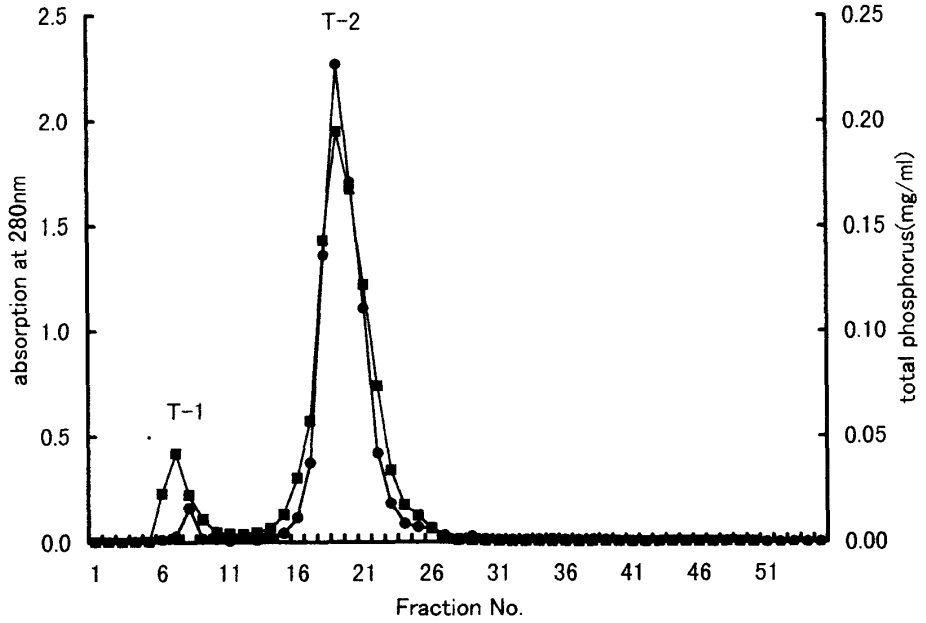

Fig. 2. Chromatographic Profile of Phosphopeptides Eluted by Sephadex G-50 Column Chromatography from the Hydrolysates Obtained by Tryptic Hydrolysis of Oyster, *Crassostrea gigas*.

The experimental details are described in the text.

●—● indicate total-phosphorus and ■—■ indicate phosphopeptides at 280nm in the collected fractions. T-1 and T-2 represent combined fractions 5～10 and fractions 15～26, respectively.

Two phosphorus-containing peaks and two peptides peaks were eluted from the column in the case of tryptic hydrolysates.

The first peak and second peak designated as T-1 and T-2, respectively.

The maximum total-phosphorus content was observed in fraction No. 8 in the case of T-1, and in fraction No.19 in the case of T-2 as shown in the Fig. 2.

In addition, the maximum absorption at 280nm was observed in fraction No. 7 in the case of T-1, and in fraction No.19 in the case of T-2. Fraction No. 5～10 of the T-1 and fraction No.15～26 of the T-2 were pooled, and analysed for phosphonate-phosphorus, amino acids and ACE inhibitory activity.

Fig. 3 shows the Sephadex G-50 column chromatogram of the chymotryptic hydrolysates of Oyster, *Crassostrea gigas*.

The experimental details are described in the text.

The entire effluent was collected in tubes on a fraction collector for quantitative

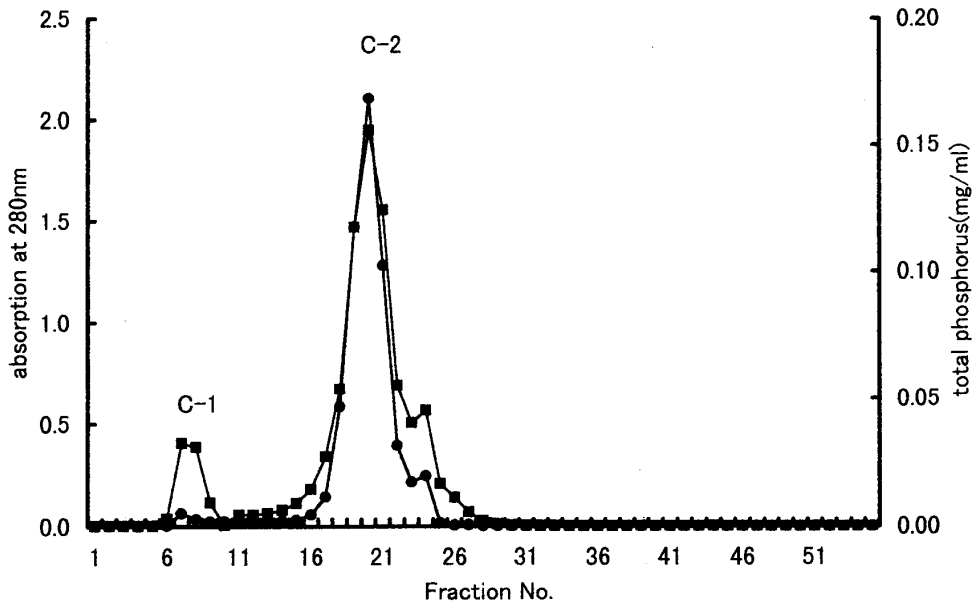

Fig. 3. Chromatographic Profile of Phosphopeptides Eluted by Sephadex G-50 Column Chromatography from the Hydrolysates Obtained by Chymotryptic Hydrolysis of Oyster, *Crassostrea gigas.*

The experimental details are described in the text.

●−●indicate total-phosphorus and ■−■ indicate phosphopeptides at 280nm in the collected fractions. C-1 and C-2 represent combined fractions 6 ~10 and fractions 16~27, respectively.

analysis of the total phosphorus and for qualitative analysis of the phosphopeptides at 280nm.

Two phosphorus-containing peaks and two peptides peaks were eluted from the column in the case of chymotryptic hydrolysates.

The first peak and second peak designated as C-1 and C-2, respectively. The maximum total-phosphorus contents were observed in fraction No. 7 in the case of C-1, and in fraction No.20 in the case of C-2 as shown in the Fig. 3.

In addition, the maximum absorption at 280nm was observed in fraction No. 7 in the case of C-1, and in fraction No.20 in the case of C-2. Fraction No. 6 ~10 of the C-1, and fraction No.16~27 of the C-2 were pooled, and analysed for phosphonate-phosphorus, amino acid and ACE inhibitory activity.

In Table 1 is reported the phosphorus contents of the P-1, P-2, T-1, T-2, C-1 and C-2 from the proteolytic hydrolysates of the Oyster, *Crassostrea gigas.*

The total-phosphorus content in the P-1, P-2, T-1, T-2, C-1 and C-2 fractions were 5.08, 714.54, 23.13, 390.71, 12.38 and 261.72μg per ml of the peaks of the *Crassostrea gigas,* respectively.

The amounts of phosphonate-phosphorus (C-P) in the P-1, P-2, T-1, T-2, C-1 and

Table 1. Phosphorus Contents of the P-1, P-2, T-1, T-2, C-1 and C-2 from the Proteolytic Hydrolysates of the Oyster, *Crassostrea gigas*.

Fraction	T-P(μg/ml)	C-P(μg/ml)	C-P / T-P(%)
P-1	5.08	4.02 (0.67)	79.13
P-2	714.54	565.86 (93.61)	79.19
T-1	23.13	2.56 (0.24)	11.07
T-2	90.71	18.41 (3.05)	4.71
C-1	12.38	1.89 (0.31)	15.26
C-2	261.72	11.76 (1.95)	4.49

Abbreviations used are:

C-P, Phosphonate-phosphorus ; T-P, Total-phosphorus ;

( ), as % of Total C-P

Table 2. Amino Acid Composition in the Acid Hydrolysates of Phosphopeptides in the P-1, P-2, T-1, T-2, C-1 and C-2 Fractions Eluted by Sephadex G-50 Column Chromatography of the Three Different Enzymatic Hydrolysates of Oyster, *Crassostrea gigas*.

Amino Acid	P-1	P-2	T-1	T-2	C-1	C-2
Asp	249.6	433.8	269.3	954.1	136.4	986.4
Thr	91.8	441.2	218.1	426.2	130.3	487.1
Ser	102.6	293.5	166.9	459.9	87.1	508.2
Glu	448.6	442.1	269.1	1,729.4	115.1	1,573.4
Gly	160.2	286.9	149.1	731.1	80.0	709.2
Ala	174.5	293.2	131.4	608.4	86.0	601.2
Cys	7.3	100.0	32.9	19.7	8.3	0.0
Val	81.2	163.9	119.1	445.6	79.8	464.0
Met	38.3	51.5	24.3	21.1	8.8	0.0
I leu	68.4	102.1	82.4	378.3	41.7	379.8
Leu	144.0	129.0	127.1	639.7	68.0	658.3
Tyr	76.3	446.6	218.1	233.5	123.9	171.9
Phe	88.0	294.7	150.0	323.9	74.8	364.1
His	52.2	232.0	122.6	334.1	50.2	381.1
Lys	137.4	679.3	119.1	977.2	71.9	911.7
Arg	123.6	200.0	55.0	396.7	32.0	601.9
Pro	140.0	393.4	143.3	724.3	73.7	812.5
2-AEP	16.1	2,263.4	10.2	73.6	7.6	47.0
Total	2,200.0	7,246.6	2,407.9	9,477.1	1,275.5	9,657.8

• Values are expressed as μg/mg Nitrogen.

• Abbreviations used are : 2-AEP, 2-aminoethylphosphonic acid.

C-2 were 4.02, 565.86, 2.56, 18.41, 1.89 and 11.76μg per ml of the peaks.

In addition, about 79, 79, 11, 4, 15 and 4% of the total-phosphorus in the P-1, P-2, T-1, T-2, C-1 and C-2 fractions was found to be phosphonate, which was primarily 2-aminoethylphosphonic acid.

The P-2 fraction contained 93.6% of the phosphonate, and the amount of phosphonate in the P-2 fraction was 141, 221, 31, 299 and 48 times higher than those in the P-1, in the T-1, in the T-2, in the C-1 and in the C-2 fractions, respectively.

Table 2 summarises the amino acid composition of the P-1, P-2, T-1, T-2, C-1 and C-2 fractions obtained under three different proteolytic hydrolysis.

Of note in P-1, P-2, T-1, T-2, C-1 and C-2 peaks is the very high acidic amino acids(Asp and Glu), Gly, Ala, Leu, Lys and Pro content, and the presence of unique amino acid, 2-aminoethylphosphonic acid.

In P-2 phosphopeptide, the 2-AEP content was high, when compared to P-2, other peaks(P-1, T-1, T-2, C-1 and C-2) had a low 2-AEP.

Cheung et.al.[21] and Matsui et.al.[6] 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 Converting Enzyme Inhibitory Activity of the P-1, P-2, T-1, T-2, C-1 and C-2 Phosphopeptides Eluted by Sephadex G-50 Column Chromatography of the Hydrolysates Obtained by Various Enzymatic Hydrolysis of Oyster, *Crassostrea gigas*

Fraction	Protein(mg/ml)	IC_{50} (mg protein/ml)
P-1	0.17	0.30
P-2	1.94	2.90
T-1	0.66	2.73
T-2	4.60	2.64
C-1	0.85	1.53
C-2	2.30	1.47

Table 3 shows angiotensin converting enzyme inhibitory activity of the P-1, P-2, T-1, T-2, C-1 and C-2 phosphopeptides eluted by Sephadex G-50 column chromatography of the hydrolysates obtained by various enzymatic hydrolysis of oyster, *Crassostrea gigas*.

The IC_{50} values for P-1, P-2, T-1, T-2, C-1 and C-2 were 0.3, 2.9, 2.7, 2.6, 1.5 and 1.5 mg protein/ml, respectively.

The pepsin treated fraction P-1 had most inhibition activity and showed 0.3 mg protein/ml inhibition against ACE at IC_{50} value, and the IC_{50} value in the P-1 fraction was 9.6, 9.0, 8.7, 5.0 and 4.7 times higher than those in the P-2, in the T-1, in the T-2, in the C-1 and in the C-2 fractions, respectively.

DISCUSSION

Our interest has been focused on the preparation and isolation of phosphopeptides from natural resources that are involved in blood pressure regulation. To date, there is still no report on the ACE inhibition activity of the phosphopeptides and phosphonopeptides in nature. This report describes the isolation of an ACE inhibitory phosphopeptides having antihypertensive effect from Oyster, *Crassostrea gigas*.

To investigate the resistance of phosphoprotein to digestion by gastrointestinal proteases, phosphoprotein was digested by various proteases: pepsin, trypsin, chymotrypsin.

In this study, in the case of peptic hydrolysates of oyster contained two major ACE inhibitors (P-1 and P-2) that were fractionated by gel filtration. The specific activity for ACE inhibition of the P-1 fraction was about 10 times higher than that of the P-2 fraction.

In the case of tryptic hydrolysates contained two major ACE inhibitors (T-1 and T-2) that were fractionated by gel filtation. The specific activity for ACE inhibition of the T-1 fraction was almost the same that of the T-2 fraction.

In the case of chymotryptic hydrolysates contained two major ACE inhibitors (C-1 and C-2) that were fractionated by gel filtration. The specific activity for ACE inhibition of the C-1 fraction was almost the same that of the C-2 fraction. The pepsin treated fraction P-1 had most inhibition activity and showed 0.3mg protein/ml inhibition against ACE at IC_{50} value.

In previous papers[15~17], author et. al. have reported that the inhibitory activity of ACE detected in several shellfishes was fractionated into two major fractions of high molecular weight and low molecular weight by gel filtration chromatography on Sephadex G-50.

On the other hand, Sugiyama et. al[22] have already reported that the ACE inhibitory activity of the alkaline protease hydrolysate from defatted sardine meal was reduced by 40% after a digestion test with gastrointestinal proteases.

Matsuda et.al.[23] and Ogihira et.al.[24] have reported that di-and tri-peptides would have low susceptibility to hydrolysis by any digestive enzymes.

Matsufuji et. al.[7] and Matsui et. al.[6] have reported that the ACE inhibitor isolated from alkaline protease hydrolysate of sardine muscle was inhibited ACE competitively.

Kinoshita et. al.[11] have reported that the inhibitory activity of ACE detected in soy sause was fractionated into two major fractions high molecular weight (HW) and low molecular weight (LW) by gel filtration chromatography on Bio-gel P-2; the HW fraction reduced the blood pressure in hypertensive rats after orally administering, while the LW fraction did not.

On the other hand, the search related to the structure and function of phosphonomacromolecules will be one of the most facinating fields. Aminophosphonic acid are present in various bound forms other than the phosphonolipids and antibiotic

tripeptides. For instant, 2 - AEP is found in *Tetrahymena* associated with macromolecular complexes which resist hydrolysis by proteolytic enzymes such as protease and trypsin[25].

Gibson and Dixon[26] isolated from the sea anemone *Metridium senile* three chymotrypsin-like protease, of which two were proved by Stevenson et. al.[27] to contain 2 - AEP. The two enzymes, protease A and B, have properties similar to the mammalian γ- chymotrypsin with respect to the active-site amino acid sequence and the mode of enzyme action.

Protease A contained 6 2 - AEP residues per 243 residues of total amino acids and protease B had 4 2 - AEP residues per 239 residues of total amino acids.

The site of attachment of the 2 - AEP to three proteases are yet unknown. Quin[28] purified a polypeptide containing 4.4% 2 - AEP by pepsin hydrolysis of the insoluble residue from alcohol and chloroform extracts of *M. dianthus*, and found that none of the 2 - AEP with the amino group free was present.

From the results above, it has been demonstrated that the isolated peptide inhibitors occurs as the phosphonate containing phosphonopeptides and phosphate containing phosphopeptides, but more experiments will be needed to prove the ACE inhibition of the phosphopeptides isolated from oyster.

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