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Inhibitory Activity of Angiotensin 1-Converting Enzyme of Phosphopeptides Obtained from Proteolytic Hydrolyzates of Oyster, *Crassostrea gigas*.

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ABSTRACT

In this investigation, the ACE inhibitory phosphopeptides of P-1 and C-2 were further purified by ultrafiltration and by Sephadex G-15 chromatography.

ACE inhibitory activity was fractionated into three major phosphopeptides fractions of P-1-1, P-1-2 and P-1-3 in the pepsin hydrolyzates of the P-1, and into fractions of C-2-1, C-2-2 and C-2-3 in the pepsin hydrolyzates of the C-2 by gel filtration rechromatography on Sephadex G-15, respectively.

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

The IC_{50} values of P-1-1, P-1-2, P-1-3, C-2-1, C-2-2 and C-2-3 of phosphopeptides for ACE were 1.11, 0.04, 0.05, 1.04, 0.72 and 0.06 mg protein/ml, respectively.

The P-1-2 fraction had the most inhibitory activity and showed 0.04 mg protein/ml inhibition against ACE at IC_{50} value.

It has been demonstrated that the P-1-1, P-1-2, P-1-3, C-2-1, C-2-2 and C-2-3 contained about 23.2%, 4.4%, 28.1%, 2.0%, 3.8% and 1.6% as phosphonate-phosphorus of total phosphorus.

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

When the ACE inhibitory phosphopeptides were analyzed by thin layer chromatography, some ninhydrine-positive spots were observed. These results suggest that the phosphopeptides are a mixture of several phosphopeptides.

The results above, the Sephadex G-15 gel filtration patterns of the active fraction obtained from the Sephadex G-50 column chromatography indicated that the molecular weight of the phosphopeptide was about 200～1000.

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 are still no reports on the ACE inhibitory activity of phosphoproteins and phosphopeptides in nature.

In previous papers[11~14], 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.

The ACE inhibitors were detected two major phosphopeptides of P-1 and P-2 in pepsin digests, T-1 and T-2 in trypsin digests, C-1 and C-2 in chymotrypsin digests by Sephadex G-50 chromatography.

The P-1 and C-2 had high inhibitory activity and showed 0.3 and 1.4 mg protein/ml inhibition against ACE at IC_{50} value.

In this investigation, therefore, P-1 and C-2 of ACE inhibitors were further purified by ultrafiltration and by Sephadex G-15 chromatography.

MATERIALS AND METHODS

Materials:

Sephadex G-15 was a product of Pharmacia. Proteolytic enzymes (pepsin, trypsin, chymotrypsin) 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[15].

For each assay, 100 μl of ACE inhibitor and 50 μl of Hip-His-Leu (2.5 mM in a borate buffer containing 200 mM NaCl at pH 8.3) were incubated with 100 μl of 2.5 mu/ml of ACE at 37°C for 45min. 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 1.25 ml from the supernatant was transfered to test tube, and then heated at 120°C for 30min., 0.5 ml 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 228 nm 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.

Preparation and purification of ACE inhibitors from pepsin hydrolyzate:

The freeze-dried powder about 450 mg of P-1 and C-2 obtained by Sephadex G-50 chromatography were dissolved in 10 ml of HCl buffer (pH 2.0), and then 10 mg of pepsin was added to the mixture, and the mixture incubated at 37°C for 24hrs. After proteolysis, the incubation mixture was adjusted with 0.1 NaOH to pH 8.4, the solution 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 2.5×40 cm column of Sephadex G-15 (prewashed with water). The column was eluted with 200 ml of water. The eluate was collected in 4 ml fractions, while monitoring the absorbance at 280 nm for peptides and at 820 nm for total phosphorus. 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[16]. Phosphonate-phosphorus was estimated by the method of Tamari et. al[17]. Amino acid analyses were carried out with a JTC-200A amino acid analyzer. The sample for amino acid analyses was hydrolyzated in 6N HCl at 110°C for 24hrs. The nitrogen and protein were analyzed in the usual way (% protein=% N ×6.25).

Thin layer chromatography:

The presence of phosphopeptide in the P-1-1, P-1-2, P-1-3, C-2-1, C-2-2 and C-2-3 was demonstrated by thin layer chromatography (Kiesel gel $60F_{254}$, 20×20 cm, thick 0.25 mm) using two different solvent systems.

RESULTS AND DISCUSSION

Fig. 1 showed the Sephadex G-50 column chromatogram of the peptic hydrolyzates of Oyster, *Crassostrea gigas*. The experimental details are described in the previous report[14]. 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 280 nm.

Phosphorus-containing two peaks and two peaks of peptides at 280 nm were eluted from the column in the case of peptic hydrolyzates. The first peak and second peak designated as P-1 and P-2, respectively. Fraction No. 6~10 of the P-1 and

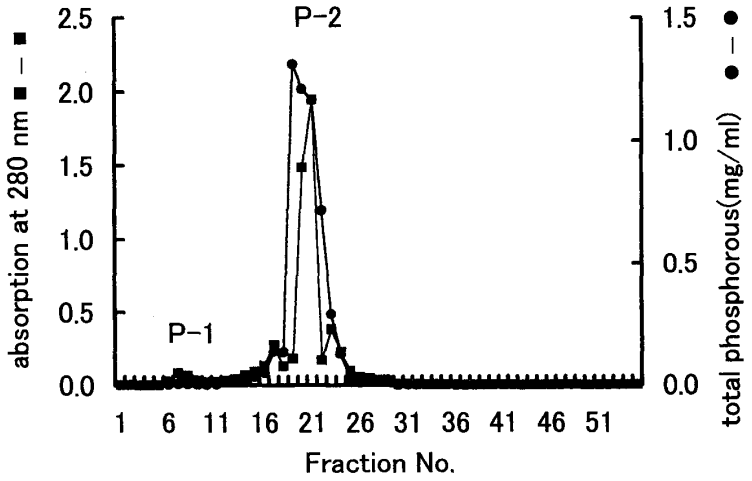

Fig. 1. Chromatographic Profile of Phosphopeptides Eluted by Sephadex G-50 Column Chromatography from the Hydrolyzates Obtained by Peptic Hydrolysis of Oyster, *Crassostrea gigas*.
The experimental details are described in the previous paper[14]. ●—● indicate total-phosphorus and ■—■ indicate phosphopeptides at 280 nm in the collected fractions. P-1 and P-2 represent combined fractions 6~10 and fractions 18~24, respectively.

fraction No. 18~24 of the P-2 were pooled, and analyzed for ACE inhibitory activity.

Fig. 2 shows the Sephadex G-50 column chromatogram of the chymotryptic hydrolyzates of Oyster, *Crassostrea gigas*.

The experimental details are described in the previous report[14].

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 280 nm.

Phosphorus-containing two peaks and two peaks of peptides at 280 nm were eluted from the column in the case of chymotryptic hydrolyzates.

The first peak and second peak designated as C-1 and C-2, respectively. Fraction No. 6~10 of the C-1, and fraction No. 16~27 of the C-2 were pooled, and analyzed for ACE inhibitory activity.

The inhibition of ACE of the P-1 and C-2 was investigated in vitro. It has been demonstrated that P-1 and C-2 had high inhibitory activity and showed 0.3 and 1.4 mg protein/ml inhibition against ACE at IC_{50} value.

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

Fig. 3 shows the chromatographic profile of phosphopeptides by rechromatography on Sephadex G-15 column of pepsin digest of the most active fraction (P-1)

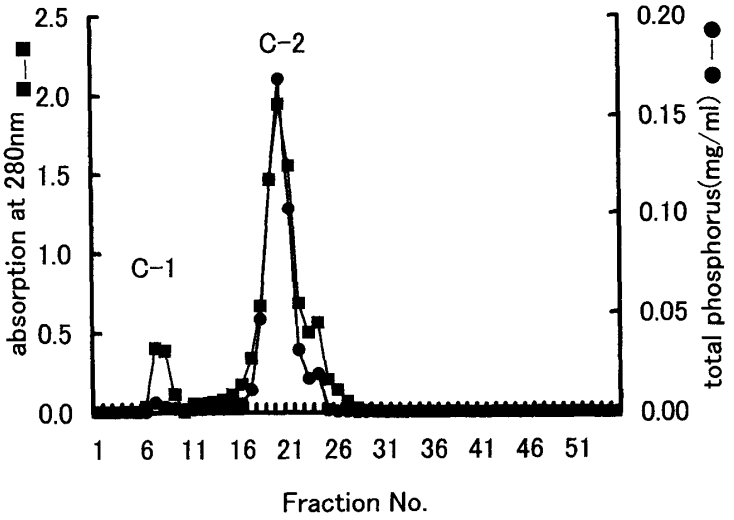

Fig. 2. Chromatographic Profile of Phosphopeptides Eluted by Sephadex G-50 Column Chromatography from the Hydrolyzates Obtained by Chymotryptic Hydrolysis of Oyster, *Crassostrea gigas*.
The experimental details are described in the previous paper[14]. ●—● indicate total-phosphorus and ■—■ indicate phosphopeptides at 280 nm in the collected fractions. C-1 and C-2 represent combined fractions 6~10 and fractions 16~27, respectively.

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 280 nm.

Phosphorus-containing three peaks and three peaks of peptides at 280 nm were eluted from the column in the case of peptic 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. 11 in the case of P-1-1, and in fraction No. 18 in the case of P-1-2, and in fraction No. 29 in the case of P-1-3 as shown in the Fig. 3.

In addition, the maximum absorption at 280 nm was observed in fraction No. 11 in the case of P-1-1 in fraction No. 18 in the case of P-1-2 and in fraction No. 32 in the case of P-1-3. Fraction No. 10~13 of the P-1-1, fraction No. 16~21 of the P-1-2 and fraction No. 31~55 of the P-1-3 were pooled, and analyzed for phosphonate-phosphorus (C-P), amino acids and ACE inhibitory activity.

Fig. 4 shows the chromatographic profile of phosphopeptides by rechromatography on Sephadex G-15 column of pepsin digest of active fraction (C-2) obtained from Sephadex G-50 chromatography. The experimental details are described in the text.

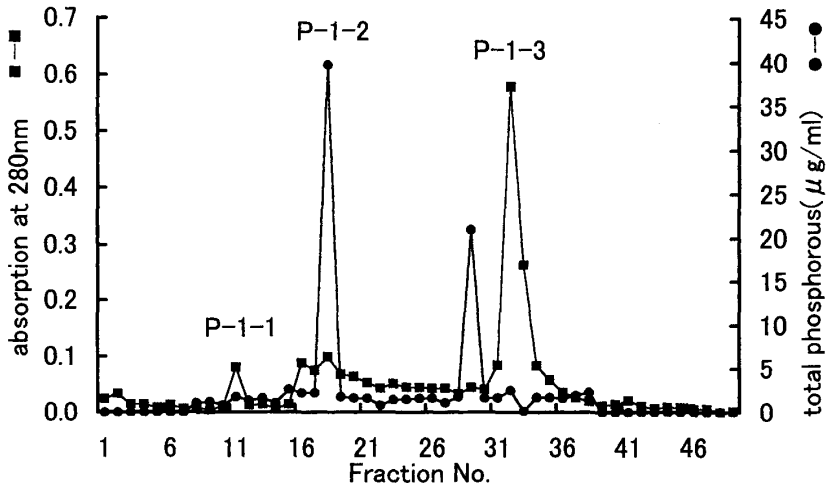

Fig. 3. Chromatographic Profile of Phosphopeptides by Rechromatography on Sephadex G-15 Column of Pepsin Digest of the Most Active Fraction (P-1) Obtained from Sephadex G-50 Chromatography.
The most active fraction (P-1) obtained from Sephadex G-50 chromatography was applied on the Sephadex G-15 column (2.5×40 cm), preequilibrated with water, and eluted with water. The eluate was collected in 4 ml fractions, while monitoring the absorbance at 280 nm for peptides and at 820 nm for total phosphorus. ●—● indicate total-phosphorus and ■—■ indicate phosphopeptides at 280 nm in the collected fractions. P-1-1, P-1-2, and P-1-3 represent combined fractions 10~13, fractions 16~21, and fractions 31~35, 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 280 nm.

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

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

The maximum total-phosphorus content was observed in fraction No. 25 in the case of C-2-1, and in fraction No. 35 in the case of C-2-2 and in fraction No. 38 in the case of C-2-3 as shown in the Fig. 4.

In addition, the maximum absorption at 280 nm was observed in fraction No. 26 in the case of C-2-1, and in fraction No. 34 in the case of C-2-2 and in fraction No. 39 in the case of C-2-3. Fraction No. 18~28 of the C-2-1, fraction No. 29~36 of the C-2-2 and fraction No. 37~41 of the C-2-3 were pooled, and analyzed for phosphonate-phosphorus, amino acids and ACE inhibitory activity.

In Table 1 is reported the phosphorus contents of the P-1-1, P-1-2, P-1-3, C-2-1,

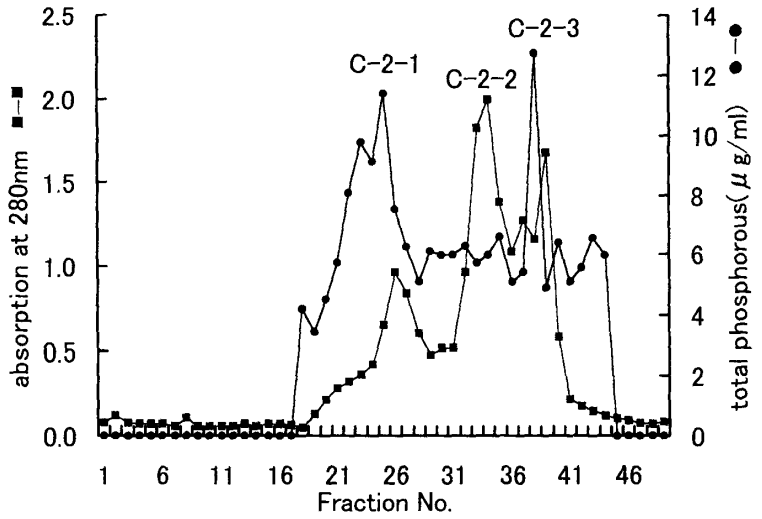

Fig. 4. Chromatographic Profile of Phosphopeptides by Rechromatography on Sephadex G-15 Column of Pepsin Digest of Active Fraction (C-2) Obtained from Sephadex G-50 Chromatography.
Active fraction (C-2) obtained from Sephadex G-50 Chromatography was applied on the Sephadex G-15 Column (2.5×40 cm), preequilibrated with water, and eluted with water. The eluate was collected in 4 ml fractions. ●—● indicate total-phosphorus and ■—■ indicate phosphopeptides at 280 nm in the collected fractions. C-2-1, C-2-2, and C-2-3 represent combined fractions 18~28, fractions 29~36, and fractions 37~41, respectively.

C-2-2 and C-2-3 eluted by rechromatography on Sephadex G-15 column of peptic hydrolyzates of P-1 and C-2.

The total-phosphorus content in the P-1-1, P-1-2, P-1-3, C-2-1, C-2-2 and C-2-3 fractions were 1.42, 8.21, 1.56, 6.81, 5.97 and 6.91 μg per ml of the fraction, respectively.

The amounts of phosphonate-phosphorus (C-P) in the P-1-1, P-1-2, P-1-3, C-2-1, C-2-2 and C-2-3 were 0.33, 0.36, 0.44, 0.14, 0.23 and 0.11 μg per ml of the fraction.

In addition, about 23, 4, 28, 2, 3 and 1% of the total-phosphorus in the P-1-1, P-1-2, P-1-3, C-2-1, C-2-2 and C-2-3 fractions was found to be phosphonate, which was primarily 2-aminoethylphosphonic acid.

Table 2 summarises the amino acid composition of the P-1-1, P-1-2, P-1-3, C-2-1, C-2-2 and C-2-3 fractions.

Of note in P-1-1, P-1-2, P-1-3, C-2-1, C-2-2 and C-2-3 peaks is the very high acidic amino acids (Asp and Glu), Gly, Ala, Val, Leu, Tyr, Phe, Lys and Arg content, and the presence of unique amino acid, 2-aminoethylphosphonic acid, (2-AEP).

In P-1-3 phosphopeptide, the 2-AEP content was the highest, when compared to P-1-1 and P-1-2, other peaks (C-2-1, C-2-2 and C-2-3) had low 2-AEP.

Table 1. Phosphorus Contents of the P-1-1, P-1-2, P-1-3, C-2-1, C-2-2 and C-2-3 by Fractionated Rechromatography on Sephadex G-15 Column of Peptic Hydrolyzates of P-1 and C-2 Obtained from Sephadex G-50 Chromatography.

Fraction	T-P (μg/ml)	C-P (μg/ml)	C-P/T-P (%)
P-1-1	1.42	0.33	23.19
P-1-2	8.21	0.36	4.43
P-1-3	1.56	0.44	28.08
C-2-1	6.81	0.14	1.99
C-2-2	5.97	0.23	3.77
C-2-3	6.91	0.11	1.59

Abbreviations used are:
C-P, Phosphonate-phosphorus; T-P, Total-phosphorus

Table 2. Amino Acid Composition of Phosphopeptides in the P-1-1, P-1-2, P-1-3, C-2-1, C-2-2 and C-2-3 Fractionated by Rechromatography on Sephadex G-15 Column of Peptic Hydrolyzates of the P-1 and C-2 Obtained from Sephadex G-50 Chromatography.

Amino acid	P-1-1	P-1-2	P-1-3	C-2-1	C-2-2	C-2-3
Asp	2.46	2.60	1.03	0.30	0.22	0.28
Thr	1.16	1.20	0.49	0.00	0.23	0.19
Ser	1.52	1.74	1.03	0.14	0.17	0.22
Glu	2.52	2.17	1.06	0.22	0.24	0.23
Gly	1.52	1.34	0.84	0.00	3.06	1.19
Ala	1.48	0.86	0.64	0.42	1.81	0.43
Cys	0.17	0.10	0.21	0.19	0.05	0.05
Val	1.17	0.72	0.82	0.01	1.15	0.42
Met	0.36	0.12	0.26	0.02	0.23	0.28
Ileu	1.23	0.83	0.62	0.00	0.73	0.37
Leu	2.29	0.98	1.11	0.05	0.83	0.49
Tyr	22.58	0.29	1.72	0.00	0.12	1.58
Phe	4.13	0.31	1.78	0.13	0.16	1.97
His	0.45	0.40	2.94	0.08	0.30	0.37
Lys	1.47	0.32	0.57	0.12	1.25	0.16
Arg	1.58	0.32	0.54	0.07	0.23	0.20
Pro	1.11	0.78	0.29	0.00	0.00	0.00
2-AEP	0.13	0.13	0.17	0.03	0.06	0.04
TOTAL	47.32	15.21	16.13	1.78	10.85	8.45

• Values are expressed as mg/mg Nitrogen.
• Abbreviations used are: 2-AEP, 2-aminoethylphosphonic acid.

Cheung et. al[18]. 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 P-1-1, P-1-2, P-1-3, C-2-1, C-2-2 and C-2-3 phosphopeptides eluted by Sephadex G-15 column chromatography and by ultrafiltration of the hydrolyzates obtained by pepsin hydrolysis of P-1 and C-2.

The IC_{50} values for P-1-1, P-1-2, P-1-3, C-2-1, C-2-2 and C-2-3 were 1.11, 0.04, 0.05, 1.04, 0.72 and 0.06 mg protein/ml, respectively.

The pepsin treated fraction P-1-2 had the most inhibitory activity and showed 0.04 mg protein/ml inhibition against ACE at IC_{50} value, and the IC_{50} value in the P-1-2 fraction was 27, 1.3, 25, 18 and 1.5 times higher than those in the P-1-1, in the P-1-3, in the C-2-1, in the C-2-2 and in the C-2-3 fractions, respectively.

Fig. 5~10 show the two dimensional thin layer chromatogram of the P-1-1, P-1-2, P-1-3, C-2-1, C-2-2 and C-2-3 obtained by rechromatography on Sephadex G-15 column of peptic hydrolyzates of the P-1 and C-2 obtained from Sephadex G-50 chromatography.

When the ACE inhibitory phosphopeptides were analyzed by thin layer chromatography, some ninhydrine-positive spots were observed.

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 occur 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.

Table 3. Angiotensin Converting Enzyme Inhibitory Activity of the P-1-1, P-1-2, P-1-3, C-2-1, C-2-2 and C-2-3 of Phosphopeptides Obtained by Rechromatography on Sephadex G-15 Column of Peptic Hydrolyzates of the P-1 and C-2 Obtained from Sephadex G-50 Chromatography.

Fraction	Protein (mg/ml)	IC_{50} (mg protein/ml)
P-1-1	0.065	1.106
P-1-2	0.069	0.041
P-1-3	0.065	0.054
C-2-1	0.113	1.041
C-2-2	0.100	0.721
C-2-3	0.069	0.060

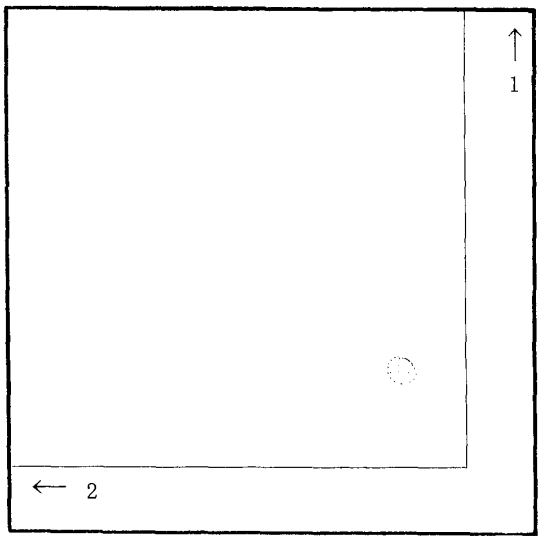

Fig. 5. Two Dimensional Thin layer Chromatogram of the P-1-1 Obtained by Rechromatography on Sephadex G-15 Column of Peptic Hydrolyzate of the P-1 Obtained from Sephadex G-50 Chromatography.
Solvents:
1 : *n*-butanol : acetone : acetic acid : 5% ammonium hydroxide : water (4.5 : 1.5 : 1 : 1 : 2)
2 : *n*-butanol : acetic acid: 5% ammonium hydroxide (5.5 : 3 : 1.5)
The spots were detected by 0.2% ninhydrin in aceton.

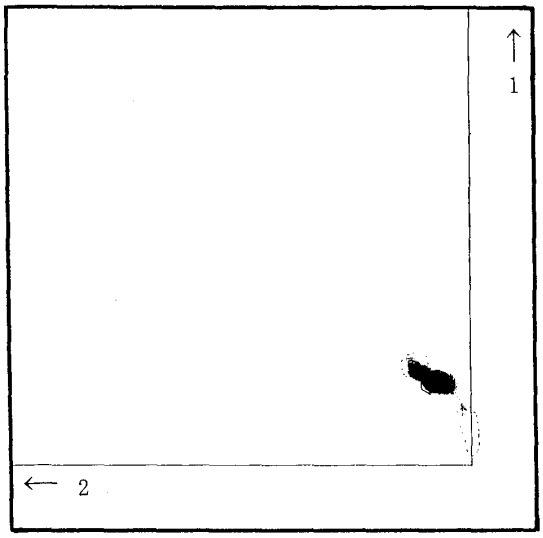

Fig. 6. Two Dimensional Thin layer Chromatogram of the P-1-2 Obtained by Rechromatography on Sephadex G-15 Column of Peptic Hydrolyzate of the P-1 Obtained from Sephadex G-50 Chromatography.
Solvents:
1 : *n*-butanol : acetone : acetic acid : 5% ammonium hydroxide : water (4.5 : 1.5 : 1 : 1 : 2)
2 : *n*-butanol : acetic acid : 5% ammonium hydroxide (5.5 : 3 : 1.5)
The spots were detected by 0.2% ninhydrin in aceton.

REFERENCES

1) G. Oshima, H. Shimabukuro and K. Nagasawa; Biochem. Biophys. Acta, **556**, (1979), 128-137
2) S. Maruyama, K. Nakagomi, N. Tomizuka and H. Suzuki; Agric. Biol. Chem., **49**, (1985), 1405-1409
3) H. Karaki, K. Doi, S. Sugano, H. Uchiwa, R. Sugai, U. Murakami and S. Takemoto; Comp. Biochem. Physiol., **96C**, (1990), 367-371
4) T. Matsui, H. Matsufuji, E. Seki, K. Osajima, M. Nakashima and Y. Osajima; Biosci. Biotech. Biochem., **57**, (1993), 922-925
5) H. Matsufuji, T. Matsui, E. Seki, K. Osajima, M. Nakashima and Y. Osajima; Biosci. Biotech. Biochem., **58**, (1994), 2244-2245
6) H. Ukeda, H. Matsuda, H. Kuroda, K. Osajima, H. Matsufuji and Y. Osajima; Nippon Nogeikagaku Kaishi, **65**, (1991), 1223-1228
7) H. Ukeda, H. Matsuda, K. Osajima, H. Matsufuji, T. Matsui and Y. Osajima; Nippon

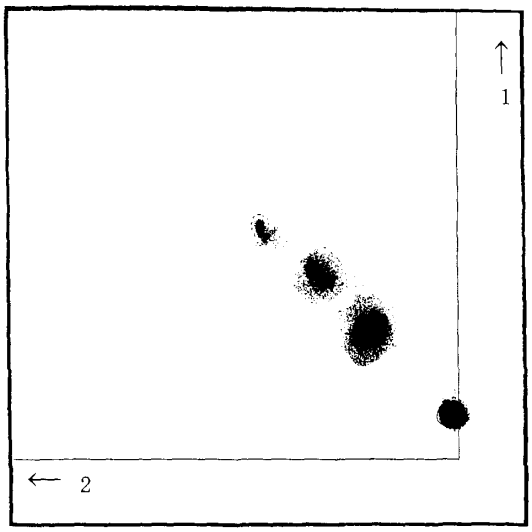

Fig. 7. Two Dimensional Thin layer Chromatogram of the P-1-3 Obtained by Rechromatography on Sephadex G-15 Column of Peptic Hydrolyzate of the P-1 Obtained from Sephadex G-50 Chromatography.
Solvents:
1 : *n*-butanol : acetone : acetic acid : 5% ammonium hydroxide : water (4.5 : 1.5 : 1 : 1 : 2)
2 : *n*-butanol : acetic acid : 5% ammonium hydroxide (5.5 : 3 : 1.5)
The spots were detected by 0.2% ninhydrin in aceton.

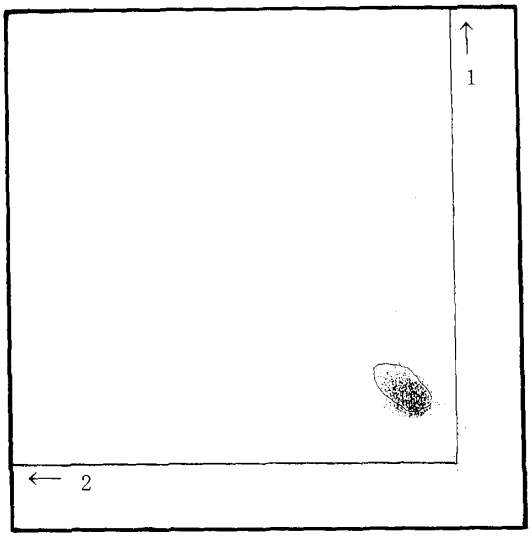

Fig. 8. Two Dimensional Thin layer Chromatogram of the C-2-1 Obtained by Rechromatography on Sephadex G-15 Column of Peptic Hydrolyzate of the C-2 Obtained from Sephadex G-50 Chromatography.
Solvents:
1 : *n*-butanol : acetone : acetic acid : 5% ammonium hydroxide : water (4.5 : 1.5 : 1 : 1 : 2)
2 : *n*-butanol : acetic acid : 5% ammonium hydroxide (5.5 : 3 : 1.5)
The spots were detected by 0.2% ninhydrin in aceton.

Nogeikagaku Kaishi, **66**, (1992), 25-29
8) T. Suzuki, N. Ishikawa and H. Meguro; Nippon Nogeikagaku Kaishi, **11**, (1983), 1143-1146
9) L. T. Skeggs, Jr., J. R. Kahn and N. P. Shumway; J. Exp. Med., **103**, (1956), 295-299
10) J. H. Laragh, L. Baer, H. R. Brunner, F. R. Buhler, J. E. Sealey and E. D. Vaughan; Jr. Amer. J. Med., **52**, (1972), 633-652
11) M. Tamari, T. Hisatomi and A. Ikeda; Sci. Bull. of Fac. of Educ., Nagasaki Univ., No. 50, (1994), 59-69
12) M. Tamari; Sci. Bull. of Fac. of Educ., Nagasaki Univ., No. 55, (1996), 37-47, 49-57
13) M. Tamari; Sci. Bull. of Fac. of Educ., Nagasaki Univ., No. 57, (1997), 51-58
14) M. Tamari and H. Kanda; Bull. of Fac. of Educ., Nagasaki Univ., Natural Science No. 61, (1999), 41-51
15) S. Yamamoto, I. Toita and K. Iwai; Nippon Kyobusikkangakkai Zassi, **18**, (1980), 297-303
16) P. S. Chen, T. Y. Tonibara and H. Warner; Anal. Chem., **28**, (1956), 1756

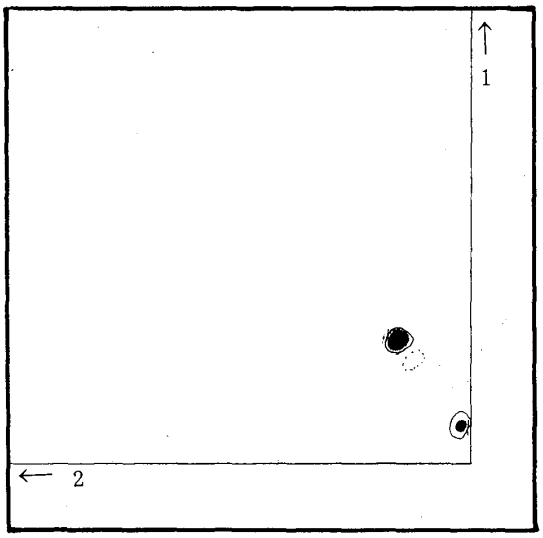

Fig. 9. Two Dimensional Thin layer Chromatogram of the C-2-2 Obtained by Rechromatography on Sephadex G-15 Column of Peptic Hydrolyzate of the C-2 Obtained from Sephadex G-50 Chromatography.
Solvents:
1 : *n*-butanol : acetone : acetic acid : 5% ammonium hydroxide : water (4.5 : 1.5 : 1 : 1 : 2)
2 : *n*-butanol : acetic acid : 5% ammonium hydroxide (5.5 : 3 : 1.5)
The spots were detected by 0.2% ninhydrin in aceton.

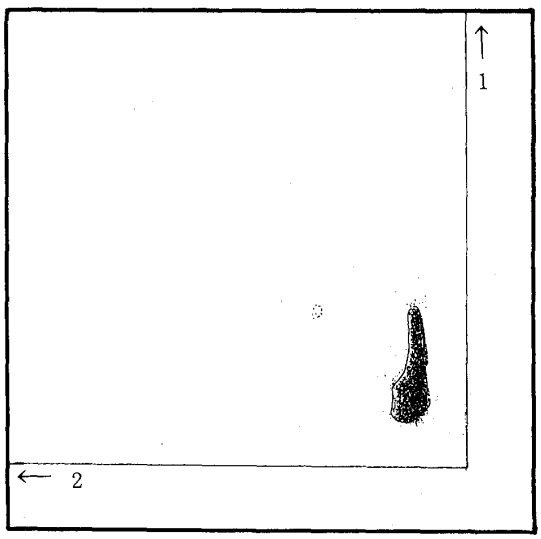

Fig. 10. Two Dimensional Thin layer Chromatogram of the C-2-3 Obtained by Rechromatography on Sephadex G-15 Column of Peptic Hydrolyzate of the C-2 obtained from Sephadex G-50 Chromatography.
Solvents:
1 : *n*-butanol : acetone : acetic acid : 5% ammonium hydroxide : water (4.5 : 1.5 : 1 : 1 : 2)
2 : *n*-butanol : acetic acid : 5% ammonium hydroxide (5.5 : 3 : 1.5)
The spots were detected by 0.2% ninhydrin in aceton.

17) M. Tamari, M. Horiguchi and M. Kandatu; Nippon Nogeikagaku Kaishi., **45**, (1971), 433
18) H. S. Cheung, F. L. Wang, M. A. Ondetti, E. F. Sabo and D. W. Cushman; J. Biol. Chem., **255**, (1980), 401-407