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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 the previous papers[1,2], we investigated the effects of ACE inhibitory activities of phosphopeptides of P-1 and C-2 which were obtained from edible oyster by proteolytic hydrolyzation.

In this investigation, the ACE inhibitory phosphopeptides of T-1 was further purified by ultrafiltration and Sephadex G-15 column chromatography.

ACE inhibitory activity was fractionated into three major phosphopeptides fractions of T-1-1, T-1-2 and T-1-3 in the pepsin hydrolyzates of the T-1 by gel filtration rechromatography on Sephadex G-15. The inhibition of ACE of the three kinds of phosphopeptides fractions (T-1-1, T-1-2 and T-1-3) was analyzed in vitro.

The IC_{50} values of T-1-1, T-1-2 and T-1-3 of phosphopeptides for ACE were 0.159, 0.095 and 0.140 mg protein/ml, respectively. The T-1-2 fraction had the most potent inyhibitory activity and showed 0.095 mg protein/ml inhibition against ACE at IC_{50} value.

It has been demonstrated that the T-1-1, T-1-2 and T-1-3 contained about 33.3%, 3.01% and 27.12% as phosphonate-phosphorus of total phosphorus.

The amino acid compositions of the phosphopeptides fractions (T-1-1, T-1-2 and T-1-3) were characterized by relatively high percentage for Tyr, Ser, Arg, Ala, Asp and Phe.

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.

INTRODUCTION

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

Many investigators have tried to prepare and insolate the ACD inhibitory peptide[3~12].

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

In the previous papers[1,2,13~15], we have investigated that the inhibitory activities of angiotensin 1-converting enzyme of phosphoproteins and phosphopeptides obtained from proteolytic hydrolyzates of edible shellfishes.

In the previous paper[1], we reported that the inhibitory activities of ACE detected from digests by proteolytic enzymes such as pepsin, trypsin and chymotrypsin of oyster extracts were fractionated into two major factions of high molecular weight and low molecular weight by gel filtration chromatography on Sephadex G-50.

The ACE inhibitors were detected two major phosphoperptides 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 column chromatography.

The P-1 had the most potent inhibitory activity and showed 0.30 mg protein/ml inhibition against ACE at IC_{50} value, and C-2, T-1 showed 1.47 and 2.73 mg protein/ml inhibition against ACE at IC_{50} value, respectively.

In the previous paper[2], we have also reported that the ACE inhibitory phosphopeptides of P-1 and C-2 were further purified by ultrafiltration and Sephadex G-15 column 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 P-1-2 fraction had the most potent inhibitory activity and showed 0.04 mg protein/ml inhibition against ACE at IC_{50} value.

When the ACE inhibitory phosphopeptides (P-1, P-2) were analyzed by TLC, some ninhydrine-positive spots were observed. These results suggested that the phosphopeptides were a mixture of several phosphopeptides.

In this investigation, therefore, T-1 of ACE inhibitors were further purified by ultrafiltration, Sephadex G-15 column chromatography and thin layer chromatography (TLC).

MATERIALS AND METHODS

Materials:

Sephadex G-15 was a product of Pharmacia. Proteolytic enzymes (pepsin, trypsin, chymotrypsin) were obtained from Boehringerr 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[16].

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℃ for 30 min. The reaction was stopped by adding 250 μl of 1 M HCl, and 1.5 ml of ethyl acetate was thenn added to the mixture, and the mixture was shaked for 30 sec. The mixture was centrifuged at 2500 rpm for 10 min., and 1.25 ml from the supernatant was transfered to test tube, and then heated at 120℃ for 45 min., 0.5 ml 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 defined as the IC_{50} value.

Preparation and purification of ACE inhibitors from pepsin hydrolyzate:

The freeze-dried powder about 160 mg of T-1 obtained by Sephadex G-50 column 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℃ for 24 hrs. After proteolysis, the incubation mixture was adjusted with 0.1 N NaOH to pH 8.4, the solution was then heated for 15 min. in a boiling-water bath. After the precipitate had been removed by centrifugation at 14000 rpm for 15 min., the supernatant was then ultrafiltrated with membrane filter (M.W. Cutoff 2000; Amicon).

The filtrate was then applied to a 2.5 x 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 phosphours, amino acid and ACE inhibitory activity.

The molecular weight distribution of peptide was estimated by using adenine dinucleotide (M.W. 830), Hippuryl-L-Histidyl-Leucine (M.W. 430) and L-tyrosine (M.W. 181).

Quantitative analysis:

Total phosphorus was estimated by the method of Chen et.al[17]. Phosphonate-phosphorus was estimated by the method of Tamari 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 M HCl at 110℃ for 24 hrs. The nitrogen and protein were analyzed in the usual way (% protein = % N x 6.25).

Thin layer chromatography:

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

RESULTS AND DISCUSSION

Fig 1 shows the Sephadex G-50 column chromatogram of the tryptic hydrolyzates of Oyster, *Crassostrea gigas*. The experimental details were 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.

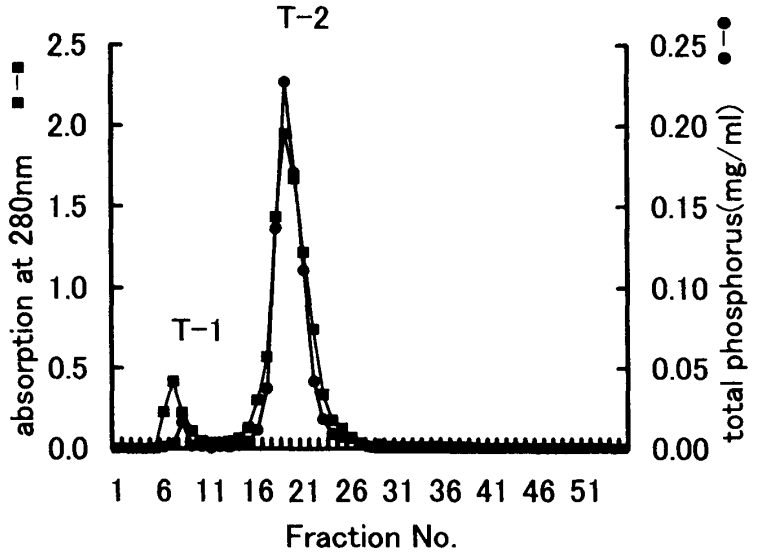

Fig. 1 Chromatographic Profile of Phosphopeptides Eluted by Sephadex G-50 Column Chromatography from the Hydrolyzates Obtained by Tryptic Hydrolysis of Oyster, *Crassostrea gigas*.
The experimental details are described in the text.
●－● indicate total-phosphorus and ■－■ indicate phosphopeptides at 280 nm in the collected fractions. T-1 and T-2 represent combined fractions No.5～10 and fractions No.15～26, respectively.

Phosphorus-containing two peaks and two peaks of peptides at 280 nm were eluted from the column in the case of tryptic hydrolyzates. The first peak and the second peak designated as T-1 and T-2, respectively. Fraction No.5~10 of the T-1, and fraction No.15~26 of the T-2 were pooled, and analyzed for ACE inhibitory activity.

Table 1. Angiotensin Converting Enzyme Inhibitory Activity of the T-1 and T-2 Phosphopeptides Eluted by Sephadex G-50 Column Chromatography of the Hydrolyzates Obtained by Tryptic Hydrolysis of Oyster, *Crassostrea gigas*.

Fraction	Protein (mg/ml)	IC_{50} (mg protein/ml)
T-1	0.66	2.73
T-2	4.60	2.64

The inhibition of ACE of the T-1 and T-2 was investigated in vitro. It has been demonstrated that T-1 and T-2 had similar inhibitory activity and showed 2.73 and 2.64 mg protein/ml inhibition against ACE at IC_{50} value (Table 1).

Therefore, the T-1 of ACE inhibitors were further purified by ultrafiltration and Sephadex G-15 column chromatography.

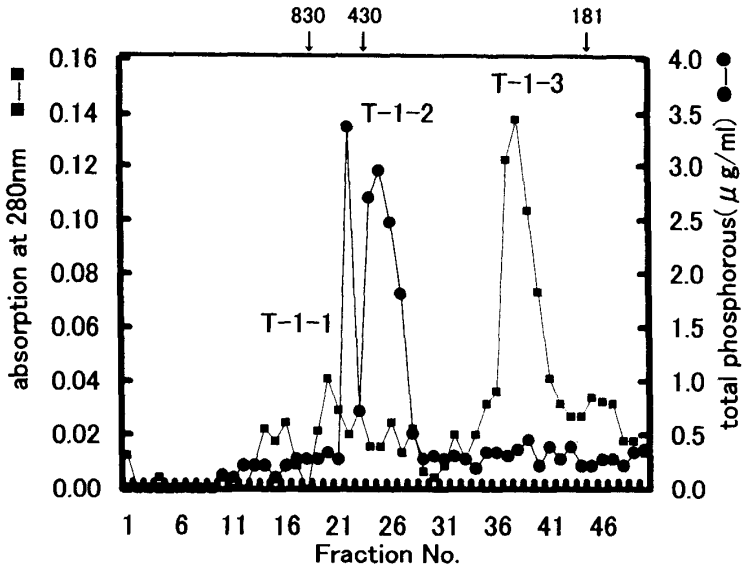

Fig. 2 Chromatographic Profile of Phosphopeptides by Rechromatography on Sephadex G-15 Column of Pepsin Digest of Active Fraction (T-1) Obtained from Sephadex G-50 Chromatography.
The active fraction (T-1) obtained from Sephadex G-50 Chromatography was applied on the Sephadex G-15 Column (2.5 x 40cm), pre-equilibrated with water, and eluted with water. The eluate was collected in 4ml fractions. ●—● indicate total-phosphorus and ■—■ indicate phosphopeptides at 280 nm in the collected fractions. T-1-1, T-1-2, and T-1-3 represent combined fractions No.13 ~ 17, fractions No.19 ~ 29, and fractions No.35 ~ 43, respectively.

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

The experimental details were 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 T-1. The first peak, the second peak and the third peak designated as T-1-1, T-1-2 and T-1-3, respectively.

The maximum total-phosphorus content was observed in fraction No.16 in the case of T-1-1, and in fraction No.20 in the case of T-1-2, and in fraction No.38 in the case of T-1-3 as shown in the Fig 2.

In addition, the maximum absorption at 280 nm was observed in fraction No.15 in the case of T-1-1, and in fraction No.29 in the case of T-1-2 and in fraction No.38 in the case of T-1-3. Fraction No.13～17 of the T-1-1, fraction No.19～29 of the T-1-2 and fraction No.35～43 of the T-1-3 were pooled, and analyzed for phosphonate-phosphorus (C-P), amino acids and ACE inhibitory activity.

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

Fraction	T-P (μg/ml)	C-P (μg/ml)	C-P/T-P (%)
T-1-1	0.84	0.279	33.25
T-1-2	5.72	0.172	3.01
T-1-3	1.36	0.370	27.12

Abbreviations used are:

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

Table 2 shows the phosphorus contents of the T-1-1, T-1-2 and T-1-3 eluted by rechromatography on Sephadex G-15 column of peptic hydrolyzates of T-1.

The total-phosphorus content in the T-1-1, T-1-2 and T-1-3 fractions were 0.084, 5.72 and 1.36 μg per ml of the peak, respectively.

The amounts of phosphonate-phosphorus (C-P) in the T-1-1, T-1-2 and T-1-3 were 0.279, 0.172 and 0.370 μg per ml of the peak.

In addition, about 33.25%, 3.01% and 27.12% of the total-phosphorus in the T-1-1, T-1-2 and T-1-3 fractions was found to be phosphonate, which was primarily 2-aminoethylphosphonic acid.

Table 3 summarized the amino acid compositions of the T-1-1, T-1-2 and T-1-3 fractions. The amino acid compositions of the phosphopeptides fractions (T-1-1, T-1-2 and

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

Amino Acid	T-1-1	T-1-2	T-1-3
Asp	0.002	0.012	0.004
Thr	0.001	0.008	0.003
Ser	0.002	0.025	0.005
Glu	0.002	0.010	0.005
Gly	0.002	0.016	0.003
Ala	0.000	0.013	0.003
Cys	0.002	0.005	0.003
Val	0.000	0.008	0.005
Met	0.000	0.000	0.000
Ileu	0.000	0.004	0.003
Leu	0.002	0.007	0.009
Tyr	0.031	0.033	0.036
Phe	0.010	0.010	0.003
His	0.005	0.008	0.003
Lys	0.000	0.004	0.002
Arg	0.002	0.005	0.019
Pro	0.000	0.004	0.000
2-AEP	0.009	0.006	0.011
Total	0.070	0.177	0.115

· Values are expressed as mg/mg Nitrogen.

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

T-1-3) were characterized by relatively high percentage for Tyr, Ser, Arg, Ala, Asp, Glu and Phe content, and the presence of unique amino acid, 2-aminoethylphosphonic acid, (2-AEP).

In T-1-3 phosphopeptide, the 2-AEP content was high, when compared to T-1-3, other peaks (T-1-1 and T-1-2) had a low 2-AEP.

Cheung et.al[19]. and Matsui et.al[6]. have reported that in was essential for strong and competitive ACE inhibition that a peptide had aromatic amino acid residues at the

Table 4. Angiotensin 1-Converting Enzyme Inhibitory Activity of the T-1-1, T-1-2 and T-1-3 of Phosphopeptides Obtained by Rechromatography on Sephadex G-15 Column of Peptic Hydrolyzates of the T-1 Obtained from Sephadex G-50 Column Chromatography.

Fraction	Inhibition (%)	Protein (mg/ml)	IC_{50} (mg protein/ml)
T-1-1	39.20	0.7780	0.159
T-1-2	65.34	0.7781	0.095
T-1-3	50.00	0.8750	0.140

C-terminal (i.e., Try. Tyr. Pro.), and hydrophobic or basic ones at the N-terminal.

Table 4 shows angiotensin 1-converting enzyme inhibitory activity of the T-1-1, T-1-2 and T-1-3 phosphopeptides eluted by Sephadex G-15 column chromatography and ultrafiltration of the hydrolyzates obtained by pepsin hydrolysis of T-1.

The IC_{50} values for T-1-1, T-1-2 and T-1-3 were 0.159, 0.095 and 0.140 mg protein/ml, respectively.

The fraction T-1-2 had the most potent inhibitory activity and showed 0.095 mg protein/ml inhibition against ACE at IC_{50} value, and the IC_{50} value in the T-1-2 fraction was 1.67 and 1.47 times higher than those in the T-1-1 and in the T-1-3 fractions, respectively.

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

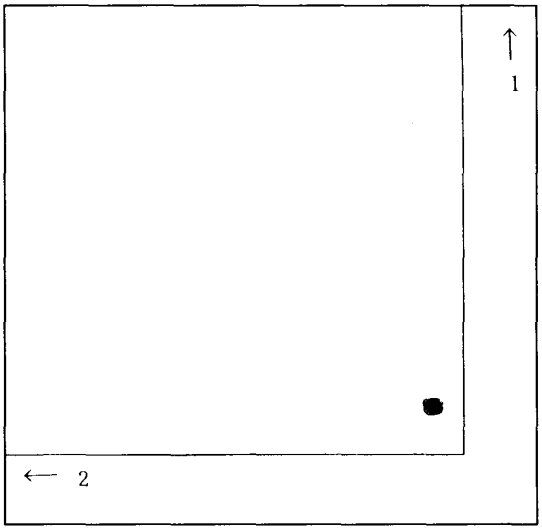

Fig. 3 Two Dimensional Thin layer Chromatogram of the T-1-1 Obtained by Rechromatography on Sephadex G-15 Column of Peptic Hydrolyzate of the T-1 Obtained from Sephadex G-50 Column Chromatography.
Solvents:
1 : *n*-butanol : acetone : acetic acid : 5% ammonium hydroxide : water (4.5 : 1.5 : 1 : 1 : 2)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

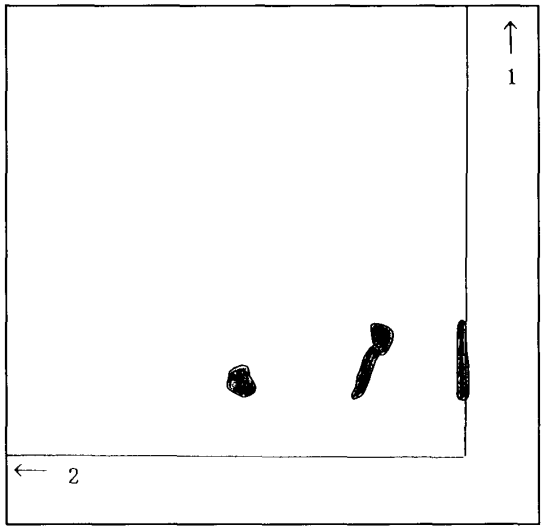

Fig. 4 Two Dimensional Thin layer Chromatogram of the T-1-2 Obtained by Rechromatography on Sephadex G-15 Column of Peptic Hydrolyzate of the T-1 Obtained from Sephadex G-50 Column Chromatography.
Solvents:
1 : *n*-butanol : acetone: acetic acid : 5% ammonium hydroxide : water (4.5 : 1.5 : 1 : 1 : 2)
2 : *n*-butanol : acetic acid: 5% ammonium hydroxide (5.5 : 3 : 1.5)
The spots were detected by 0.2% ninhydrin in aceton.

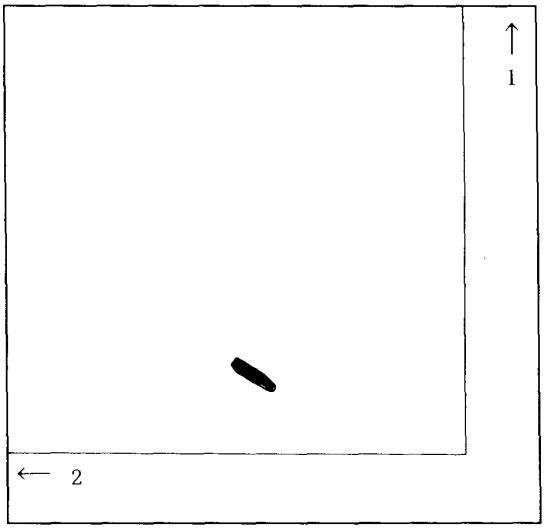

Fig. 5 Two Dimensional Thin layer Chromatogram of the T-1-3 Obtained by Rechromatography on Sephadex G-15 Column of Peptic Hydrolyzate of the T-1 Obtained from Sephadex G-50 Column Chromatography.
Solvents:
1 : *n*-butanol : acetone : acetic acid : 5% ammonium hydroxide : water (4.5 : 1.5 : 1 : 1 : 2)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

In addition, the results above, it has been demonstrated that 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 phosphopeptides were about 200 ~ 1000, and that the isolated peptide inhibitors occur as the phosphonate-containing phosphonopeptides and phosphate-containing phosphopeptides, however more experiments will be needed to prove the ACE inhibition of the phosphopeptides isolated from oyster.

REFERENCES

1) M.Tamari and H.Kanda; Bull. of Fac. of Educ., Nagasaki Univ., Natural Science **No.61**, (1999), 41-51
2) M.Tamari and H.Kanda; Bull. of Fac. of Educ., Nagasaki Univ., Natural Science **No.62**, (2000), 29-40
3) G.Oshima, H.Shimabukuro and K.Nagasawa; Biochem. Biophys. Acta, **556**, (1979), 128-137
4) S.Maruyama, K.Nakagomi, N.Tomizuka and H.Suzuki; Agric. Biol. Chem., **49**, (1985), 1405-1409
5) H.Karaki, K.Doi, S.Sugano, H.Uchiwa, R.Sugai, U.Murakami and S.Takemoto; Comp.

Biochem. Physiol., **96C**, (1990), 367-371
6) T.Matsui, H.Matsufuji, E.Seki, K.Osajima, M.Nakashima, and Y.Osajima; Biosci. Biotech. Biochem., **57**, (1993), 922-925
7) H.Matsufuji, T.Matsui, E.Seki, K.Osajima, M.Nakashima and Y.Osajima; Biosci. Biotech. Biochem., **58**, (1994), 2244-2245
8) H.Ukeda, H.Matsuda, H.Kuroda, K.Osajima, H.Matsufuji and Y.Osajima; Nippon Nogeikagaku Kaishi, **65**, (1991), 1223-1228
9) H.Ukeda, H.Matsuda, K.Osjima, H.Matsufuji, T.Matsui, and Y.Osajima; Nippon Nogeikagaku Kaishi, **66**, (1992), 25-29
10) T.Suzuki, N.Ishikawa and H.Meguro; Nippon Nogeikagaku Kaishi, **11**, (1983), 1143-1146
11) L.T.Skeggs, Jr.,J.R.Kahn and N.P.Shumway; J.Exp.Med., **103**, (1956) 295-299
12) 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
13) M.Tamari, T.Hisatomi and A.Ikeda; Sci. Bull. of Fac. of Educ., Nagasaki Univ., **No.50**, (1994), 56-69
14) M.Tamari; Sci. Bull. of Fac. of Educ., Nagasaki Univ., **No.55**, (1996), 37-47, 49-57
15) M.Tamari; Sci. Bull. of Fac. of Edue., Nagasaki Univ., **No.57**, (1997), 51-58
16) S.Yamamoto, I.Toita and K.Iwai; Nippon Kyoubusikkan Gakkai-zasshi, **18**, (1980), 297-303
17) P.S.Chen, T.Y.Tonibara and H.Warner; Anal. Chem., **28**, (1956), 1756
18) M.Tamari, M.Horiguchi and M.Kandatu; Nippon Nogeikagaku Kaishi., **45**, (1971), 433
19) H.S.Cheung, F.L.Wang, M.A.Ondetti, E.F.Sabo and D.W.Cushman; J.Biol.Chem., **255**, (1980), 401-407